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Development of Immune Reactions in the Absence or Presence of an Antigenic Stimulus

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The aim of this work was to differentiate serum factors with some immune activites, which develop spontaneously without dependence on an antigenic stimulus, from antibodies which arise in response to the injection of antigen. Under normal conditions(conventionally reared animals) antigenic stimuli are practically uncontrollable; they act in the organism, such as bacteria colonizing the intestine, respiratory tract and surface of the body. Moreover, food contains antigenic substances which are also a source of antigenic stimuli. Antibodies arising in response to them, without interference and control, are called natural antibodies. On the other hand, natural factors are present in the serum which have the capacity of interacting with macromolecular substances (e.g. alpha macroglobulin reacting with insulin, natural conglutinin with zymosan (1)). In this communication, the role of antigen in developing of the serum factors above mentioned is studied.

Experimental model. The newborn animals contain antibodies transferred from the mother and a great number of antigenic substances stimulating the maturing of its own immune mechanisms. The level of transferred antibodies does not usually permit detection of antibodies formed by the young animal itself. It is, therefore, of advantage to use a type of animal whose placental barrier does not permit the transfer of antibodies into the circulation of the foetus. If such animals (e.g. piglets) are artificially fed and do not obtain maternal colostrum, we do not find antibodies

passively transferred from the mother in their serum. In order further to exclude contact of the newborn animal with microbial antigens, the foetus is removed from the mother before birth under sterile conditions and put into an incubator where it is excluded from bacterial contamination.(Figs 1, 2, 3). However, the rearing of animals without microorganisms does not fully guarantee that antigenic stimuli are completely eliminated, because of content of dead bacteria and other antigenic components in food. We have attempted to reduce this source of antigenic stimuli by using a nonantigenic diet (mixture of amino acids and vitamins) and also by observing the development of immune factors in the early postnatal period, when immune response to small doses of antigen is reduced. We consider that studies of natural and immune factors in animals which have not obtained antibodies by transfer from the mother and are reared under sterile conditions in the early stages of ontogenesis, is the best model for solving these questions at the present time.

The characterization of some serum fractions of sterile precolostral piglets. The electrophoretic pattern of piglet sera shows, in agreement with published results, that the newborn do not possess serum γ-globulin. After concentration, however, protein has been detected in the region corresponding to γ-globulin (2). Sera of precolostral piglets contain approximately 40 µg per ml. of this protein. The incorporation of methionine into the γ-globulin fraction of the newborn was demonstrated by the use of labeled methionine S^{35}. The rate of increase in radioactivity indicated the typical process of synthesis (3). The nature of γ-globulin in the newborn was studied by physico-chemical and immunochemical methods. Two fractions were separated on DEAE cellulose. A fraction (I) of the neonatal γ-globulin is not precipitated and has a sedimentation coefficient of 2.7 S. The other fraction (II) is precipitated

with antiserum against the γ-globulin of adult pigs and has a sedimentation coefficient of 5.1 S (4). This fraction bears no relation to Bence Jones protein but a relation between 5 S neonatal γ-globulin and H chains of adult pig γ-globulin was determined by the fingerprint technique (5).

The evidence that antibodies are not present in precolostral piglet. Antibodies have not hitherto been demonstrated by any serological reaction to any bacterial, virus, phage or tissue antigens. No antibodies were detected by the agglutination reaction to the O and H antigens of various Gram-negative bacteria, nor by passive haemagglutination with antigens isolated from bacterial strains and bound on to sheep erythrocytes (6).

Passive haemolysis was carried out with erythrocytes, to which different antigens were adsorbed, not only with usual erythrocyte concentration (1%) but with progressively decreasing concentrations of erythrocytes (0.001%) (Fig.4). In this way the amount of antibodies necessary to produce haemolysis was greatly decreased, to the level 10^{-6} µg N/ml (7). The bactericidal reaction for which strains in the S-form were selected, has the same degree of sensitivity (8). Even detection of antibodies by opsonization of strains in the S-form (at the level of 10^{-6} µg N/ml) with sera of newborn precolostral piglets was negative. The sera do not display neutralization activity to phage (the experiments made by Dr.Trnka) neither neutralization activity against poliomyelitis viruses (Dr.Slonim). Antibodies were not only not found in the serum but also not in the concentrated γ-globulin fraction even on using passive haemagglutination and passive haemolysis with adsorbed diphtheria toxoid as in the experiments of Segre (9). We conclude, therefore, that the protein of the γ-globulin type in precolostral newborn piglets has not any detectable antibody activity.

Although antibodies were not detected in newborn piglet sera, a certain amount of complement was demonstrated immediately after birth by the haemolytic reaction (about 2 $C'H_{50}$ units). The amount of complement increased gradually in animals reared under sterile conditions; 20-day old piglet had 15 - 20 C'H units/ml. (9).

The immunological properties of piglet sera without the presence of antibodies. Some changes in antigen suspension similar to antigen-antibody reaction can be observed without the presence of antibody: bacterial agglutination at low pH, haemolysis by complement of erythrocytes previously treated with tannic acid, etc. The data will be presented that complement can act on certain bacterial surfaces even in the absence of antibodies.

a. Bactericidal reaction: In experiments using strains in the typical S-form, e.g. strain of Salmonella paratyphi B, we never detected bactericidal activity in precolostral piglet sera. On testing a larger group of Gram-negative bacteria, however, we found that these sera exert a bactericidal effect on some bacterial strains. These strains, which were sensitive to piglet complement, were in the typical R-form. On absorbing sera at 0° with R strains the bactericidal effect of serum was not abolished; it was abolished, however, if any components of complement was inactivated. In selected strains it was found that there is a direct relationship between the character of their surfaces (R-form) and the degree of dilution of test serum still capable of bactericidal activity (Table 1). We further demonstrated that there is a dependence of the amount of piglet complement, the character of the bacterial surface and the bactericidal effect: sera with a small amount of complement only have an effect on strains in the R-form (Table 2). Higher complement levels (5 $C'H_{50}$ units) act against strain 346 which has the characteristics of bacteria in the S-form and which is not sensitive to sera with

a lower complement level. Bactericidal activity is thus determined by the surface of the bacteria and not by its specific antigenic structure (the series included strains of coli, S.typhi, Shigella shigae, etc.). At the same time as using precolostral piglet serum we also worked with precolostral calf sera. It was shown that their bactericidal effect against some strains, e.g. Shigella shigae, is higher than would have been expected from the relationship between calf and piglet serum (Fig. 5). We therefore used different serological methods to determine whether antibodies to the Shigella strain were not present in the calf serum. We found that with O antigen adsorbed to erythrocytes (antibodies to this antigen are responsible for the bactericidal reaction) and passive haemolysis done with minimal numbers of erythrocytes (0,001%), the demonstration of antibodies by this method is more sensitive than the bactericidal reaction (T.3). We conclude that if antibodies are demonstrated in newborn serum by the bactericidal reaction only and not by the more sensitive passive haemolysis with minimal amounts of erythrocytes, precolostral serum probably does not contain antibodies. We further found that the bactericidal activity of precolostral serum can only be abolished if it is absorbed with zymosan in the presence of complement. If native calf serum is treated with zymosan, the bactericidal action disappears; after adding piglet complement to the absorbed calf serum in a concentration which does not itself have a bactericidal effect, bactericidal activity was not changed and the bactericidal effect was not present. On the contrary, in calf serum first inactivated at 56°C and treated with (Fig.6) zymosan to which the same amount of piglet complement had been added after inactivation, bactericidal activity was almost fully restored. Our contemporary programme is to establish whether absorption of sera does not affect some components of complement which were restored on adding neonatal complement or whether precolostral sera contain

a substance with properties such as properdin or natural conglutinin.

b. Opsonizing effect of precolostral piglet serum: A study was made of the opsonizing activity of neonatal precolostral piglet serum to strains in the S- and R-form. Phagocytosis was determined by perfusion of the isolated liver 10) and by the method of determining the time course of bacterial clearance from the circulation in neonatal piglets. Strains, which are in the typical S- and R-form were selected for the study of opsonization (Table 1). The concentration of the bacterial suspension for opsonization was 5×10^4 bacteria/ml. 0.1 ml of this suspension was mixed with 0.9 ml. test serum and left for one hour in the refrigerator. Bacteria were introduced into the afferent cannila leading to vena portae and the number of bacteria not taken up by the liver was determined in samples taken off from the inferior vena cava. Opsonization was carried out in the same way in clearance tests in vivo and 10^5 bacteria/kg. body weight were injected into the blood stream of piglets. The bacterial count was determined in blood samples obtained by cardiac puncture at different time intervals after injection.

In the first experiments on the isolated rat liver it was found that the uptake of E.coli suspended in Ringer solution is dependent on the character of the bacterial surfaces. An average of only 10% of the S-form are retained, whereas in the R-form the number retained amounts to 55 - 64%. In experiments in which E.coli (S-form) were opsonized with neonatal serum containing 2 - 3 units of $C'H_{50}$ complement, it was found that complement has an opsonizing effect and increases the number of bacteria retained from 10 to 48 %. Any inactivation of complement components abolished the opsonizing activity (Table 4) (11).

In further experiments phagocytosis was determined by the method of clearance of bacteria from the blood stream in neonatal precolostral piglets. Evidence was provided that

E.coli in the S-form is not phagocytosed in the newborn (Fig.7). On the other hand if the R-form of the strain is injected into neonatal piglets it is effectively cleared from the blood stream (Fig.8). In order to determine the minimal amount of antibodies for opsonization and phagocytosis of coli in the S-form, we mixed a bacterial suspension in the S-form with different amounts of rabbit antibody. It was found that dilutions of 10^{-8} - 10^{-9} of antiserum still produce a full opsonizing effect displayed by the complete clearance of the injected bacteria from the blood stream. Dilutions of 10^{-10} and 10^{-11} lead to only a partial and transient decrease of the number of circulating bacteria (Fig.9). Since the same strain mixed with neonatal serum containing a small amount of complement did not have an opsonizing effect it shows that the precolostral sera of neonatal piglets does not contain antibodies detectable by this very sensitive test (by which antibodies are determined in a concentration of about 10^{-6} µg N/ml). This experiment, therefore, shows that the sera of animals in which no antibodies have been demonstrated by any method, contain complement which acts on some bacterial surfaces and is thus able to imitate immune processes for which the presence of antibodies has hitherto been considered to be necessary.

The development of antibody formation in sterile animals after immunization with different antigens:
The study of antibody formation in sterile precolostral piglets has a number of advantages. Since there is no transfer of γ-globulin from the mother and there is no level of natural and passively acquired antibodies, the first antibodies detected are formed by the infant animal itself. Since the serum contains only a small amount of neonatal γ-globulin of low molecular weight, the character of the first antibodies can be well determined.

a. Time course of onset of antibody formation after immunization with different antigens: Newborn precolostral piglets were given an injection of S.paratyphi B and antibodies detected mainly using the bactericidal reaction permitting detection of 10^{-6} µg of antibody N/ml. The newborn animals were given i.p. injections of the maximum dose of antigen that they tolerate, i.e. 2.5×10^{10} bacteria in 5 ml. The first antibodies were but rarely detected 5 days after immunization. The amount of antibody increased to the 10 - 15th day after the injection of antigen. If sheep erythrocytes are injected (20% suspension in amounts of 10 or 20 ml) antibodies appear earlier, in most animals on the fifth day after the injection of antigen. However, we did not succeed in demonstrating antibody on the third day after injection, even using very sensitive tests. For demonstrating antibodies by the haemagglutination reaction we use a 0.1% suspension of erythrocytes, for demonstrating antibodies by the haemolytic reaction a 0.001% suspension. Very similar results - i.e. negative demonstration of antibodies on the third day after the injection of antigen and reliable demonstration on the fifth day - were obtained after immunization with the corpuscular antigen of phage T 2 and virus (Sabin attenuated vaccine strain). A comparison of the results after immunization with the different antigens is given in the table 5, Fig. 10. It is evident that the smallest immunizing effect was displayed by the bacterial antigen S.paratyphi B. If it cannot be objected that the different results in the formation of antibodies express different sensitivity of the serological reactions to all antigens (we used tests whose sensitivity was at the level of 10^{-6} µgN/ml) then it is necessary to consider the quality and quantity of injected antigen.

It is very difficult to compare the amount of antigenic substance injected in the complex structure of corpuscular antigens since haemolytic, haemagglutinating and virus and phage neutralizing antibodies only react with a certain

part of the whole injected corpuscle. We, therefore, wished to know whether the previously observed finding that increasing the amount of antigen speeded up the onset of antibody formation in young animals (12) would be valid for neonatal piglets. From the results (Fig.11) it is evident that increasing the amount of injected erythrocytes and phage (Fig.10) led to the earlier onset of antibody formation and also to a higher resulting titre.

The effect of the passive transfer of antibodies for the onset of antibody formation in infant animals has often been discussed and investigated in a number of works. Most work has shown that the transfer of antibodies delays the onset of immunization processes. It was found only rarely that the passive transfer of antibodies has a stimulating effect. In the work of Segre (13) on the same model of precolostral piglets, the passive transfer of antibodies was considered to be the basis of a good onset of an immunity response in infant animals. In all the antigens used we obtained a good response in precolostral piglets. We made a comparison of the immunizing effect of the same amount of erythrodyte antigen in piglets reared conventionally with the mother and in sterile precolostral piglets. The table shows that the passive transfer of antibodies inhibits to some extent the onset of the actual immunizing process in infant animals (Table 5, Fig. 12).

We further wished to find out the earliest onset of antibody formation and whether there is an increased immune response to different antigenic stimuli during the maturing of the individual in infant animals reared non exposed to antigens. We were unable to detect antibody formation after immunizing the foetus in utero one month before term (gestation lasts three months in pigs) using S.paratyphi B and Brucella suis as antigens; these results were published in 1960 (6). In recent years we have used sheep erythrocytes for intrauterine immunization and have demonstrated antibody formation immediately after birth in piglets immunized one month before term. In some animals, however,

antibodies were not detected at birth after intrauterine immunization. This could have been because the level of antibodies formed was not high enough or because antibodies which could have been detected during the month disappeared before birth. If such an animal is given the same injection of antigen immediately after birth as is given to newborn animals not immunized in utero, the onset, course and level of antibody formation has the typical character of a secondary reaction (table 7). If the same dose of antigen as the first immunizing dose (10 ml. of 20% sheep erythrocytes) is injected into young animals of various ages reared under sterile conditions, we do not observe a significant increase in the immunizing effect which would show that marked changes in the ability to respond to antigen during ontogenesis occur without an antigenic stimulus. This finding has obviously a parallel in the number of antibody producing cells detected, as will be reported later.

b. Characterization of the first antibodies formed: A determination was made of the molecular weight of the first antibodies in the sera of precolostral sterile piglets. Antibodies to erythrocytes, phage and strain S.paratyphi B were centrifuged in a sacharose gradient in a Spinco rotor 40 centrifuge at 35,000 r.p.m. for 16 hours. Six layers were successively separated in which the bactericidal, haemolytic and neutralizing activity of antibodies were tested. In all samples, antibodies were found in the last, i.e. the fifth and sixth fractions at the bottom of the tube. These tests confirmed a whole series of similar work that the first formed antibodies in newborn animals are of the macromolecular type (14,15).

The first detectable antibodies, however, are not characterized by this property only, but also by serological properties. If antibodies to erythrocytes are determined by the test described, in which the sensitivity is increased

by lowering the erythrocyte concentration, we do not find that the increased sensitivity of the serological reaction has a linear dependence as in the sera of hyperimmune adult animals (Fig.13). The results show that infant antibodies have a different binding capacity from that of the antibodies of hyperimmune adult animals. This could be due to the fact that they are mainly 19 S antibodies. We therefore isolated antibodies of the 19 S character from adult hyperimmunized animals on Sephadex G-200 (16) and determined the dependence of the amount of antibody essential for 50% haemolysis at different concentrations of erythrocytes. In 19 S antibodies of adult animals we found a linear dependence, i.e. within the class of one molecular type of antibody the binding capacity changes. This was also confirmed for 7 S antibodies. The antibodies formed immediately after birth during the secondary reaction, according to their sedimentation characteristics in a sacharose gradient, are antibodies of the 7 S type. These antibodies however, when tested for binding capacity by the test using a decreased number of erythrocytes, also failed to show a linear dependence. Again the reverse to 7 S antibodies of adult animals.

Differences in the binding capacity in the first antibodies of immunized piglets were found with anti-phage sera. It was found that the antibodies have a very small neutralizing capacity, which, however, is considerably increased if complement is added to the test. The source of complement was the serum from precolostral non immunized piglets which itself has no neutralizing activity to phage. Here complement evidently acts as a cofactor which strengthens the binding of easily dissociable antibodes on the phage particle similarly as in the first antiviral antibodies described by Dulbecco (17).

A further characteristic property of the first antibodies in newborns is their different sensitivity to mercaptoethanol (ME). Hyperimmune sera were tested and it was found that different concentrations of ME used by some authors (18, 19), for the inactivation of 19 S antibodies (1 M, 0.1 M, 0.05 M) do not exert a different effect. After the action of these concetrations we found the same titres, i.e. the same decrease in the initial haemagglutination reaction. If, however, we investigate the first antibodies of infant animals, the serological activity of antibodies disappeared after treatment with the given concentrations of mercaptoethanol. If sera obtained from infant animals reacting after birth with a secondary reaction (i.e. those which were immunized in utero) are treated with 0.05 M ME, the titre of antibodies is only partly decreased. However, 1 M concentration of ME, which has the same effect on hyperimmune sera as 0.05 M ME completely abolishes the serological activity of infant 7 S antibodies.

The results obtained with immunization of the foetus and newborn with different antigens provide evidence that the character of the antigen is of importance for the stimulation of the antibody response as well as the amount of antigen injected. Unlike the proteins found in the serum of nonimmunized newborn piglets in the γ-globulin zone, which have a low molecular weight of about 5 S, the first antibodies formed are macromolecular antibodies with different binding capacities from those of adult animals. Newborn piglets are able to react with a secondary reaction immediately after birth if the first dose is given in utero. These antibodies appear to be of the 7 S type. But their properties still show some characteristics of primitive antibodies (low binding capacity, sensitivity to mercaptoethanol). It can therefore be assumed that the change in molecular type, like increased resistance to ME and changes in binding capacity, will not occur at once

but that there will evidently be further antibodies of intermediate molecular characteristics.

The present results permit a more exact distinction between the antibody and nonantibody natural components of sera. Antibody is not only substance bound to a certain group of proteins of sera (according to present knowledge to γ-globulin) but is the result of adaptation processes in the organism after contact with antigen. The adaptation process is characterized by the formation of molecules of increasing fitness and effectiveness in their reaction with antigen. It is just these properties that distinguish antibody from the nonantibody components of the serum which are present and have a certain binding capacity with some macromolecular substances - even imitating the reaction of antigen with antibody - but which are formed spontaneously and their properties do not undergo any change after antigen injection.

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Table 1

		Coli strains in S-form				Coli strains in R-form		
		378	346	055	322	16	3A	288
Unstability in suspension heated 100°C - 1 hr		-	-	-	-	+	+	+
Agglutination in acriflavine solution 1 : 500		-	-	-	-	+	+	+
Electrophoretic migration velocity mm/3 min.		24	20	20	61	55	62	80
Phagocytosis: % of bacteria removed by liver perfusion		11	16	30	14	64	55	89
Dilution of serum for 50% bactericidal effect	precolostral piglet	0	0	0	0	1:128	1:128	1:8
	precolostral calf	1:1	1:2	1:1	0	1:256	1:256	1:64

Table 2

Dependence of bactericidal activity of piglet sera containing different amounts of complement on the surface character of bacterial strains

$C'H_{50}$ units/ml of piglet sera	S-form → R-form					
	coli 127	coli 346	coli 378	Shig.	coli3A	coli 16
1	0	0	0	1:1	1:8	1:8
1,5	0	0	0	1:1	1:8	1:16
2,2	0	0	0	1:2	1:8	1:16
3,1	0	0	0	1:4	1:32	1:32
5	0	1:1	1:1	1:6	1:64	1:64

Table 3

Demonstration of antibodies to Shigella by different methods

	reaction		dilution of serum
anti-Shigella rabbit serum	bacterial agglutination		1 : 1280
	passive hemagglutination 0,1%		650.000
	passive hemolysis	0,25%	65.000
		0,1 %	280.000
		0,001 %	1,500.000
	bactericidal reaction		500.000
newborn calf sera	bactericidal titer		32, 16, 8, 64
	passive hemagglutination 0,1%		0
	passive hemolysis 0,001 %		0
newborn piglet sera	bactericidal titer		1, 2, 1, 1, 1
	passive hemagglutination 0,1%		0
	passive hemolysis 0,001 %		0

Table 4

Uptake of E.coli 346 by the perfused rat liver suspended in Ringer-Locke and after opsonization by complement of newborn colostrum free piglets

Experimental conditions	Total number of experiment	% of bacterial uptake average	maxim.&minim. values
0,1 ml. E.coli 346 + 0,9 ml. Ringer-Locke	14	10	(28 - 0)
0,1 ml. E.coli + 0,9 ml. native piglet serum (p.s.)	8	48	(63 - 30)
0,1 ml. E.coli + 0,9 ml. p.s. inact.30 min. at 56°C	5	9	(24 - 3)
0,1 ml. E.coli 0,9 ml. p.s. + EDTA	5	12	(21 - 5)
0,1 ml. E.coli + 0,9 ml. p.s. absorbed by zymosan	5	10	(18 - 0)
0,1 ml. E. coli + 0,9 ml. p.s.treated with NH_3	3	12	(23 - 2)

Table 5

Antibody formation in precolostral sterile piglets immunized with different antigens immediately after birth

The type and amount of antigen		Days of life 3	5	7	10	
Salmonella paratyphi B $2,5 \times 10^9$ heat inact. microbes		0	0	0	1:1	≠
		0	0	0	1:1	
		0	0	0	2	
		0	0	0	8	
		0	0	1:1	10	
Sheep red blood cells 20 w/v	10 ml	0	0	16	256	
		0	2	16	64	≠ ≠
	20 ml	0	4	16	256	
		0	2	16	64	
T 2 phage particles	10^7	0	40	320	320	≠ ≠ ≠
	10^9	0	80	640	1280	

≠ 50 % bactericidal reaction with S. paratyphi B estimated at 3 hours after incubation at 37°C

≠ ≠ Hemagglutination with inactivated sera (for 30 min. at 56°C) with 0,1 % suspension of sheep red cells

≠ ≠ ≠ 50% inhibition test in the presence of precolostral calf serum complement diluted 1 : 5

Table 6

Comparison of formation of antibodies to sheep erythrocytes in precolostral and colostrum-fed piglets

	20% suspension of sheep erythrocytes	Days after immunization				
		0	3	5	7	9
Pre-colostral	10 ml	0	0	0	1:16	1:256
	10 ml	0	0	1:16	1:16	1:256
	20 ml	0	0	0	1:16	1:64
	20 ml	0	0	1:2	1:16	1:64
Colostrum-fed	10 ml	1:16	1:16	1:8	1:16	1:32
	10 ml	1:128	1:8	1:8	1:16	1:8
	20 ml	1:32	1:16	1:8	1:8	1:8
	20 ml	1:32	1:16	1:16	1:8	1:8

Table 7

Primary and secondary response to sheep red cells in newborn piglets

Primary				Secondary			
age in days	serum	serum treated with ME 0,05 M	1 M	age in days	serum	serum treated with ME 0,05 M	1 M
RBC 0	0	-	-	RBC 0	0	-	-
→5	0	-	-	→5	0	-	-
9	2	-	-	9	16	0	0
10	2	-	-	10	1024	512	0
11	4	-	-	11	8192	2048	0
13	8	-	-	13	8192	1024	0
15	128	0	0	15	8192	2048	0
17	256	0	0	17	4096	512	8
19	128	0	0	19	2048	-	-
21	256	0	0	21	4096	-	-

Table 8

Effect of complement on 50 % phage neutralization

	days after immuniz. with 10^7 T 2	serum dilution									
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
compl. 1:5 added	5	11%	15%	28%	34%	82%					
	10	14%	17%	15%	22%	28%	38%	50%	67%	67%	71%
	15	6%	17%	20%	21%	20%	22%	42%	62%	82%	98%
heat inact. compl. 1:5	5	33%	75%								
	10	30%	58%	69%	103%						
	15	33%	69%	112%							

The Inductive Phase of Antibody Formation Studied with Isolated Cells

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At the Symposium in 1959 we summed up experimental results which made it possible to reach the conclusion that the first phase of antibody formation can be considered to be functionally distinct /processes taking place during that phase/ from the later phase - the actual production of antibodies /15/. This conclusion was based on observations that the initial phase of antibody formation is much more sensitive to certain forms of interference /X-irradiation /6, 23/, vitamin deficiency /1/, the action of hormones /3/, and particularly on data obtained in experiments with the transfer of isolated spleen cells to young homologous recipients /13//. We showed that if spleen cells are cultivated with the antigen in vitro antibody formation does not occur; cells potentially capable of antibody formation /immunologically competent/ only survive in tissue culture and the process of differentiation for antibody formation starts only when they are transferred from tissue culture to tissue culture in vivo in a newborn recipient /14/. Using this method of transfer we showed that the smallest number of spleen cells capable of forming a sufficient level of antibodies in young recipients for detection by the agglutination reaction /i.e. about 0.1 /ug N antibody/ml./ is 10^6. We further found that of this number probably only in the order of 10^3 cells participate

in actual production, i.e. about 0.1%. On the basis of these quantitative findings we were able to exclude the simplest version of the clonal selection theory, by which antigen acts directly by the proliferation of cells already producing the given type of antibody. Our calculations showed that on this assumption and with our methods of detecting antibodies, they should have been detectable sooner than they actually were experimentally /20/. This was the basis which led to our arriving at the conclusion that actual production is preceded by a qualitatively different, inductive phase of antibody formation whose basis is a change in biochemical and morphological properties during the process of differentiation /15/.

In the most recent years we have made further observations in support of these conclusions. With a model of transfer of isolated cells we showed that the duration of the inductive phase can only be little shortened but that it cannot be eliminated /17/ if an increasing number of spleen cells is used for transfer. We used a whole spectrum of inhibitors of nucleic acids and found that only a few inhibit antibody formation even if those which do not affect antibody formation have marked antimitotic activity /18/. The nucleic acid inhibitors, e.g. 6-mercaptopurine and 6-thioguanine, act only during the inductive phase of antibody formation./16/. On a similar model, the transfer of spleen cells to lethally irradiated isologous recipients, Makinodan et al. /9/ reached the same conclusion, i.e. that the first phase of antibody formation /the latent phase/ actually exists and can be explained in terms of disorganization and reorganization of the germinal centre.

There are, however, certain data, obtained for the most part

with phage and virus antigens /12, 24, 5/, which are at variance with these results and conclusions. These authors found increasing titres of antibodies in the serum very soon after giving antigen and by the interpolation of the results almost eliminated the possibility of the existence of a period during which antigens were not formed. However, since in our experiments, particularly with the immunization of young animals, we continue to reach the opposite conclusion /19/ we attempt in the experiments submitted to provide further evidence.

Direct proof of the existence of an inductive phase would be provided if, using a population of a sufficient number of cells /i.e. at least 10^7 - 10^8/ and a sensitive method, we could demonstrate directly on the cellular level that antibody producing cells do not appear for a short period after the administration of antigen, or if such cells are already present in a given number, that this number does not increase during a short period after antigen injection. We selected the plaque technique of determining antibodies which was introduced by Jerne/8/ as most suitable in resolving our problem and modified it by using agarose, a substance forming a gel medium without an anticomplementary effect /4/. The test was further modified to permit morphological and autoradiographic observations of antibody producing cells /22/.

Method: Cells isolated from the spleen or lymph nodes were washed three times and diluted to a given concentration in Parker solution with 0.5% HSA and immediately before mixing temperated at 42°C. One part of cells was added to two parts of agarose containing washed sheep erythrocytes /3 parts 1% agarose in Parker solution with HSA + 1 part 6% erythrocytes in Parker solution with HSA/ which was also temperated to 42°C. The mix-

ture of cells, erythrocytes and agarose was pipetted drop by drop from a height of about 60 cm. into a Petri dish or onto a slide. Drops with a diameter of 18 mm. and single layers of erythrocytes and lymphatic cells are formed. If the drop is dried it has a thickness of about 20 /u. The drops are incubated at 37°C in a moist atmosphere with 5% carbon dioxide for 6 - 20 hours. After incubation drops are overlayered with guinea pig complement absorbed with sheep erythrocytes. One ml. of complement used contained 20 units. The plaques are counted after incubation at 37°C for 1/2 hour, and the complement then removed by veronal buffer and the drops fixed in formalin vapour. After fixation they are washed in distilled water and dried. The preparations were stained with Giemsa-Romanowsky diluted 1 : 20 for 3 - 5 min. for the morphological determination of the lymphatic cells. For autoradiographic determinations they were covered with stripping film Kodak AR10.

We first determined the dynamics of the increase in the number of antibody producing cells after i.v. immunization of mice of a noninbred H strain /weight approximately 20 g./ with 0.5 ml. 1% suspension of sheep erythrocytes. Before the addition of antigen we find an average of 65 antibody forming cells from the total number of 10^8 cells /minimal 10, maximum 140/; 24 hours after the addition of antigen we find, on an average, double the number. This increase, however, is only found in some individuals; in others the number remains within the limits of the initial value after 24 hours and may even approximate to the minimal number of cells determined before immunization /17 to 10 cells/ 10^8/. In other animals after 24 hours we found the highest values which were found in non-immunized animals /110, 129, 159/10^8/,

in some, however, the number was double the highest values before immunization /377, 315/10^8/. A uniform increase in the number of antibody-producing cells of an average of 1,741 occurs only after 48 hours and the highest values are obtained after 4 and 5 days and after that the number of cells producing haematolytic antibodies in the spleen decreases. Since the largest number of cells forming haematolytic antibody was found on the fourth day we investigated the morphological characteristics of antibody producing cells at this time. The cells were classified according to the usual convention /10/ and we found that 25% of antibody forming cells were small lymphocytes, 47% were medium-sized lymphocytes and 27.5% large lymphocytes. Cells which would probably gradually be transformed into typical plasmocytes were seen only rarely. The greatest increase in the number of antibody producing cells was between the third and fourth day; we wished to determine whether the increase in the cell population at this time was due mainly to mitotic activity. It would also be possible for the quantitative increase to be due to unequal contact with antigen and variations in the time during which cells develop into antibody producing cells under the influence of antigen. If the first theory were correct most of the cells would incorporate labeled thymidine between the third and fourth day. We first used thymidine-H^3 but it was found that most of the cells remain deeper that 2 u below the surface in the thin layer of agarose so that labeled cells were not detected. We were more successful on using thymidine-C^{14} whose effect penetrates to a distance of 90 μ /2/. In the first experiment thymidine-H^3 in amounts of 10 μC was injected i.p. into mice three times at intervals of 6 hours between the third and fourth day after immunization. Only 10% of the centric /antibody producing/ cells in the plaques

were labeled with thymidine. In the subsequent experiment we therefore administered thymidine from the injection of antigen every 8 hours to 72 hours, i.e. a total of 10 doses /one mouse received a total of 50 μC/. In these experiments we found that the total number of labeled small, medium-sized and large lymphocytes was 25%, the large lymphocytes being labeled 100%. The same percentage, i.e. 25% was labeled in smears and in agarose if the drops were fixed immediately without incubation. Of a total of 50 examined centric cells only 40% had incorporated thymidine. If we add 10% of cells which could have acquired labelling between the third and fourth day, during which thymidine was not given in this experiment /on the basis of the preceding experiment/, we consider it to be definitely established that not all cells detected as antibody producing cells, arise by mitotic division. The experiments determining the time course of the increase in the number of antibody producing cells in mice make it clear that we are dealing with a most heterogeneous population with a diverse individual history. In nonimmunized adult mice there is not only variation in the number of cells in different individuals before immunization, but after giving antigen the rate of onset of antibody formation which is very marked already after 24 hours, shows individual differences. We consider that the individual heterogencity is the result of the diverse immunization history in adult animals and it is probably the reason for the diverse results obtained when immunizing adult animals.

On the other hand our conclusions on the time course of the onset of antibody formation are mainly based on developmental studies during ontogenesis. We have repeatedly confirmed /21/ that a real primary reaction can be expected with most antigens only in newborn animals. We therefore determined the number of

antibody forming cells in newborn rabbits from the first to the 30th day of life, both in nonimmunized animals and in newborn animals injected i.p. at different ages with 1 ml. 10% sheep erythrocytes. Shortly after birth we did not find a single cell in lymphatic tissue which formed antibodies. Only from the 20th day did we find very small numbers of cells producing antibody to sheep erythrocytes /Tab.2/ in nonimmunized rabbits. On the other hand, as found previously by Říha /11/, if sheep erythrocytes are injected into newborn rabbits, antibodies are already formed on about the fifth day of life. In accord with this, antibody producing cells are detected at this time by the plaque method /Fig.3/. It is also evident from the experiments on rabbits that the number of cells detected at the same hours after antigen injection increases continuously with age. Whether this was due to the spontaneous maturation of lymphatic tissue or to antigens of the intestinal microflora or food antigens which have chemical groups in common with the antigens of sheep erythrocytes /7/, we attempted to decide by further experiments.

To solve this question we used once again the model of sterile piglets which were fed on a nonantigenic diet, as described previously. If we determine the appearance of producing cells in normally reared piglets /which received colostrum and were reared with the mother under normal conditions/ we do not find antibody forming cells immediately after birth or on the seventh day of life, but already on the 14th day there are 9 antibody forming cells per 10^8 spleen cells,/i.e. 63 cells in the whole spleen/, in another animal of the same age we found 277 cells calculated to the whole weight of the spleen. These results stand in sharp contrast with those obtained in piglets

reared under sterile conditions on a nonantigenic diet. Up to one month, i.e. for the whole period sterile artificially fed piglets were reared, we did not detect one antibody-forming cell to sheep erythrocytes if antigen had not been given. On the other hand, if the piglets are given an injection of sheep erythrocytes /10 ml. 20% suspension of sheep erythrocytes i.p./ on the first day of life, the first antibodies can be detected after 72 hours /Tab.3, Fig.4/. Whether the number of cells detected at first /72 hours after immunization/ changes with age is difficult to decide on account of the small number of results. It would seem, however, that it is more affected by individual factors than by maturation, as we thought originally /18/.

The results show explicitly, that antibody formation does not start spontaneously during individual development if the individual is protected from antigenic stimuli. The proof that the so-called "spontaneous" development of antibody forming cells in normally reared piglets is due to antigens encountered by the animal, appears to be given by the experiment in which the number of antibody forming cells was determined in sterile piglets fed on an antigenic diet - degraded cow 's milk. On the 30th day of life 7 plaques were detected in one animal and 10 in another. In both cases, however, not a single antibody forming cell was present in the lymph nodes /Tab. 4 /. The difference between the onset of antibody formation in normally reared rabbits and sterile piglets /Fig.5/ is probably that in the first case antigen acts during development while in the second andantigenic stimulus is not present.

However, there are several conclusions that are common both for rabbits and sterile piglets. On the basis of the data obtained in 8 - 10-day rabbits it can be excluded that a previously exist-

ing cell in the organism at the time of immunization could give rise by proliferation to the number of cells determined after the negative period. If we assumed that the number of producing cells arose by proliferation, then at zero time in the rabbit there would have to be only 1/250 antibody producing cell for the total 10^8 spleen cells. One antibody-forming cell would only be present in about 10^{11} lymphatic cells, which is much more than the young organism actually contains /this amount would correspond to about 10 - 100 kg. rabbit/. The situation in piglets is similar to that in infant rabbit. In this case the onset of antibody formation is a little later and therefore in the zero hour, only 1/1000 of a cell from the entire population of 10^8 lymphatic cells would be able to take part in antibody formation. This would correspond to the presence of one cell in 10^{11} lymphatic cells, thus again a number greater than that present in a newborn piglet. The second possibility that at zero hour one cell is present which reaches the number determined in 72 hours by the very slow process of proliferation in untenable since at 24 and 48 hours the producing cells could actually be detected in both models /piglets and newborn rabbits/. If an estimation is made of the doubling time in both models the basis of experimental data obtained between 72 and 96 hours, it comes to 5 hours. On the basis of the above calculation and the very short doubling time we are of the opinion that producing cells do not arise by a process of proliferation of preformed cells. It would certainly be correct if we could provide direct proof in the newborn that the production cell which is first detected does not arise by division and does not incorporate thymidine-C^{14}. We have no such proof at present for technical reasons. In order

to detect the very small number of cells appearing in the newborn it is necessary to use concentrated cell suspension, 10^8 cells per 1 ml. If cells are added in this concentration to agarose, the area of the plaque of Ø 1 mm. contains approximately 4,000 lymphatic cells. It is therefore impossible in such a concentration to estimate what cells are centric, i.e. productive.

The second more probable conclusion which is also in keeping with the previous findings with the isolated cell transfer method assumes that for a certain time after antigen injection during the primary reaction the cell passes through the inductive phase and does not produce antibodies and that processes take place during this time which are distinct from the later process of actual antibody production. It is possible that part of the cells which are capable of responding to antigen /competent cells/ divide already in the course of functional transformation when antibody is not yet produced. However, on the basis of experiments with the incorporation of thymidine into antibody producing cells it must be accepted that at least a part of competent cells is transformed into producing cells without mitotic division.

We will attempt to treat the experimental results in the light of the basic unresolved questions of the origin of antibodies. If we consider the theory of the existence of multipotent stem cells capable of reacting with different antigens, then at the time of administration of the antigen only an unprobably small amount of stem cells would be present in a physiological condition allowing differentiation according to the type of stimulus. If we find that out of a total of 10^7 lymphatic cells only one cell would be just in the physiological

state permitting it to react to any antigen, then this hypothesis seems highly unprobable. In adult animals the ratio of the total number of cells to the producing cells shifts to the side of the producing cell. In our experiments, we have shown that this occurs under the influence of antigen. It cannot, therefore, be doubted that there is a certain form of branching process in cell population capable of responding to a corresponding antigen. It would seem, therefore, that the quantitative results obtained in newborn developing animals point to the hypothesis of the selection type in which antigen would act either on certain cells genetically preformed or arising by a mutation process and selected by the antigen. These immunologically competent cells, differentiate biochemically and morphologically during the inductive phase into antibody producing cells.

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Table 1.

Number of antibody producing cells in individual mice immunized with sheep erythrocytes

Days after immunization	Number of plaques per 10^8 spleen cells	Average number of antibody producing cells per 10^8 spleen cells	Percentage of antibody producing cells per 10^8 spleen cells
Non-immunized	10, 10, 23, 23, 59, 60, 65, 107, 140	55	0,00005
1 day	10, 17, 110, 129, 159, 315, 377	159	0,00016
2 days	356,1237, 2530, 2843	1741	0,0017
3 days	2131,5747,7833,9750	6.115	0,0061
4 days,	21300, 32600, 35500, 46200	33 900	0,034
5 days	16000, 25976, 38192	26723	0,026
7 days	9667, 16580	13 084	0,013
8 days	10050, 12870	11 460	0,011
9 days	4920, 16117	10 519	0,01
10 days	4557, 8687	6 622	0,0066
11 days	3617	3 617	0,0036
13 days	833, 3793	2 313	0,0023

Table 2.

Number of antibody producing cells /per 10^8 spleen cells/ in newborn rabbits immunized with sheep erythrocytes

Age of rabbits at the time of immunization days	Non-immunized	After immunization 24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.
3				0		
4			0 0			
5		0 0			203	
6	0			234		
7			0 0			192
8		0 0			1773	
9	0		0	335		
10		0	0 4	11		
11	0		9			
12	0	3				
13	0 0			444		
16	0					
20	8 1					
26				336, 302		

Table 3

Number of antibody producing cells /per 10^8 spleen cells/ in newborn precolostral sterile piglets reared on non-antigenic diet

Age of piglets at the time of immunization days	Non-immunized	After immunization			
		48 hrs.	72 hrs.	96 hrs	120 hrs.
1	0 0	0,0,0,0,	0,0,37,37,50	18,797,1450	719
6		0	6,53		
8	0				
13			283		
17	0				
18		0	16		
19	0,0,0,0,0				
29		0	2		

Table 4

Number of Antibody Producing Cells in One-month Old Piglets reared Sterile and Fed on Conventional Diet

		weight in g	total number of cells	total number plaques	percentage of productive cells
Piglet 3	spleen	5,17	$6,1 \times 10^8$	10	0,00000164
	lymph node	3,29	$1,5 \times 10^8$	0	0
Piglet 5	spleen	7,43	$9,5 \times 10^8$	7	0,00000073
	lymph node	3,07	$2,34 \times 10^8$	0	0

On the Role of Charge and Optical Configuration in Antigenicity

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The availability of synthetic polypeptide antigens /1, 2, 3/ permit a systematic inquiry into the structural basis of antigenicity. Knowing the chemistry of these compounds it is possible, through a study of copolymers showing only limited variations in their chemical formaulae, to arrive at conclusions concerning the role of various structural features in their antigenic function. From earlier studies /4/ it was concluded that the immunogenically important area of the molecule must be accessible to the site of the biosynthesis of the antibody, that the overall shape of the molecule does not seem to be an important factor in determining immunogenicity, and that synthetic materials with molecular weights as low as 4,000 may be good immunogens. Through appropriate chemical modification, non-antigenic macromolecules may be converted into antigens, while antigenic ones may become non-antigenic. The polypeptides investigated contain determinants of well-defined and rather narrow specificity /5/.

In this paper we describe and discuss experiments planned to elucidate whether the presence of electrical charges on a macromolecule is a minimal requirement necessary to endow it with immunogenic properties, and whether amino acids of the D-optical configuration - which normally are not present in proteins - influence the immunological response. Thus, we inquired whether the attachment of peptides containing D-tyro-

sine to a poor antigen-gelatin - may enhance its antigenicity and whether a similar attachment to a non-antigenic macro-molecule-multichain poly-DL-alanine - may convert it into an immunogen. The study was extended to macromolecules composed exclusively of D-amino acids.

Experimental

Gelatin /Gel/, U.S.P. granular, was obtained from Fischer Scientific Co., Pittsburgh, Pa. Poly-L-tyrosyl gelatin /p-L-TyrGel/, sample 240, and poly-D-tyrosyl gelatin /p-D-TyrGel/, sample 246, were prepared according to Arnon and Sela /6/ from gelatin and the respective N-carboxytyrosine anhydrides. The chemical characterization of the two polytyrosyl gelatins is given in Table 1.

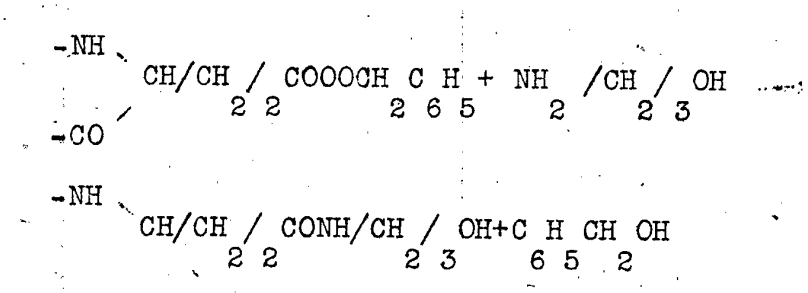
The nomenclature used in this paper for linear and multichain copolymers of α-amino acids is that described previously /4/, except that the latter defining the optical configuration of an amino acid residue precedes the abbreviation of the amino acid. The linear copolymers 42,p/L-Tyr,L-Glu,L-Ala/, and 102,p/L-Tyr,L-Glu/, were described in a previous paper /4/. The other copolymers listed in Tables 2 and 3 were prepared in complete analogy with similar polymers described by Sela et al. /4/.

The sedimentation and diffusion coefficients of some copolymers investigated are given in Table 4. For calculation of the average molecular weights, the partial specific volumes of the copolymers were computed from the partical specific volume of the amino acid residues and their proportion by weight in the polymer /4/.

A multichain copolymer was prepared in which peptides of

L-tyrosine and N^{5}-/3-hydroxypropyl/-L-glutamine are attached to multichain poly-DL-alanine. The designation of this substance is p/Tyr,Hpg/-pAla-pLys. It was synthetized as follows:

Multichain poly-DL-alanine /pAla-pLys/ was reacted as usual /4/ with the N-carboxyanhydrides of L-tyrosine and benzyl-L-glutamate. The product was reacted with propanolamine, according to Lupu et al. /8/. Under these conditions the benzylglutamate residues were converted quantitatively to N^{5}-/3-hydroxypropyl/-glutaminyl residues /Hpg residues/, according to the following scheme:



The resulting 218,p/tyr,Hpg/-pAla--pLys, had a tyrosine content of 12.5% and an average molecular weight of 30,400 calculated from a sedimentation coefficient of $s_{20,w}$ = 2.1 S, a diffusion coefficient of $D_{20,w} = 5.6 \times 10^{-7}$ cm^2/s and a partial specific volume of 0.70.

An uncharged multichain copolypeptide /designated des-p/Tyr,Hpg/-pAla-pLys/ was obtained from the above polymer upon desamination /Fig.1/. The p/Tyr,Hpg/pAla--pLys /1 g/ was desaminated with nitrous acid /500 ml 0.0625 N sodium nitrate and 120 ml 2.5M acetic acid/ for 1 hour at 37° C. The dialyzed and lyophilized product contained no amino groups /Van Slyke analysis/.

Methods of immunization of rabbits, precipitin and inhibition tests as well as physico-chemical measurements used in this

study are those described previously /4,5/. When antigens were reacted with antisera at different pH values, the following procedure was used: the antiserum was dialyzed for 48 hours against two changes of a tris-malonate buffer of the desired pH and an ionic strength of 0.15 /sodium chloride was added to 0.05M tris-malonate to adjust it to a constant ionic strength/; at the end of this period the contents of the dialysis bag were centrifuged and the supernatant was used in precipitin tests.

Antigenicity of an unchanged synthetic polypeptide

Electrical charges on antigenic determinants have been assigned a crucial role in defining the antigenic specificity, affecting the charge distribution on the combining sites of the antibodies, and contributing in an important way to the forces of the specific interaction between an antigen and an antibody. Singer /9/ has reported that in each antigen-antibody bond in several different systems there is critically involved a single pair of oppositely charged groups. Kabat /10/ has questioned the direct role of the charged group in the antibody combining site, emphasizing that charged groups cannot play a significant role in the specificity of uncharged carbohydrate haptens. In a recent paper by Wofsy and Singer /11/ it has been concluded that lysine residues cannot be vital components of the reactive sites of antibodies to either negatively charged antigenic determinants or to a neutral hapten.

Studying the effect of attachment of peptides of various amino acids upon the immunogenicity of gelatin, we observed that while some amino acids - such as tyrosine or tryptophan -

enhanced the formation of antibodies, the ionized amino acid residues /either lysine or glutamic acid/ has no significant enhancing ability /12/, though they exercised considerable influence on the specificity of antigens /6/. This has been observed also with synthetic polypeptide antigens /4, 5/. On the other hand, the attachment of mixed peptides of positively and negatively charged amino acid residues /lysine and glutamic acid/ enhanced the antigenicity of gelatin and converted the non-antigenic multichain poly-DL-alanine /p-DL-Ala-pp-L-Lys/ into an immunogen /13/. This is in agreement with the observation that, while neither poly-L-glutamic acid /14/ nor poly-L-lysine /15/ are immunogenic, some linear copolymers of L-lysine and L-glutamic acid possess the capacity to elicit antibodies /16,17/.

The qestion arose whether electrical charges are at all necessary to endow a macromolecule with immunogenic properties. Detran and levan are devoid of charges and are immunogenic in humans /10/. We have endeavoured to prepare a synthetic polypeptide that would be water-soluable, non-ionizable and immunogenic. A preliminary report of our work has been published recently /18/.

The synthetic multichain polypeptide antigen, p/L-Tyr,L-Glu/-p-DL-Ala--p-L-Lys /1,4/contains both negatively charged carboxylate ions of the glutamate residues, and positively charged ammonium ions at the termini of the polymeric side chains. The polymer p-L-Tyr--p-DL-Ala--p-L-Lys contains no carboxylate ions, but the removal of the ammonium ions by desamination with nitrous acid converts it into a water-insoluble product. In order to obtain an uncharged water-soluble analog of p/L-Tyr, L-Glu/--p-DL-Ala--p-L-Lys, multichain poly-DL-alanine

/p-DL-Ala--p-L-Lys/ was reacted as usual with the N-carboxyanhydrides of L-tyrosine and benzyl-L-glutamate, but instead of removing the benzyl groups with anhydrous hydrogen bromide to yield free carboxylate ions, the benzylglutamate residues were reacted with propanolamine to yield N^5-/3-hydroxypropyl/ glutaminyl residues. The product, p/L-Tyr,L-Hpg/--p-DL-Ala--p-L-Lys which contains many hydroxyl groups, is soluble in water and still contains ammonium ions at the termini of the polymeric side-chains. This substance is antigenic: in a typical experiment 160 /ug of antibody were precipitated when 100 /ug antigen was added to 1 ml antiserum.

The above polymer was desaminated with nitrous acid for 1 hour at 37° C. The dialyzed and lyophilized product contained no amino groups /Van Slyke/. The reaction is illustrated schematically in Fig.1. The desaminated polymer is devoid of charged groups. Nevertheless it is water-soluble, due to the introduction into the molecule of many hydroxyl groups upon the reaction with propanolamine.

The desaminated polymer elicited antibodies /300 /ug/ml serum/ in rabbits immunized in Freund's adjuvant, as checked by homologous precipitin reaction /Fig.2/. The antiserum cross-reacted to a smaller extent with the non-desaminated polymer /which carries some positive charges/, and gave only a poor cross-reaction with the highly charged p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys /Fig.2/. The last finding suggests that no conversion of hydroxypropylglutaminyl to glutamyl residues had occurred in vivo between the time of injection and the time of the "imprint" at the biosynthetic site.

It may be concluded from the above experiments that a completely uncharged synthetic polypeptide possessing the necessary immunogenic features /in this case tyrosine/ is capable

of eliciting antibodies in rabbits and, therefore, that the presence of charged groups in the molecule is not always essential for immunogenicity.

In view of the fact that a molecule completely devoid of charge may not only induce the formation of antibodies, but also precipitate with them immunospecifically, it seems of interest to elucidate in this case the nature of the forces between the antigen and the antibody, as electrostatic interactions are obviously excluded. Preliminary experiments /in collaboration with Mrs.E.Hurwitz/, comparing the pH dependence of the homologous and heterologous reactions of the desaminated polymer and of p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys with antisera to these two antigens are illustrated in Fig.3 and Fig.4.

No significant differences are observed between the two systems. In both cases the amount of precipitate decreases when the pH is either raised or lowered from the neutral region. As the pH could not influence the ionization of the uncharged antigen, the changes in the precipitin reaction upon varying the pH must have been due to intramolecular changes within the γ-globulin molecule. One could extrapolate from the above to conclude that even in the case of charged antigens and their antibodies, the dependence of the precipitin reaction on the pH does not necessarily result from changes in the ionization of the interacting groupings on the antigen or antibody.

Antigenicity of macromolecules containing D-Tyrosine and some L-Amino acids

Differences between optical isomers of organic compounds may be detected by immunological methods /19,20,21/. The distinct serological specificity of antigenic determinants of different

optical configurations has been demonstrated also for optical isomers of amino acids /22,23/. The question we asked ourselves in the study reported here was not primarily whether - and to what extent - the different optical isomers of amino acids contribute to antigenic specificity, but rather to what extent they are capable of contributing to the immunogenicity of a molecule. The amino acid tyrosie was chosen for these experiments as it was known that the attachment of peptides of L-tyrosine, or of L-glutamic acid, enhanced the immunogenicity of gelatin /12/, and converted multichain poly-DL-alanine from a non-antigen into an immunogenic molecule /1,4/.

Attachment of peptides of D-tyrosine resulted in a definite increase in antigenicity as compared with unmodified gelatin /Fig.5/. The homologous system of poly-L-tyrosyl gelatin is given in the same figure for comparison. Antibodies to p-L-TyrGel cross-reactdd only to a small extent with p-D-TyrGel, and the same situation obtained in the reverse case.

In analogy with the synthetic immonogen p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys, we have prepared a synthetic multichain polypeptide p/D-Tyr,L-Glu/--p-DL-Ala--p-L-Lys and tested whether it is able to elicit an immunoe response. In Fig.6 the homologous precipitin reaction of p/D-Tyr,L-Glu/--p-DL-Ala--p-L-Lys is given. Thus, the attachment of peptides of D-tyrosine and L-glutamic acid converted multichain poly-DL-alanine into an antigenic molecule+. The attachment of peptides of L-glutamic acid alone does not confer immunogenic properties

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+The finding was discussed in a preliminary form /24/.

on multichain poly-DL-alanine /4/. The cross-reactions of the antisera against p/D-Tyr,L-Glu/--p-DL-Ala--p-L-Lys and against p/L-Tyr,,L-Glu/--p-DL-Ala--p-L-Lys with the antigens containing tyrosine of the opposite optical configuration are illustrated in Fig.7. The contribution of the optical configuration to the specificity of these antisera is apparent also from the fact /Fig.7/ that the antiserum to p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys reacts to a much greater extent with p/L-Tyr,L-Glu,L-Ala/ than with p/D-Tyr,L-Glu,L-Ala/.

The antigenicity of the linear polypeptide p/D-Tyr,L-Glu, L-Ala/ is shown in Fig.6 and compared with that of p/L-Tyr, L-Glu,L-Ala/ in Fig.8. The respective cross-reactions are also given in Fig.8. Neither of the two antisera cross-precipitated with the polymer composed exclusively of D-amino acids, p/D-Tyr, D-Glu,D-Ala/.

It may be concluded from the above experiments that introduction of D-tyrosine into macromolecules containing also L-amino acids, and which are either poor antigens or non-antigenic, may result in an increase of antigenicity or conversion into an antigenicsimilarly to the introduction of L-tyrosine.

Immunological studies on macromolecules composed exclusively of D-amino acids

In view of the results obtained with polymers containing both D-tyrosine and L-amino acids, it was of interest to enquire whether synthetic polypetides containing exclusively D-amino acids - and among them D-tyrosine which was shown to be an immunogenic factor - may elicit an antibody response in rabbits.

We have prepared several linear and multichain polypetides composed exclusively of D-amino acids. One linear polymer, 237,

contained only D-tyrosine and D-glutamic acid. Two other linear polymers, 236 and 247, contained D-tyrosine, D-glutamic acid and D-alanine. A multichain polypeptide was prepared from poly-D-lysine and N-carboxyanhydrides of D-tyrosine and benzyl-D glutamate, and converted into 248,p/D-Tyr,D-Glu/--p-D-Lys. All these polymers are described and characterized in Tables 2,3 and 4. Analogous polymers of the L-series, which were shown previously to be potenti antigens /4/, are also included in these tables.

The above four D-amino acid polymers were tested for their antigenicity in rabbits. Each polymer was injected into eight rabbits, in an intensive course of immunization /4 injections, every 10 days, in complete Freund's adjuvant followed by 4 intravenous booster injections/. The sera were examined with the homologous polypeptides and with L-analogs, before each injection as well as for three months after the last injection, and found to be in all cases completely negative as followed by precipitin reaction. The sera pooled at the end of the immunization period were checked also by passive cutaneous anaphylaxix in guinea pigs /we are grateful to Dr. F. Borek and Miss J.Stup for this experiment/ and found to be nggative both with the polymers composed of D-amino acids and those composed of L-amino acids.

These results are in agreement with these reported recently by Gill et al. /25/ and by Maurer /26/. Gill et al. have found that a copolymer of D-glutamic acid and D-lysine did not elicit antibodies in rabbits, even though and L-copolymer of a similar composition was immunogenic /25/. Maurer has reported the lack of antigenicity of a copolymer of D-glutamic acid and D-alanine and of a copolymer of D-tyrosine, D-glutamic acid and D-alanine

/26/.

In this connection it is pertinent that, while an azobenezenarsonate derivative of poly-L-tyrosine induced delayed hypersensitivity in guinea pigs /27/, a similar derivative of poly-D-tyrosine was immunologically inert in this species /Borek, Stup, and Sola, unpublished results/. An azobenzenearsonate derivative of a copolymer of D-tyrosine, D-glutamic acid and D-alanine was also shown recently to lack the capacity to induce an immune response in guinea pigs /28/.

It is apparent that polypeptides composed exclusively of D-amino acids differ basically from those containing L-amino acids in their capacity to elicit an immune response. It is not at all clear whether this difference is due to the lack of proteolytic enzymes capable of splitting peptides of D-amino acids /this assumption would imply that at a certain stage of the immunization process such a step is necessary/, or whether the optical configuration plays a role in a specific fit with a complimentary area at some stage of the immunization. A suggestion has also been made /29/ that the non-antigenicity might be due to a continuous excess of the undigestable D-antigen.

Gill et al. /30/ have succeeded recently in eliciting in rabbits the formation of antibodies against a copolymer of D-tyrosine, D-glutamic acid and D-lysine. /We wish to thank Dr. Gill for the opportunity of reading a manuscript of his, prior to publication/. It seems, thus, that some D-amino acid copolymers may be immunogenic in rabbits. It would follow that different chemical criteria govern the immunogenic capacity of polypeptides of the L- and D-series. On the other hand, the immunological behaviour of polypeptides of opposite optical configurations might also depend on the genetic make-up of

the test animals.

Concluding remarks

We have demonstrated that a completely uncharged macromolecule - a synthetic protein model composed of amino acid residues - possessing the necessary immunogenic features may elicit antibodies in rabbits, and therefore that the presence of charged groups is not essential for immunogenicity.

Peptides containing D-tyrosine possess immunogenic capacity similar to that of peptides containing L-tyrosine, being able to convert non-antigenic molecules containing also L-amino acids into immunogens. On the other hand, all efforts to elicit antibodies to four preparations of polypeptides composed exclusively of D-amino acids were unsuccessful until now in our laboratory.

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Table 1

Polytyrosyl gelatins

A Gelatin derivative	B Percentage of tyrosine residue in the original gelatin [a]	C Percentage of tyrosine residue in the gelatin derivative	D Enrichment [b] $\frac{C - B}{100-C} \times 100$
240,p-L-TyrGel	0.21	13.5	15.4
246,p-D-TyrGel	0.21	13.2	15.0

[a] From Eastoe /7/.

[b] Calculated assuming gelatin as 100%.

Table 2

Linear copolypeptides.

No. and designation of sample	Molar ratio of N-carboxyamino acid anhydrides in the polymerization mixture	Molar ratio of amino acid residues in the copolymer	Weight /%/ of tyrosine residues in the copolymer
102,p/L-Tyr,L-Glu/[a]	1:1	1:1	56
237,p/D-Tyr,D-Glu/[a]	1:1	1:1.2	52
42,p/L-Tyr,L-Glu,L-Ala/	1:5:4	1:5.4:3:9	15
213,p/D-Tyr,L-Glu,L-Ala/	1:5:4	1:6:8:3.7	12.4
236,p/D-Tyr,D-Glu,D-Ala/	1:5:4	1:5.8:5.1	12.5
247,p/D-Tyr,L-Glu,D-Ala/	1:5:4	1:5.6:4.4	13.8

[a] Number average degree of polymerization of these samples was calculated from amino nitrogen determinations, and found to be 85 and 93 for samples 102 and 237, respectively.

Table 3

Multichain copolypeptides

No. and designation of sample	No. and designation	Weight /g/ and volume /ml/ of buffer[a]
210, p/L-Tyr, L-Glu/-p-DL-Ala--p-L-Lys	208, p-DL-Ala--p-L-Lys[c]	5; 350
211, p/D-Tyr, L-Glu/p-DL-Ala--p-L-Lys	208, p-DL-Ala--p-L-Lys[c]	2; 140
249, p/L-Tyr, L-Glu/--p-L-Lys	p-L-Lys	0.7; 70
248, p/D-Tyr, D-Glu/--p-D-Lys	p-D-Lys	0.7; 70

Amount /g/ of N-carboxyanhydrides of		Molar ratio of amino acid residues in the copolymer				Weight /%/ of tyrosine residues in the copolymer
Tyr[b]	-Benzyl Glu[b]	Lys[b]	Tyr[b]	Glu[b]	DL-Ala	
1.75	2.5	1	1.65	2.1	17.2	14.3
0.7	1.0	1	1.3	1.5	18.3	11.6
0.45	2.4	1	0.25	1.9	-	9.8
0.45	2.4	1	0.28	2.3	-	9.8

[a] Phosphate buffer /4/.

[b] D- or L- form

[c] In a molar ration of Lys to Ala of 1:19.4; average molecular weight 27,800.

Table 4

Physicochemical properties of some copolymers.

No. and designation of sample	Partial specific volume	Sedimentation coefficient /$s_{20,w}$ in Svedberg units/	Diffusion coefficient /10^7 x $D_{20,w}$/	Average molecular weight
42,p/L-Tyr,L-Glu,L-Ala/	0.63	0.9	14.4	4,100
213,p/D-Tyr,L-Glu,L-Ala/	0.61	1.6	4.65	21,400
236,p/D-Tyr,D-Glu,D-Ala/	0.63	1.75	3.4	33,800
247,p/D-Tyr,D-Glu,D-Ala/	0.63	1.5	5	19,700
210,p/L-Tyr,L-Glu--p-DL-Ala--p-L-Lys	0.70	2.25	5.5	33,200
211,p/D-Tyr,L-Glu/--p-LL-Ala--p-L-Lys	0.70	2.38	7.3	26,400

Legends to Figures

Fig.1. Schematic presentation of the desamination reaction leading to the uncharged synthetic antigen.

Fig.2. Extinction at 2800 Å of solutions in 0.1N sodium hydroxide of precipitates obtained by the addition to an antiserum to the uncharged desaminated polymers, of: o____o, the desaminated polymer; c———c, p/L-Tyr,L-Hpg/--p-DL-Ala--p-L-Lys; ▫____▫, p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys.

Fig.3. The pH dependence of the precipitates obtained in the equivalence zone upon reacting antisera to the desaminated polymer /----/ and to p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys /———/, with the desaminated polymer / O / and with p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys / O /. The antisera were brough to the desired pH values as described in 0.1N sodium hydroxide and the extinction of the solution was read at 2800 Å.

Fig.4. Data of Fig.3 normalized to give the same maximal precipitation for the two homologous systems. ----, the desaminated polymer and its antiserum; -.-., p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys and the antiserum to the desaminated polymer; ———, p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys and its antiserum; ·····, the desaminated polymer and the antiserum to p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys.

Fig.5. Extinction at 2800 Å of solutions in 0.1N sodium hydroxide of precipitates obtained by the addition of /left/ p-D-TyrGel / ○ / and p-L-TyrGel / ◑ / to the antiserum against p-D-TyrGel; /right/ p-L-TyrGel / ● / and p-D-TyrGel to the antiserum against p-L-TyrGel.

Fig.6. Homologous precipitin curves of 211,p/D-Tyr,L-Glu/--p-DL-Ala--p-L-Lys, / ● / and 213,p/D-Tyr,L-Glu,L-Ala/, / ○ /. The amount of antibody was obtained from the extinction at 2800 Å after deducting the calculated extinction of the antigen. The amount of antigen in the precipitate was obtained from radioactivity data.

Fig.7. Extinction at 2800 Å of solution in 0.1N sodium hydroxide of precipitates obtained by the addition of: A ; - 210,p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys, / ● /, 211,p/D-Tyr,L-Glu/--p-DL-Ala--p-L-Lys, / ○ /, 42,p /L-Tyr,L-Glu,L-Ala/, /◬/ and 213,p/D-Tyr,L-Glu, L-Ala/, /◮/ to antiserum against p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys- B; 211,p/D-Tyr,L-Glu/--p-DL-Ala--p-L-Lys, /◑/ and 210,p/L-Tyr,L-Glu/--p-DL-Ala--p-L-Lys.

Fig.8. Extinction at 2800 Å of solutions in 0.1N sodium hydroxide of precipitates obtained by the addition of: A; 42,p,p/L-Tyr,L-Glu,L-Ala/, /◬/ and 213,p /D-Tyr, L-Glu,L-Ala/, / ○ / to antiserum against p/L-Tyr, L-Glu,L-Ala/. B;- 213,p/D-Tyr,L-Glu-L-Ala/, / ○ / and 42,p/L-Tyr,L-Glu,L-Ala/, /◮/ to antiserum against p/D-Tyr,L-Glu,L-Ala/.

Studies on the Nature of Immunogenity Emploing Soluble and Particulate Bacterial Proteins

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Many examples are now known in nature of particulate preparations, composed wholly or mainly of protein, whose basic structure can be described as an orderly aggregation of small building blocks. A well documented example is the protein shell of tobacco mosaic virus. The basic subunit of this structure is a protein of low molecular weight. At weakly acid pH, the protein molecules will aggregate to form hollow cylinders, similar in dimension and appearance to the original virus particle from which the protein sub-units can be obtained. Other viruses seem to be constructed in a comparable fashion and it is becoming clear that this pattern of construction is widely used in nature. For example, some years ago it was shown that the flagella of certain microorganisms were also built up as linear polymers of small protein units, and this soluble protein was called flagellin /1/. It seemed to us that a situation such as this offered an opportunity to study the antibody response to natural antigens of similar chemical properties but of varying size.

Proteins of both viral or bacterial origin are known to be powerful antigens. Bacterial flagella and derivatives made from them were chosen for our study, not only for this reason but also for their ease of preparation in a relatively pure state.

Preparation of three flagella antigens /2/

Flagella were prepared from cultures of Salmonella adelaide. This organism was chosen as its occurrence in nature is rare and

high yields of flagella are readily obtained. The flagella are sheared off the bacterial bodies which are then deposited from solution by low speed centrifugation. The flagella are sedimented from this solution by centrifugation at higher speeds and are purified by means of several cycles of differential centrifugation until free low molecular weight protein. Such preparations were used as the first antigen. They are freshly prepared or after storage at -20°C.

Disaggregation of flagella can be made to occur in many ways /3/ but we have used treatment of the preparation with weak acid /0.05N-HCl, 30 min., 20°C/. Centrifugation of this solution at high speed leaves the soluble protein, flagellin, in the supernatant and deposits about one per cent of the original flagella as an acid insoluble residue. The supernatant fluid is neutralized with alkali and passed through a sterilizing filter. The filtrate is clear and colourless. Addition to this solution of a concentrated salt solution causes an immediate turbidity. A convenient way to achieve this is to bring the solution to 60 per cent saturation with ammonium sulphate and, after standing, to dialyze the solution against distilled water until salt free. Such a solution is opaque and viscous. It is termed polymerized flagellin and is used as the second antigen.

The third antigen used in these studies is the soluble protein flagellin. This can be prepared as described above directly from flagella. We, however, have chosen to prepare it by acid treatment of the polymerized flagellin, as flagellin so prepared is less likely to be contaminated with the acid insoluble material present in the flagella particle; it seems likely that the process of polymerization depends upon the specific configuration of the protein molecule so that the polymerization itself would act as a puri-

fication procedure. Such preparations of flagellin, particularly if the protein concentration is high, will readily repolymerize and to minimize the chances of this happening, flagellin solutions were injected or studied as soon as possible after preparation.

Properties of flagella, flagellin and polymerized flagellin /2/

Physical and chemical properties. When flagellin is treated with strong salt solutions, polymerization occurs and the resulting polymer, when examined in the electron microscope, is seen to consist of rod-like structures which are similar in appearance to particles of flagella. Though not examined in great detail, both preparations consist of rods of varying length and of varying curvature. This variation in size can also be demonstrated by studying the sedimentation of either preparation in a sucrose gradient. Both preparations show a distribution of proteins which extends from the top to the bottom of the gradient. When flagellin preparations are similarly examined, the protein is found hardly to penetrate the top sucrose layer. Examination of freshly prepared solutions of flagellin in an analytical rotor in the ultracentrifuge show the presence of one slowly sedimenting peak of protein. The protein probably exists as dimer with a molecular weight about 30,000, so that an average size particle of flagella might contain about 300 such units. Flagellin elutes as a single peak when chromatographed on a column of hydroxyl apatite.

Amino acid analysis of flagellin from S.adelaide shown a pattern of amino acids content similar to that for other strains /4/. No cysteine is present, and there are 3 residues of tyrosine per 215 residues of amino acids. The N-terminal amino acid is

alanine and the amount found suggests that the protein consists of a single polypeptide chain of molecular weight about 28,000. The major chemical difference between the antigens is the presence in flagella of the acid-insoluble moiety. This is rich in phosphorus and carbohydrate. A portion of the fraction, containing some phosphorus, is soluble in lipid solvents. These properties are summarized in Table 1.

Serological properties. Hyperimmune sera to each of the three antigens were prepared in rabbits. The resulting sera had high titres as tested in the bacterial immobilization test of Nossal /5/. Furthermore, each antigen in this test was capable of neutralizing antiflagellar antibody prepared against any of the three antigens. When expressed on a weight basis, flagellin was slightly less efficient as an inhibitor of anti-flagellar antibody than flagella or polymerized flagellin. Gel diffusion showed ane main common antigen and a second antigen which was present in small amounts.

Experiments to test immunogenicity

Three general types of experiments were carried out. Two of them involved the injection of antigen into the hind footpads of mature rats /Wistar, albino rats, weight 150-250 g/. The third type of experiment involved intraperitoneal injection of antigen to neonatal rats and at various intervals thereafter. When mature, these animals were challenged with antigen injected via the hind footpads. The amount of antigen injected varied over a 10^9 fold range, from 10 picogram to 10 mg. /1 mg = 10^{-3}g; 1/ug = 10^{-6}g; 1 nanogram /ng/ = 10^{-9}g; 1 picogram /pg/ = 10^{-12}g./. A minimum of three rats was used for each time point.

Rats were bled from the tail at appropriate intervals up to 36 weeks after the first injection. Serum samples were titrated for antiflagellar /anti-H/ antibody using the immobilization technique described previously. Initial dilutions were usually 1/5 /occasionally 1/2/. Pre-bleeds from rats were tested and all showed absence of circulating antibody /titre < 1/5/.

Sera were tested for their susceptibility to the action of mercaptoethanol /0.1M,. 37°C, 1 hour/. In separate experiments /6/, it was demonstrated that mercaptoethanol sensitive antibody sedimented in a sucrose gradient at a rate close to that estimated for a 19 S protein, whereas mercaptoethanol-insensitive antibody had a sedimentation coefficient of 7 S. Thus, for the purposes of this article, gross sensitivity to mercaptoethanol is regarded as indicating 19 S antibody.

Antibody response to a primary injection /6/

Flagella. For doses in an intermediate range, the characteristic finding was a lag period of 3 days followed by a sharp rise in antibody titre over a 3-4 day period. An apparent short lag period was followed by a long slow rise in titre which reached a peak at about 6 weeks. Thereafter, antibody levels stayed high or decreased only slightly for periods up to 34 weeks after the injection. With low doses, for example, 1-10ng, little or no antibody was detected in the first two weeks and only moderate titres were found thereafter. Antibody was not detected in rats receiving 10 pg of antigen but some /6/45/ formed low levels following injection of 100 pg. The lowest uniformly immunogenic dose was 100 ng. Doses higher than this merely increased the rate at which maximum titres were obtained.

Most antibody formed in the first week was macroglobulin.

Antibody present at three weeks and thereafter was 7 S. Whereas there was little evidence of a dose response relationship over the range 100 ng to 1 mg for 7 S antibody production, there is a dose response relationship for 19 S antibody production in the range of 10 ng to 10 /ug antigen. Figure 1 illustrates some of these results.

b/ Polymerized flagellin. The general features of the antibody response to polymerized flagellin were similar to those described above for flagella and are not recorded here in detail. Early production of macroglobulin was followed by high and prolonged titres of 7 S antibody. The major difference was that with lower antigen doses, about ten times as much polymerized flagellin was needed to achieve the response resulting from a given dose of flagella. Thus, 1 /ug of the polymer, compared with 100 ng of flagella, was the smallest uniformly immunogenic dose and the smallest dose causing significant 19 S antibody production.

c/ Flagellin. The response to injection of flagellin, studied over the range 10 pg to 10 mg, differed significantly from the response to the previous two particulate antigens. Figure 2 shows the antibody titres obtained following injection of doses between 10 ng and 10 /ug. Points to be stressed are: there was no dose response relationship over this range. The lowest uniformly immunogenic dose was 10 ng, but 4 out of 5 rats injected with 1 ng also responded. Peak titres were not as high as those given by the particulate antigen. After injection of 100 /ug or less, antibody was first detected more than a week later and it was mercaptoethanol resistant. Injection of flagellin in doses higher than this caused the early production of small amounts of macroglobulin. The necessity to use high concentrations of antigen to chieve this response suggested that it was due to a contaminating material,

possibly polymerized flagellin. The evidence appears to indicate that soluble flagellin per se does not cause the production of macroglobulin.

In this connection it is worth noting that injection of each antigen caused the formation of some anti-O antibody and this was macroglobulin. Titres reached a peak in one week and then slowly declined. As no dose-response relationship was found, it is difficult to compare each antigen in this respect.

Antibody response to a second injection

The general procedure followed was to inject mature rats with antigen via the hind footpad, and six weeks later to give a second injection of antigen by the same route. Blood samples for antibody estimation were taken at intervals, particularly immediately prior to and after the second injection. Using suitable controls, a rapid response and increased titres were taken as avidence of a secondary response. It was hoped to answer the following types of question. In view of the prolonged response to a primary injection of these antigens, can a second injection give enhanced antibody titres and if so, what are the relative amounts of antigen needed to achieve this? Can an enhanced response be achieved if the primary injection is a "sub-immunogenic" dose? What type of antibody is formed in a secondary response? Is flagella more efficient than flagellin in causing an enhanced response? Can flagellin enhance a response initiated by flagella? Not all these questions can as yet be answered in detail. Attention so far has been concentrated on experiments using flagella and to a lesser extent, flagellin.

The results using two doses of flagella fall into two

groups. In those animals which received an initial dose of 100 ng or more of flagella, the serum titres at the end of six weeks were already substantial /about 1000/ and were due to 7 S antibody. Challenge with a dose of flagella smaller than this resulted in titres which were in the same range as the control values. Higher titres were obtained only if the challenge dose was equal to a greater than the dose of flagella previously injected. This is shown in two experiments quoted in Table 2 /lines 4 and 5/. Because of the high initial 7 S antibody titres, we have not been able to determine how much, if any, of the new antibody formed was macroglobulin. The situation was more clear cut when the initial dose of flagella resulted in either trace amounts or no antibody titres being detected at the six week period. Table 2 /lines 1-3/ shows the results of two such experiments. The initial dose was 1 ng in each case and the second dose, either 1 ng or 10 /ug. In both experiments, there was an immediate marked response to the second injection. Furthermore, all the antibody produced up to 5-7 days after the second injection was macroglobulin. Rats given 1 or 10 pg an initial dose of flagella did not show any enhanced response upon challenge. Results obtained using 100 pg of flagella as the primary dose, have been very variable. In one experiment, challenge with 10 /ug of flagella or polymerized flagellin gave rapid, enhanced responses and the antibody formed was mercaptoethanol-sensitive. Other experiments of a similar nature did not show such an enhanced response.

Experiments using flagellin are not as far advanced as those reported above. It is clear that rats given a prior injection of flagellin /10 /ug/ will show a very marked response when challenged with a similar dose of flagellin, polymerized flagel-

lin or flagella /Table 3/. Experiments using small doses of flagellin in the initial injection followed by varying doses of flagella or flagellin are in progress.

Acquired tolerance to bacterial protein antigen. The condition of tolerance is recognized as a continued failure of the host animal to produce detectable antibody to injected antigen. The classical procedure for producing such tolerance is to inject in single or multiple doses substantial amounts of antigen into the animal either prior to or shortly after birth. Tolerance to many antigens has been achieved, but no one has yet reported complete tolerance to well defined proteins of bacterial origin. We have attempted to achieve tolerance to the antigens described above. In most experiments to date, the procedure has been to inject rats on the day of birth and at frequent intervals thereafter. After some weeks of such a course, the animals have been rested and then challenged by one or the other antigen. Control experiments involved challenge of normal 100 week old mice with comparable amounts of antigen. Table 4 shows the results of experiments in which rats were given at birth and then twice a week, 10 /ug doses of flagellin, polymerized flagellin or flagella. After 14 to 16 weeks of such treatment, no antigen was given for 8 weeks and the rats were then challenged with a signle dose of the same antigen. Sera were taken throughout this whole period and afterwards for the estimation of antibody.

The results show beyond doubt that rats can be made tolerant to flagellin and at this dose levelm remain in a tolerant state for some weeks after antigen injection has stopped. Some remain tolerant even after a further dose of antigen is given. Rats given polymerized flagellin develop good partial tolerance which stands up well to subsequent challenge. On the other hand, rats given

flagella from birth rapidly develop high antibody titres. Despite this, rats receiving 10 /ug of flagellin twice a week, when challenged with 100 /ug flagella at 11 weeks, produce a small amount /titre 10 to 160/ of macroglobulin only and this disappears rapidly from the circulation.

How much antigen is necessary to confer a tolerant state in rats? Rats were treated in the same way as above but the dose of flagellin varied from 100 ng to 1 mg twice a week. Injection of 100 ng quantities led to high antibody titres in 6 out of 7 rats. Good tolerance was achieved with the other dose levels. At the higher dose levels, particularly 1 mg, there was early production of small amounts of antibody and this was 19 S. Rats treated at the 100 /ug level until 16 weeks old remained almost completely tolerant, even when challenged at 24 and 32 weeks with 10 /ug doses of flagella. Similar experiments with polymerized flagellin are under way and have already shown that at the lower dose levels /1 ng, 100 ng/, injection of this antigen leads to high antibody production.

Finally, a further development is the determination of the amount of antigen which will induce tolerance to flagellin in the rat given as a single injection on the day of birth. Such information will allow a meaningful study to be made of the distribution and fate of the antigen in these circumstances.

Discussion

The experiments reported in this paper are part of a programme to study the induction of antibody formation by small amounts of powerful antigens. Bacterial antigens were chosen for this reason and also because a study of their behaviour has special relevance to practical immunology. Three related bacterial antigens were

prepared and characterized; flagellin, a soluble protein of low molecular weight; polymerized flagellin, a linear polymer of this protein; and flagella, the naturally occurring particle from which the other two antigens were derived. Though each preparation caontained the same protein unit, there were major differences in their size and structural organization. As an initial step in the overall programme, the effect of these variable on the ability of each antigen to cause antibody formation after a primary and secondary injection and to induce immunological tolerance, has been investigated.

A first major finding was that with each antigen, a single injection of a remarkably small amount caused the production of detectable antibody. As these proteins are bacterial products, an immunological response to such small amounts raised the question whether in fact we were studying a primary response or a secondary, enhanced response following a primary natural infection. Since specific antibody has not been detected in a pre-injection bleed of any rat, three pieces of evidence presented in the paper support the primary response hypothesis. 1. Injection of antigen results in very prolonged antibody production. 2. Injection of doses of flagella many times less than the immunogenic does does not result in immunological memory. 3. Tolerance has not been achieved with any dose of intact flagella or with micro-doses of flagella derivatives so that a natural, early infection should have yielded detectable antibody titres. Two other points are worthy of special comment. The particulate antigens produced early 19 S antibody and subsequently, high titres of 7 S antibody. In view of the chemical similarity between flagellin and polymerized flagellin, size of the antigen appears to be a major factor in this connection.

The second point is that though the particulate antigens produced higher 7 S titres than did flagellin, lower doses of the latter were more consistently immunogenic. This was particularly so when flagellin was compared with polymerized flagellin where the difference was about 100-fold. That is, in this respect one molecule of flagellin seemed to be equivalent to one particle of the polymer. It is known from autoradioagraphic studies /7, 8/ that flagellin diffuses more readily through the popliteal node than do the other preparations so a low moleculare size may be of particular importance in this respect.

The studies reported on the secondary response to these proteins are incomplete so discussion will be confined to those obtained using flagella as antigen. Two points are of particular interest. Current results indicate that an enhanced secondary response was obtained only if the challenge dose was equal to or greater in amount than that given in the first injection. This is turn suggests that to elicit such a response, the level of antigen is the lymph nodes must be substantially increased. It is known from studies with I^{131} labelled flagella that six weeks after the first injection, the level of antigen in the popliteal node is about 25% of that present in the node 24 hours after injection /9/. This may mean that an increase of about 10 fold in the amount of antigen in the node has given an enhanced response. The second point is the clear demonstration of immunological memory for 19 S antibody when flagella was used as antigen. This was shown when the initial injection of antigen was a dose near or slightly below the minimum immunogenic dose so that either no or very low levels of antibody was detected at the six week period. Bauer, Mathies and Stavitsky /10/ did not observe this effect in studies with Salmonella nor did Uhr and Finkelstein /11/ using

bacterial virus as antigen. Uhr and Finkelstein suggested antigen depletion as a possible reason for the failure of the cell responsible for 19 S antibody formation to develop persisting immunological memory, but until information becomes available on retention in lymphoid organs of the responsible antigen in the bacterial virus, the two systems cannot be compared in this respect.

The achievement of tolerance in rats to a purified, highly immunogenic bacterial protein opens the way for meaningful investigation into the mechanisms of tolerance, the fate of the protein in tolerant and normal animals and in respect to the present thesis, the antigenic complexity of the reagents. The results presented are incomplete but several observations can be made. Though complete tolerance to flagellin has been achieved in rats for extended periods, injection of polymerized flagellin over a range of doses has yielded good but not complete tolerance. Again the effect of size of the antigen is demonstrated. Can flagellin, because of its small size, gain access more readily to all the critical sites in the body? Both preparations when given in frequent large doses after birth cause the formation of small amounts of macroglobulin. An explanation for this may be the presence in both samples of trace amounts of a substance/s/ present in greater amounts in the flagella. The failure to obtain tolerance to flagella is of great interest. First, it seems not unlikely that this is of considerable biological significance with respect to the maintenance of the animal's integrity. Secondly the finding that animals tolerant to flagellin are essentially tolerant to flagella - a small amount only of 19 S antibody is formed - indicates clearly the similarity of the main antigenic grouping in each preparation. Perhaps the failure to achieve tolerance to intact flagella is due to an adjuvant action of

substances in the acid-soluble component. Further studies may show this.

In conclusion, the results reported have shown clearly different effects given by soluble and particulate forms of an antigen. Many of the questions asked however remain unanswered. One which is at the basis of all this work, and is particularly intriguin is - why is an apparently simple protein, such as flagellin, such a powerful immunogen?

Summary

1. The preparation and properties of three related bacterial antigens has been described. Flagella, obtained from Salmonella adelaide were broken down to form the soluble protein, flagellin, which inturn was readily aggregated to form polymerized flagellin. Flagellin and polymerized flagellin were chemically similar but flagella contained in addition a substance rich in carbohydrate and phosphorus.

2. Each preparation was a powerful immunogen in rats. The particulate antigens, flagella and polymerized flagellin, caused after one injection early 19 S antibody formation and then high and prolonged titres of 7 S antibody. The soluble antigen, flagellin, caused only 7 S antibody formation and lower titres than those caused by the other preparation. In contrast, a smaller dose of flagellin than of flagella or the polymer caused consistently an immunological response.

3. A second injection of flagella or flagellin caused an enhanced response. Persistent immunological memory to 19 S antibody formation was demonstrated when the primary injection of flagella was a dose near or slightly below the minimum immunological dose.

4. Complete tolerance could be obtained to flagellin and good.

partial tolerance to polymerized flagellin. In constrast, a similar course of injection of flagella caused high antibody titres in the recipient rats; it was of particular interest however, that rats made tolerant to flagellin were also essentially tolerant to flagella.

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Table 1

Properties of three related bacterial antigens.

Antigen	Size	Protein	Composition Carbohydrate %	Phosphorus %
Flagellin	Soluble, low mol.wt.	+	< 0.2	< 0.0025
Polymerized flagellin	Particulate, rods of varying length	+	< 0.2	< 0.0025
Flagella	Particulate, rods of varying length	+	1.1	0.03

Table 2

Antibody titres of the sera of mature rats given two doses of flagella.

Each value is the geometric mean of three results.

Primary		Secondary			
Dose injected	Antibody titre at six weeks /prior to second injection/	Dose injected	Antibody titre after injection		
			4 days	5 days	7 days
1 ng	5	1 ng	25		140
1 ng	not detected	10 /ug	400	1,280	
NIL	not detected	10 /ug	40	100	
100 ng	800	10 /ug	2,560		6,400
10 /ug	2,400	10 /ug	16,000		32,000

Table 3.

Antibody titres of the sera of mature rats given an injection of flagellin, and six weeks later given an injection of flagellin, polymerized flagellin or flagella.

Each value is the geometric mean of these results.

Primary		Secondary			
Dose injected	Antibody titre at six weeks	Dose injected	Antibody titre after injection 4 days	5 days	7 days
10 /ug	80	10 /ug flagellin	1,280		7,680
		10 /ug polymerized flagellin	4,000		20,000
		10 /ug flagella	2,400	5,120	

Table 4

Immunological response of rats injected since birth with flagella, polymerized flagellin or flagella.

Rats were given twice weekly doses of antigen for 14-16 weeks - Period 1.

No antigen was then given for a period of 8 weeks - Period 2.

One further injection /10 /ug/ of the same antigen was then given and antibody titres estimated 2-4 weeks later - Period 3.

Titres quoted are the geometric mean of values from 3 or more rats - the range of titres is given in brackets.

Antigen	Dose	Antibody titres Period 1 10 weeks	Period 1 14 weeks	Period 1 16 weeks	Period 2	Period 3
Flagellin	10/ug	<5	-	<5	5	20 /<5-280/
Polymerized flagellin	10/ug	80 /15-480/	20 /<5-60/	-	10 /<5-20/	15 /< 5-80/
Flagella	10/ug	1,280 /600-3,200/	-	-	-	-

Legends to figures

Fig.1. Serum antibody titres following injection of various doses of flagella /6/. Anti-H antibody was measured by a microimmobilization test. The titre refers to the reciprocal of the dilution of serum giving an end-point.

Fig.2. Serum antibody titres following injection of various doses of soluble flagellin /6/.

The Role of Peptidoglycolipids of M.tuberculosis and Related Organism in Immunological Adjuvance+

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This communication deals with the morphological and chemical components of tubercle bacilli and related organisms which can act in a Freund-type adjuvant mixture /antigen dispersed in the aqueous phase of a water-in-mineral oil emulsion/ to facilitate a variety of immunological results. All such organisms contain a wax-like entity, extractable by neutral organic solvents, which has been referred to in previous communications as wax D /1/ and which can be defined as material insoluble in 1:1 ether-alcohol mixture, soluble in chloroform, insoluble in methanol /wax B being soluble/ and insoluble in hot acetone /wax C being soluble/.

The main component of such wax D is a macro-molecular lipid of highly unique chemical structure. This molecule is readily hydrolysed by alkalis to yield 50 per cent of mycolic acid and 50 per cent of a water-soluble polysaccharide moiety. Mycolic acids are typical constituents of mycobacteria /6/ and those from human and bovine strains have the approximate formula

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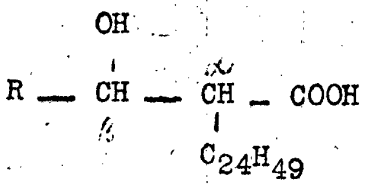
$C_{88}H_{176}O_4$ and the general structure



where R is a radical containing about 60 carbons with one oxygen function /OH or OCH_3/ and three chains /possibly 2 x C_{16-18} and 1 x C_{24-26}/. Mycolic acids from avian and saprophytic Mycobacteria /M.smegmatis, M.phlei/ are somewhat smaller molecules with R a radicle $C_{58}H_{117}$ with one hydroxyl group. Mycolic-type acids from Nocardia and Corynebacterium spp. are a good deal smaller molecules and R from nocardic acids is $C_{24}H_{45}\pm$ 3 CH_2 and for corynomycolic acid is $C_{15}H_{31}$ with the side chain at of $C_{14}H_{29}$ /5/.

The hydrosoluble portion of the molecule consists of a polysaccharide composed of the sugars D-arabinose, D-galactose and D-mannose. In wax D derived from human strain of Mycobacterium tuberculosis, but not generally in the case of other mycobacteria /bovine type of M.tuberculosis, M.avium, M.phlei, M.smegmatis/, the polysaccharide has an attached peptide composed of meso-α, α'-diaminopimelic acid /DAP/, D-glutamic acid and D- and L-alanine. Wax D from human strains of M.tuberculosis may therefore be referred to generally as peptido-glycolipids whereas wax D from other mycobacteria are normally found to be glycolipids.

The biological activity of such wax D fractions as well as whole heat-killed bacteria have been tested, in general, by five different methods for "adjuvant activity".

1. By their ability to increase precipitin levels of anti-ovalbumin, when they are mixed in the oil phase of a water-in-

oil emulsion containing ovalalbumin in the watery phase, and injected into one hind footpad of a guinea pig. An active organism or wax D therefrom will give an "adjuvant effect" so that the sera collected at 3 weeks have 3-4 mg/ml of anti-ovalbumin as opposed to the sera of controls injected with similar mixtures but lacking micro-organism or wax which have 0-0.5 mg/ml of anti-ovalbumin. The test dose of micro-organism or wax is usually 200 /ug.

2. By their ability to increase delayed type hypersensitivity. For this, the main reliance is placed on the corneal test, done with ovalbumin at 19 days after injection and read one and two days later. In a strongly positive result the cornea is greyish-white, opaque and thickened throughout the whole of its extent. Control animals usually show no detectable opacity of the cornea at 24 and 48 hours after a similar infection.

3. By their ability to induce a qualitative change as well as a quantitative change in the serum antibody in samples taken at 3 weeks after one injection. Control sera at this time which were analysed by immuno-electrophoresis show the presence of anti-ovalbumin in precipitin arcs of γ/or β_2/ mobility only /see Plate 1/. An "adjuvant-active"substance will induce an additinal component of γ_2 /or γ_{SS}/ mobility /Plate 1/.

4. By their ability to induce in a high percentage of injected guinea pigs, severe or fatal allergic disseminate encephalomyelitis at between 12 and 24 days after injection of a mixture of the test material in mineral oil containing homologous guinea pig cerebrum.

5. By their ability to induce an epithelioid and giant-cell granulomatous change locally in the injected foot, regionally in the draining group of lymph nodes and systemically as multiple

miliary granulomata throughout the lungs. The mineraloil in the injection mixture received by the control group of animals will produce a perceptible enlargement of the footpad, with some increase in foam cells /but not epithelial cells/ in histological sections. This is easily differentiated from the grossly distended foot of an animal receiving an "adjuvant-active" substance which is occupied by a massive proliferation of epithelioid cells. The regional lymph nodes show even more striking differences between test and control animals. The controls usually show a barely perceptible enlargement and some increase of foamy macrophages within the sinuses on histological section. The nodes of a positive group of animals are 4-6 times as large as those of the controls and are seen on histological section to be largely occupied by epithelioid cell granulation tissue.

On the basis of these tests for biological activity it was possible to divide wax D extracts broadly into active waxes which derived from human strains of M.tuberculosis and inactive waxes which derived from bovine strains of M.tuberculosis, M. avium, from saprophytic mycobacteria anch as M.phlei and M.smegmatis and from a Nocardia spp. /N.asteroides/. The inactivity of the latter group was something of a surprise since heat-killed whole organisms from the parent species /as well as heat-killed, human type M.tuberculosis/ were all found active at the same dose level /200 /ug per guinea pig/. The main difference between the two groups of compounds then known was that "human"-type wax D fractions all contained a peptide of meso-α, α'-diaminopimelic acid, D-glutamic acid, and D- and L-alanine, whereas, in general, wax D fractions derived from bovine strains of M.tuberculosis, M.avium, saprophytic mycobacteria and Nocardia spp. lacked such amino acids.

It is necessary to add the fact that other types of lipid extract from mycobacteria /phosphatide fraction, mycolic acid, cord-factor //trehalose di-mycolate//, methyl mycolate, phthiocerol di-acetate and waxes B and C/ were all found inactive in the above tests for biological activity /all tested at 200 /ug level/. These facts all atttested to the specific activity of wax D peptidoglycolipids. However, such wax fractions were realised to be inhomogeneuus and a method was sought for the preparation from them of purer peptidoglycolipids. Recently, fractionation of the crude wax D has been achieved by ultracentrifugation of a suspension in ether /4/. This is done in the angle head /No.40/ of a Spinco preparative ultracentrifuge at 50,000 G_{av}. This centrifugation is continued for 15, 35, 70 and 150 minutes and the deposits after each period were collected /the D_p fractions/, as well as the material which was still suspended after 150 minutes of centrifugation /the D_s fraction/. Treated in this way, wax D fractions of human strains yield 90 per cent approximately of peptide-containing D_p fractions and 3-7 per cent of a non-peptide-containing D_s fraction /Table 1/. It is to be noted that the molar proportions of the three amino acids: alanine, glutamic acid and di-aminopimelic acid remain fairly constant at 3.0 : 2.0 : 2.0. The glycine content is constant but quite variable in amount. It is also apparent that the proportion of total peptide in wax D varies progressively and is greatest in the rapidly sedimenting D_{p15} fraction.

Biological activity of D_p and D_s fractions of human type M. tuberculosis

In Table 1 those D_p fractions marked by an asterisk were all found to possess biological activity in respect of adjuvant effect

on serum antibody levels, adjuvant effect on corneal response, to induce γ_{ss} type precipitin response and in case of the total fraction of D_p $H_{37}R_vS_r$, D_{p150} of $H_{37}R_vS_r$ and D_{p70} of test to be active in the production of allergic encephalomyelitis with homologous brain. Table 2 illustrates in detail, the results with the above D_p fractions of various strains of human type M. tuberculosis /biologically active/, with the D_s fraction /lacking amino acids/ /biologically inactive/. It is further shown that the products of hydrolysis of the D_p fraction by treatment with 2 per cent alcoholic KOH, i.e. the peptide-bearing polysaccharide and mycolic acid are both biologically inactive.

Morphological studies on D_p and D_s centrifugal fractions from human-type M. tuberculosis.

The whole wax D and the centrifugal fractions derived therefrom have been studied by means of negatively stained preparations using the electron microscope /Table 3/. For this purpose wax fractions were added to distilled water, dispersed by ultrasonic vibration, transferred to the surface of a specimen grid, and allowed to dry. The dispersed wax was negatively stained on the specimen grid by a modification of the method of Brenner and Horne /2/ employing a saturated solution of sodium tungstate to which saturated phosphomolybdic acid was added until the pH was 7.0. The preparations were micrographed at a magnification of 30,000 using a Siemens Elmiskop I electron microscope at 80 kV.

Plate 2 shows a negatively stained, sonicated preparation of wax D of the human strainBrévannes which has the appearance of curved filaments of uniform width /133 Å/ which do not show obvious branching or evidence of iner subunits. The filaments were apparently not of a high degree of rigidity since sharply angled bends

could occur along their length, and they do not show frequent examples of cross fractures such as occur in preparations of flagella. The surface is presumably strongly hydrophobic since single filaments do not protrude or dissociate themselves from the edge of the clump. This is in contrast to the appearance of comparable clumps of fimbriae or flagella.

Other wax D preparations from the human strains of M. tuberculosis "Canetti" yielded filaments of very similar appearance and identical width /133 Å/. Peptidoglycolipid from the whole D_p centrifugal fraction of the human strain of M.tuberculosis $H_{37}R_vS_r$ appeared identical in all respects to the two previous /Plate 3/.

However, the D_s centrifugal fraction of $H_{37}R_vS_r$ possessed a distinct different appearance. None of the foregoing broad filaments were seen, the specimen being made up of intersecting bundles of very elongated parallel fine fibrillae 70 Å wide approximately. Groups of individual fibrils appeared to branch off from one bundle and merge with the fibrils of a neighbouring bundle. These appearances /as seen in Plate 4/ were strikingly similar to those of plant cellulose when prepared in the same manner.

Biological activity of D_p and D_s fractions of wax D from bovine type M.tuberculosis, M.avium, saprophytic mycobacteria and an atypical mycobacterium M.kansasii

The results of the centrifugal fractionation of an ether suspension of wax D from bovine type M.tuberculosis, M.avium, M.phlei, M.smegmatis and M.kansasii is shown in Table 3. In these examples, the D_s fraction makes up 45-80 per cent of the whole wax D, except for the case of M.kansasii, in which the pattern of

centrifugal fractions resembles that of human type M.tuberculosis. The yield of D_p fractions is in general much lower than from human type M.tuberculosis, and they are far more heterogeneous in respect of their component amino acids. The D_s fractions are all found to be devoid of amino acids. The D_p fractions usually contained amino acids in trace amounts and in large variety: some or all of the following being represented - aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, isoleucine, leucine and an unidentified base. Some of the D_p fractions /e.g. M.phlei D_{35}, M.avium D_{p70} and M.kansasii D_{p35} contain alanine, glutamic acid, DAP and in the case of the D_{p35} fraction of M.phlei the molar ratios ala:glu:DAP were found to be 3:1.8:1.4 which is close to that of wax D of human type M.tuberculosis.

Table 6 includes the results of biological tests of the centrifugal fractions of wax D from strains other than human type M.tuberculosis. These results show that the D_s and D_{p150} fractions of the bovine strain "Marmorek" and the D_{p35} fraction of the strain "Dupré" were all inactive in respect of adjuvant effect on serum anti-ovalbumin levels, on corneal reactivity to ovalbumin, and on the induction of allergic encephalomyelitis in guinea pigs injected with homologous brain. None of these fractions contain all of the three amino acids: alanine, glutamic acid and DAP. The D_s fraction of the bovine strain "Marmorek" is devoid of amino acids of any kind. In contrast to these findings, the D_{p35} fraction of M.phlei was found to be highly active in our tests for biological activity. This fraction caused raised anti-ovalbumin levels in serum, induced γ_{1A} and γ_{ss} precipitin arcs in the immuno-electrophoresis, induced high

levels of corneal reactivity to ovalbumin, as well as inducing encephalomyelitis in all of five guinea pigs injected with homologous brain emulsion. Moreover, the injected footpad and draining lymph nodes showed the characteristic macroscopial and microscopial changes of epithelioid formation. This fraction, as stated above, yielded on hydrolysis a qualitative and quantitative pattern of amino acids which was very similar to those of the human-type wax D hydrolysates. Also, the D_{p35} fractions of two strains of M.kansasii 4 and 21 were found to possess similar biological activity. These fractions also contained alanine, glutamic acid and DAP in molar proportions close to 3:2:2. Finally, the D_{p70} fraction of M.avium wax D was also found to give some evidence of adjuvant activity. In this case, the presence of alanine, glutamic acid and DAP in hydrolysates was established by paper chromatography, but insufficient material was available for a quantitative determination of aminoacids.

Morphological studies on wax D and centrifugal fractions from M.phlei, M.smegmatis, M.avium and bovine type M.tuberculosis

Table 6 gives the description and dimensions of wax D fractions, prepared by the method of negative staining for the electron microscope. The predominant element in the biologically inactive waxes was bundles of fine, long fibrillae 70 Å wide. This was indistinguishable from the material of the D_s fractions of human type M.tuberculosis. It bore a striking resemblance to plant cellulose and will be referred to subsequently as "cellulose-like structure". In the case of the two biologically active centrifugal fractions: D_{p35} from wax D of M.phlei and D_{p35} M.kansasii /No.21/ broad filaments of width 130 Å were found. In the case of M.phlei it is of interest that the whole wax D

/biologically inactive at dose of 200 /ug per guinea pig/ was made up mainly of "cellulose-like" structural elements.

Relative biological activity of whole heat-killed M.tuberculosis and centrifugal fractions

In the foregoing experiments an absolute correlation was noted between biological activity and the presence of a peptide of alanine, glutamic acid and di-aminopimelic acid. The content of peptide components is highest in the rapidly sedimentating fractions from ether and it was of interest to determine if these /e.g. D_{p70}/ had higher levels of biological activity as compared with the slowly sedimentating fractions /e.g. D_{p150}/.

Table 7 records the incidence of experimental allergic encephalomyelitis in guinea pigs injected with 16 mg of a saline suspension of homologous cerebral tissue and 100, 300, 900 /ug of two different centrifugal fractions from human type M.tuberculosis, as well as whole bacterial cells, which were added to the mineral oil of a water-in-oil emulsion. A single injection was given into a footpad. For the purpose of recording the incidence of encephalomyelitis, total paralysis of both hind limbs had to be present. This was sufficient to render both hind limbs useless for forward or backward progression. The brain and spinal cord were removed, placed in fixative, and examined histologically at a later date.

The results of Table 6 are sufficient to suggest that the rapidly sedimentating D_{p70} fraction is about ten times more active than the slowly sedimentating D_{p150} fraction, and is several times more active than heat-killed, dried, whole M.tuberculosis bacilli. The lowered activity at the higher dose level /900 /ug of D_{p70} and whole bacilli/ is a characteristic finding and recorded else-

where.

Morphological and chemical structure of biologically active peptido-glycolipid fractions of Mycobacteria

Investigation of the surfaces of various Mycobacteria: human type M.tuberculosis, strain $H_{37}R_a$, bovine type M.tuberculosis BCG and M.phlei by the same negative staining technique in the electron microscope has revealed the presence on the surface of the cell wall of these organisms of fibrils /133 Å average width/ /Plates 5 and 6/ which bear a striking resemblance to the broad fibrils /133 Å wide/ which appeared in electron microscopy as the main component in biologically active wax D fractions.

In regard to chemical structure, all of the wax D fractions with adjuvant activity in its various aspects have been shown to be peptido-glycolipids and to show a striking analogy, at least in the hydrosoluble part of the molecule, with the muco-peptide element of the cell wall of Gram-positive bacteria /9/. The muco-complex or "basal" cell wall is characterized by the following organic constituents: hexosamine, comprising glucosamine, muramic acid and sometimes galactosamine; peptide made up of alanine, glutamic acid and either diaminopimelic acid or lysine; usually also a polysaccharide containing not more than four different sugar residues. All these elements are present in the biologically active peptido-glycolipids previously described. Such wax D peptido-glycolipids also resemble bacterial cell walls in their having "unnatural" amino acids of the D-configuration: D-alanine and D-glutamic acid, which are also restricted in nature to the cell walls or extra-cellular products of bacteria such as various antibodies and slime layers.

The main component of the D_S fractions is the biologically inactive glycolipid which is composed of mycolic acid joined by an ester to a polysaccharide composed of D-arabinose, D-galactose and D-mannose./amino sugar component is very low or absent/. This material has such a resemblance morphologically to cellulose that it must be considered as a likely structural component of the cell wall. Wax D peptido-glycolipids differs from this material in its extra possession of hexosamines /muramic acid, glucosamine or galactosamine/ and amino acids. The most likely role of these would be to form cross links between the polysaccharide linear polymer elements. Possibly according to the degree of cross linking and mycolic acid contribution, such material could assume the structure either of the chloroform soluble, surface broad fibrils of peptido-glycolipids or the basal muco-complex of the cell wall.

Summary

The adjuvant active material of various mycobacteria is extractable in the wax D fraction. Wax D from human-type M. tuberculosis is separable into two morphologically and chemically distinct fractions. These consist of peptido-glycolipid in the configuration of worm-like cylinders 133 Å wide in electron micrographs and glycolipid, which appears as cellulose-like fibres 70 Å wide. The previous discovery of biological activity in wax D from human-type M.tuberculosis is explained by the high content /over 90 per cent/ of peptido-glycolipid in this. The low adjuvant activity of the wax D from other types of mycobacteria is explained by the fact that glycolipid forms the main bulk of this material, and peptido-glycolipid resembling that from human-type M.tuberculosis is present in low proportion and

irregularly. By the technique of centrifugation in ether it is possible to divide all wax D fractions investigated into two distinct morphological entities, one with and the other lacking biological adjuvant activity. These correspond to peptido-glycolipid and glycolipid components respectively.

Chemically the peptido-glycolipid is analogous to the structurally important elements of the bacterial cell wall or muco-complex. Morphologically it appears to correspond with a network of projections /133 Å wide/ which ramify over the surface of intact mycobacteria. Since bacilli which are thoroughly extracted with neutral solvent still possess adjuvant activity it is presumod that another morphological element, presumably the muco-complex of the bacterial cell wall, also possess such biological activity. It can be shown that treatment of mycobacterial cell walls with alcoholic-KOH results in loss of activity, presumably since ester-bound mycolic acid is split off.

On the evidence given, both mycolic acid and peptide components of the peptido-glycolipid appear essential for activity. Possibly the former is essential for the avid uptake by macrophages. Possibly the high content of unnatural or D-amino acids of the peptide confers resistance to attact by mammalian enzymes.

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Table 1

Ultracentrifugation of wax D from human strains of M.tuberculosis. Yield and amino acid composition /molar ratios/.

Strain	D_{p15}	D_{p35}	D_{p70}	D_{p150}	Total D_p	D_s	Total	Amino acid compositon of D_p fractions
Canetti	17.6[a]	43.2[a]	6.8	11.5	79.1	6.9	86.0	Ala/3.0/Glu/1.8/ DAP /1.7/ Gly /0.15-0.85/
$H_{37}R_vS_r$	6.6	30.7	36.1	9.6[a]	83.0[a]	3.0[a]	86.0	Ala/3.0/ Glu /1.9/ DAP /1.6/ Gly /0.4/
$H_{37}R_a$	-	-	-	-	90.0	5.0	95.0	Ala/3.0/ Glu /1.8/ DAP /1.6/ Gly tr.
Brévannes	49.8	26.2	14.5	4.6	95.1	3.6	98.7	Ala/3.0/ Glu /1.9/ DAP /1.7/ Gly tr.
Test	13.6	37.3	37.2[a]	4.2	92.3	4.8	97.1	
Peptide proportion of wax D /Canetti/	6.34	5.75	-	1.95	-	0		

[a] Denotes material which was tested for biological activity.

Table 2

Effect of wax D fractions of human type M.tuberculosis on serum anti-ovalbumin precipitin levels, delayed-type hypersensitivity /corneal test/ and induction of encephalomyelitis by homologous brain.

No. in group	Fraction and dose	Anti-ovalbumin /ug N/ml serum Mean	Range	Corneal reaction at 48 hours	Encephalomyelit
5	200 /ug.total D_p wax D of human type "$H_{37}R_vS_r$"	510	341-621	3, 2.5, 2.5, 3	4/5
5	Contemporaneous controls	153	24-303	0.5, 0, 0, 0, 0	0/5
5	200 /ug D_s of human type "$H_{37}R_vS_r$"	81	60-175	0, 0, 0, 0, 0	0/5
5	Contemporaneuous controls	40	0-112	0, 0, 0, 0, 0	0/5
4	200 /ug hydrosoluble moiety from D_{p35} of human type "Canetti"	95	57-152	0, 0, 2, 0	0/5
4	Contemporaneous controls	79	52-124	0.5, 0, 0, 0	0/5
4	200 /ug mycolic acid from human type "Canetti"	100	47-152	0, 0, 0, 0	
4	Contemporaneous controls	88	57-139	0, 0, 0, 0	
4	200 /ug D_{p35} Human type "Canetti"	366	330-421	3, 3, 2, 2	
4	Contemporaneuus controls	122	34-232	0, 0, 0, 0	
4	200 /ug D_{p150} human type "$H37R_vS_r$"	440	350-600	3, 3, 2, 2	
4	Contemporaneous controls	122	34-232	0, 0, 0, 0	
4	200 /ug D_{p70} human type "Test"	389	304-590	3, 3, 3, 3	
4	Contemporaneous controls	122	34-232	0, 0, 0, 0	

Table 3

Negative stain preparations of whole and centrifugal fractions of wax D from human strains of M.tuberculosis.

Reference number	Wax D or centrifugal fraction	Result and dimensions
WL 52	Whole Wax D: Canetti	Broad filaments: 133 Å wide
WL 53	Whole Wax D: Brévannes	Broad filaments: 133 Å wide
WL 54	Centrifuge fraction: Whole D_p: $H_{37}R_vS_r$	Broad filaments: 130 Å wide
WL 55	Centrifuge fraction: D_s: $H_{37}R_vS_r$	Fine long filaments: 70 Å wide.

Table 4

Ultracentrifugation of wax D from bovine strains of M.tuberculosis, M.avium, saprophytic and atypical mycobacteria.

Bacterial strain	D_{p15}	D_{p35}	D_{p70}	D_{p150}	D_s	Total
M.tuberculosis, bovine type, BCG	2.3	0	0	36.7	54.9	93.9
M.tuberculosis, bovine type, Marmorek	0	2.4	3.2	34.3[a]	44.8[a]	84.7
M.tuberculosis, bovine type, Dupré	5.5	2.9[a]	0	12.5	78.9	99.8
M.avium, No.802	2.1	0	34.2	0	48.5	84.8
M.phlei	3.5	4.5[a]	2.9	8.1	69.7	85.8
M.smegmatis	0	0	8.3	12.9[a]	78.4	99.6
M.kansasii, No.4	6.7	57.8[a]	44.3	4.0	0.8	93.6

Table 5

Effect of centrifugal fractions of wax D from bovine type M.tuberculosis, M.avium, saprophytic Mycobacteria and M. kansasii on serum precipitin levels, delayed type hypersensitivity to ovalbumin /corneal test/ and induction of encephalomyelitis by homologous brain.

No. in group	Fraction and dose	Anti-ovalbumin gN/ml serum/ Mean	Range	Corneal reaction at 48 hours	Encephalomyelitis
4	200 g D_{p150} M.tuberculosis, bovine "Marmorek"	96	16-242	0.5, 0, 0, 0	
4	Contemporaneous controls	79	52-124	0.5, 0, 0, 0	
4	200 g D_s M.tuberculosis, bovine "Marmorek"	96	16-242	0.5, 0, 0, 0	
4	Contemporaneous controls	79	52-124	0.5, 0, 0, 0	
4	200 g D_{p35} M.tuberculosis, bovine "Dupre"	55	0-130	1, 0, 0, 0	
4	Contemporaneous controls	122	34-232	0, 0, 0, 0	
5	200 g D_{p70} M.avium 802	166	13-284	2.5, 0, 0, 1, 3	
4	Contemporaneous controls	122	34-232	0.5, 0, 0, 0	
5	200 g D_{p35} M.phlei, saprophytic	359	278-414	2, 3, 3, 2, 2	5/5
5	Contemporaneous controls	39	0-112	0, 0, 0, 0, 0	0/5
4	200 g D_{p150} M.smegmatis, saprophytic	102	45-142	0.5, 0, 0, 0.5	
4	Contemporaneous controls	79	52-124	0.5, 0, 0, 0	
4	200 g D_{p35} M.kansasii No.4 atypical photochromogenic	321	227-383	3, 2.5, 2, 2	
4	Contemporaneous controls	122	34-232	0.5, 0, 0, 0	

Table 6

Electro-microscopical morphology of wax D from M.phlei, M.smegmatis, M.kansasii.

Reference number	Wax D or centrifugal fraction	Result and dimensions
WL 25	Wax D of M.phlei	"Cellulose" type; fine, long fibrils, 70 Å wide
WL 55	Fraction D_s from M.phlei	"Cellulose" type; fine, long, fibrils, 70 Å wide
WL 56	Fraction D_{p35} from M.phlei	Broad filaments, 133 Å wide
WL 29	Wax D of M.smegmatis	"Cellulose" type; fine, long, fibrils, 70 Å wide
WL 82	Fraction D_{p35} M.kansasii No.4	Broad filaments, 133 Å wide
WL 44	Wax D bovine type M.tuberculosis strain "Marmorek"	Partly "cellulose" type; fine, long, fibrils, 70 Å wide with some broad filaments, 130 Å wide.

Table 7

Relative biological activity of whole bacilli /heat killed/ and centrifugal fractions D_{p70} and D_{p150} in the production of encephalomyelitis in guinea pigs.

Centrifugal fraction	Dose /ug/	Incidence of encephalomyelitis	P^a_{50} dose
D_{p70} /WL 80/	900	2/5	
M. tuberculosis human	300	5/5	50
	100	4/5	
D_{p150} /WL 79/	900	5/5	
M. tuberculosis human	300	2/5	500
	100	1/5	
Whole	900	3/5	
M. tuberculosis human	300	5/5	200
	100	0/5	

[a]Dose calculated to cause paralysis in half of the animals.

S-sulphonated Anti-dinitrophenyl Antibodies. Some Specific Features of the Interaction between Isolated H and L Subunits.

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The existence of an active site on the protein molecule composed of several types of subunits gives rise to the question as to which type is the carrier of the active site and whether it is one type or whether more types participate. If we consider antibodies, which are known to made up of two types of subunits, H and L, it is clear that there are three basic possible answers to this question. The carrier of the active can be subunit H, subunit L or both of these subunits can participate in its formation. These basic alternatives comprise within themselves further cases expressing the possibility of the interaction of several subunits of the same type or the differentiation of the role of carrier of specificity and activator. It is not difficult to consider these possibilities and to discus. their consequences, but such considerations do not of themselves lead to any advance in the solving of the problem: no possibility can a priory be designated as impossible. In 1961, on the basis of the electrophoretic analysis of different guinea pig antibodies, Edelman et al. /1/ expressed the opinion that antibody specificity is given by the L chain. This opinion, still not supported experimentally by testing activity, was maintained by Edelman and Benacerraf in a further publication /2/ in 1962, in which other possibilities are discussed, e.g.

the interaction of two different or similar L chains. Before this in 1962 Porter /3/ has already published experimental results placing the antibody active site into the H chain. Our work published at the beginning of 1963 /4 - 7/, however, pointed to a different conclusion. No appreciable activity was found in any of the isolated chains but reappeared after mixing them. On mixing H and L chains, the activity of antibodies of different specificities was also covered but only partically and the hybrid possessed a specificity corresponding to the H chain used. From these results we reached the conclusion that the participation of both H and L chains is needed for the formation of the active site and that specificity is determined by the H chain, whereas the L chains may be partly replaced in their function by the L chain from another antibody. A very important finding was that isolated H and L chains are capable of mutual interaction even without the formation of covalent bonds. The model of equine antitoxin used, however, was not ideal from all aspects. Although the recovered activity was equal to the activity of the preparation which was subjected to the same treatment without separation of the chains, this activity was very low, maximally 5% of the protein in the sample was active. Apart from that the determination of activity by means of an immunosorbent did not permit the extension of the study to the measuring of the physico-chemical parameters of the interaction.

For the further study of this problem we chose antibodies to the dinitrophenyl group, whose preparation holds out the possibility of a high yield with a relatively narrow specificity. Big animals-bulls and pigs - were chosen as immunization objects, partly because the amount of material obtained was sufficient

for a large series of experiments and partly because it permits working with antibodies from one individual so that some aspects of heterogenicity can be excluded. The immunizing antigen was gamma-globulin, modified to a high degree by 2,4-dinitrobenzene sulphonic acid /8/, was given in adjuvant. Bulls were immunized with pig gamma-globulin, pigs with bovine gamma-globulin. In pigs a single immunization with 100 mg. antigen 2 - 3weeks before slaughter proved satisfactory. The concentration of antibodies obtained was 0.1 - 1.0 mg./ml. In bulls an antibody concentration of 0.1 - 1.0 mg./ml. was attained two weeks after one dose of 200 mg.antigen per animal. Repeated doses of antigen in adjuvant always produced approximately the same increase in the concentration of antibodies so that blood could be collected after each reimmunization containing antibodies in concentrations which were suitable for isolation.

We considerably modified the method used by Farah et al. /9/ for the isolation of antibodies. After absorbing the antibody to the carrier protein, antibodies to the hapten were precipitated by dinitrophenylated gamme globulin and the washed precipitate was dissolved with 0.05 M dinitrophenol in 0.1 M phosphate buffer, pH 7, containing 0.5 NaCl. The solution containing antibody, antigen and hapten was applied on a DEAE-Sephadex column equilibrated with the same buffer without dinitrophenol. Antigen was adsorbed firmly on the top of the column whereas antibody ran through without adsorption and was thus separated from the dinitrophenol whose zone moved down the column very slowly. The antibody yield on the DEAE-Sephadex reached up to 75% of antibodies determined in the serum by the quantitative immunosorbent method. In other cases the precipitate was dissolved with the buffer which contained

1×10^{-4} M ε-DNP-lysine. Antibody was separated from hapten and antigen in the same way as from dinitrophenol. However, it was only possible to remove excess hapten, a portion of the ε-DNP-lysine remained bound to the antibody.

The chemical character of bovine antibodies is 7 S gamma-globulin as determined by starch gel electrophoresis and by immunoelectrophoresis. At the same time the extent of electrophoretic heterogeneity is less than that of 7 S gamma-globulin. It displays no indication that the antibody could be present in two types, as was reported., for example, in guinea pigs /10/. The average association constant of the interaction of bovine antibodies with ε -DNP-lysine was within the range of 10^5 to 10^6 litre/mole in various preparations. It was determined by equilibrium dialysis and by polarography. The polarographic mthod of determining the association constants of antibody to the dinitrophenyl group was found very useful. ε -DNP-lysine gives a well measureable polarographic double-wave. By adding gelatine to the analysed mixture the height of the ε-DNP-lysine wave is made directly proportional to the concentration of free ε -DNP-lysine bound ε-DNP-lysine being not registered polarographically. The great advantage of the polarographic method is that it gives results in the course of a few minutes.

The breaking down of the disuphide bond was done by S-sulphonation. On the basis of previous work on the reactivity of the disulphide bonds /11/ and on the nature of the intermediates of limited cleavage of disulphide bonds /12/ we worked at a pH 8.6, i.e. under conditions in which 8 disulphide bonds in the molecule are cleaved in pig gamma globulin. As the second alternative we selected S-sulphonation at pH 5.7, at which fewer disulphide bonds are split and a greater percentage of higher

subunits - intermediates of the reaction - which contain both H and L chains /12/, remain in the reaction product. The electrophoretic pattern on starch gel shows that in pig antibodies there is no difference in the position of the zones or in the relative amounts between nonspecific gamma-globulin and antibodies. In bovine antibodies the mobility of the two types of chains is somewhat less than in nonspecific gamma-globulin, the width of the diffusion zone of the L chains is smaller and in addition to this there is more incompletely split material in the resulting preparations /Fig.1/. S-sulphonation was also carried out in the presence of excess of dinitrophenol or ε-DNP-Lysin. As seen from Fig.2 the hapten bound on the antibody does not affect the splitting of interchain disulphide bonds. The electrophoretic pattern of subunits is the same as after S-sulphonation in the absence of hapten. Fig.3 confirms that neither on S-sulphonation at pH 5.7 is the character of antibody subunits significantly different from the character of subunits of nonspecific gamma-globulin.

None of the electrophoretic patterns obtained in urea starch gel at pH 3 - 4 showed banding of the L chain S-sulpho antibodies which were described by Edelman et al. /1/ in pure guinea pig antibodies. Banding did not appear in bovine or pig antibodies even when they had been subjected to reduction and alkylation, exactly according to the procedure described by Edelman and Poulik /13/. The large amount of antibody from single bulls made it possible to examine the heterogenity of L chains coming from different individuals by electrophoresis in starch gel at pH 8 /14/, where sharp bands actually appear. Fig.4 shows that L chains of antibodies yield a smaller number of bands and that there is hardly any difference among individuals.

As in the previous work Sephadex G-100 in 0.05 M formic acid with 6M urea was used for the separation of subunits. Fig.5 shows the distribution of protein after the separation of bovine S-sulpho gamma-globulin and antibody on a Sephadex column. The peak of higher subunits appears first overlapping partly with the peak of the H chains, which is followed by a well resolved peak of L chains. Under the conditions of separation any bound hapten was completely released from the protein and moved as a peak very distant from the L chains. The fractions containing subunits which were taken into the experiment were pooled and concentrated with dry Sephadex G-25. The subunits were then transferred into 0.1 M borate buffer pH 8.0 using a Sephadex G-25 column. All subunits were soluble in this buffer.

Binding activity was expressed by the quantity r denoting the number of moles of hapten bound by a mole of protein, and for comparison, in cases not stated otherwise, the molecular weight of protein was taken to be 160,000, i.e. even in the case of isolated H or L chains which actually have a lower molecular weight. r was always measured at a concentration of free ε-DNP-lysine of 1×10^{-5} M and at a temperature of +4°C. The binding capacity of nonspecific bovine gamma globulin and its subunits corresponded under the given conditions to r = 0.1. The expression of nonspecific binding capacity on the basis of weight was found to be most suitable since the binding capacity of the L chains or from that of their mixture, the nonspecific binding of ε-DNP-lysine on bovine gamma globulin antibody and its subunits was therefore eliminated from the results of measurements by deducting the value 0.1 from the activity of antibody sub-

units expressed as r.

We first determined the value of r for native antibody and for S-sulphonated antibody without separation of the chains. Table 1 shows that S-sulphonated antibody which was subjected to the action of the separation medium has its activity, expressed as r, decreased to about one half of that of its original activity. In further work we investigated whether there was any residual binding activity in the individual subunits arising from S-sulphonation at pH 8.6 and 5.7. Higher subunits which do not differentiate one from another on the Sephadex column were taken together for analysis. In Fig.6 the r of the individual subunits is denoted over the corresponding fractions. They represent the average of at least three experiments and are corrected for nonspecific adsorption. Among the subunits of S-sulphonated antibodies, it is only the higher subunits whose binding activity is preserved after separation in considerable degree. Free H and L chains taken without mutual contamination have no significant binding activity demonstrable by equilibrium dialysis.

In several pilot tests we confirmed that activity was covered by mixing H and L chains, in the same way as in equine antitoxin where it was first observed by us. We therefore undertook a study on the conditions and quantitative relationships of the recovery of antibody activity. We sought the conditions promoting the recombination of the subunits. We have found that the final activity was not influenced by whether H and L chains were mixed in acid separating solution, after neutralization or even after transferring the separate chains into borate buffer. Recombination of the chains thus occurs in a neutral medium in

the absence of urea and the history of the subunits before this time has no significant effect on recombination.

The average of 10 experiments showed that the recovered activity corresponded after deducting nonspecific adsorption to $r = 0.68$. If we wish to know the yield of recombination of chains we must compare the recovered activity with the activity of S-sulpho antibodies which were not subjected to separated chains.

Table 1 shows that preparation corresponding to this requirement possesses $r = 0.86$. The recovery of binding activity after separation and recombination of the chains was thus almost 80%. If, however, we wish to compare the activity of recombined complex with the activity of native antibody, the recovery of activity is about 40%.

We mixed H and L chains in different weight ratios keeping the total weight concentration constant. If the chains recombined in stoichiometric relationship and united with a bond whose firmness was comparable with the covalent bond with which they were bound in the native molecule, the activity found in our experimental set-up would follow the broken curve shown in the upper part of Fig.7 with a sharp maximum corresponding to the composition by weight of H and L chains. In actual fact, the activity of the mixture was different as shown in the lower part of the Fig.7. The activity has no sharp maximum but is almost constant in a wide range of ratios of H : L. We shall attempt to give an explanation of this parabolic shape of the curve on Fig.7 when explaining other findings.

In our next experiments we wanted to elucidate the role of hapten in the formation of complexes of H and L chains, i.e. the determine whether the active complex of chains is formed up

in borate buffer in absence of hapten or only after its addition. It is obvious that equilibrium dialysis cannot provide an answer to this question. The measurement of increase in the binding of hapten by dialysis stopped after given time intervals gave results which were not reproducible although we tried to standadize all procedures. Polarography proved much more suitable here, because instantaneous states of decrease of free hapten can be followed. We have investigated the course of the decrease of free hapten which was added in a concentration of 1.4×10^{-5}M on the one hand to the native antibody, on the other hand to the mixture of H and L chains in borate buffer prepared fresh and to a mixture which had been left to stand for 20 hours. We confirmed that native antibody binds hapten so quickly that at the moment of recording the first data, i.e. several minutes after the addition of hapten, equilibrium has already been reached and the height of the wave of free hapten shows no further change. The curve on Fig.8 shows that ε-DNP-lysine passes into bound form very slowly with recombined complexes of H and L chains. In our conditions it took at least 50 hours. Hapten reacts with a greater initial velocity with preparations in which the chains were mixed and incubated for a long period before the addition of hapten. We assume that this finding suggests that H and L complex with a specific active site is formed in the mixture of chains before the addition of hapten.

The determination of specificity can also be studied on antihapten antibodies. We combined chains from bovine antibody on the one hand with chains of nonspecific bovine gamma-globulin, on the other hand with chains of pig antibody to the same determinant group. No hybrid of bovine and pig antibodies, i.e. neither

the mixture of bovine H and pig L, nor the mixture of pig H and bovine L, bound ε-DNP-lysine specifically. No aspect other than this binding was investigated so that we cannot say whether the chains recombined at all or whether possible recombined complex was inactive. The activity of hybrids of specific and nonspecific chains are given in Table 2. Hybrids of specific H chains with nonspecific L chains also attain a certain activity, even though lower than the complex with specific L chain, whereas in the hybrid of specific L chain with nonspecific H chain activity is not significantly increased.

In order to appreciate the role of the individual chains in determining specificity and in the formation of the active site, we elaborated an auxiliary concept, in which we considered alternately one chain as a sort of "inactive form" of the active site carrier and the second chain as its "activator". This concept empowers us to evaluate the activity by the quantities r_H and r_L which represent the number of moles of ε-DNP-lysine bound to a "mole" of chain considered as the inactive form. It is 107,000 g. for H and 53,000 g. for L chains in this ratio the mole of antibody, i.e. 160,000 g. is composed. In cases where H and L chains are mixed in the same proportions as present in the native molecule, the quantities r_H and r_L are equal to the quantity r calculated in relation to total protein.

The quantities r_H and r_L calculated for the data in Fig.7 increase in direct proportion to the presence of excess of the component considered as the "activator" in the mixture. An adequate explanation for this finding is evidently the fact that the active complex of H and L chains cannot be compared in its firmness with native antibody since it shows considerable dis-

sociation. It follows from this, of course, that the quantity r is not suitable for expressing recovered activity since it will be dependent on the concentrations of protein components and that it will be necessary to choose a different way of evaluation in further experiments. A confirmation of this explanation can only be given when data on the binding of hapten on complex H and L chains in a wide range of protein concentrations will be available. The data from such an experiment would also finally decide how much of the decrease of activity found in S-sulpho antibodies or in complex of antibody chains in comparison with native molecule /see Table 1/ should be ascribed to irreversible inactivation and how much is due to the fact that in the case of the complex we are dealing with a multicomponent dissociating system. It will also be necessary to obtain experimental data on the basis of which a standpoint could be taken up towards the alternative explanation of the parabolic shape of the curve in Fig. 7, that various active complexes of H and L chains are formed in which the ratio of H : L is not in all cases the same as in the native molecule.

The different role of the two chains in determining specificity cannot be displayed examining the combination of chains originating from the same antibody. This question can only be decided by determining whether and to what degree some chain can be substituted by a chain from nonspecific gamma-globulin. The different role was best displayed where excess nonspecific chains were added /see Table 2/. These experiments show that the H chain, considered to be the "inactive form" can be activated by excess nonspecific L chains to a greater degree than by an adequate quantity of specific chains. The reverse conception, however, fails: L chain as the "inactive form" is not "activated" by nonspecific H chains. Increased activity could not be demonstra-

ted as against the residual activity of the L chain alone. The creation of a concept of H subunits as "inactive forms" and L chains as "ativator" for the illustration of the unequal importance of H and L chains in the active complex does not mean that we consider the synthesis of antibody to take place via an inactive form. The signifiance of H chains can be compared with the significance of inactive forms of other biologically active substances. The significance of the L chain is best understood by the term "activator", the effect of its addition can best be compared with the activation of enzymes by the addition of various coenzymes, metal ions etc. It remains to explain why the effects of L chains of nonspecific gamma-globulin are not quantitatively identical with the effect of chains of antibody. L chains are undoubtedly heterogeneous. It appears that the extent of the heterogeneity of the L chains of antibody is less than that of the L chains of nonspecific gamma-globulin. Let us assume that the ability to "activate" specific H chains is a property of only certain L chains from a whole set which is found in nonspecific gamma-globulin or that this ability is not present in all L chains in the same degree. At the same time it need not be assumed that the ability to "activate" the H chain of a certain specificity is necessarily related to specificity to hapten. The "activating" effect of L chains of nonspecific gamma-globulin is therefore less than that of L chains of antibody. If added in excess, however, "activation" takes place to the same or to a higher degree.

Using the model of antibody to the dinitrophenyl group we succeeded in confirming all the findings which we were the first to establish on the model of equine antitoxins. We confirmed that isolated subunits H and L do not display any appreciable binding activity, and that residual binding activity is only attributable to

to higher subunits - incompletely split antibodies. The finding that the binding activity is recovered by mixing H and L chains although restitution of the covalent bond between them did not occur, was convincingly confirmed. Finally, it was confirmed the H chain plays the decisive role in determining the specificity of the recombined complex.

The model working with two easily soluble high molecular components /H and L chains/ and a low molecular hapten made it possible to study interactions by physico-chemical methods. We found that the interaction of H and a L chains takes place in a neutral medium even without the presence of hapten and so slowly that the reaching of equilibrium is a question of days. Findings on the role of the chains in determining specificity were supplemented by a number of findings. L chains obtained from nonspecific gamma globulin can produce the same "activation" of the antibody H chain as L chain from antibody, if the nonspecific L chain is added in excess. On the other hand, the L chain from antibody cannot "activate" nonspecific H chains even when added in excess. Nor did we succeed in obtaining active hybrids from the chains of bull and pig antibodies even when the antibodies were directed against the same determinant group. The new data led us to the hypothesis of the mechanism of interaction of H chains carrying potential specific binding activity with heterogeneous mixtures of L chains from which only some provide an active complex. It would appear at the same time that this complex is considerably dissociated. Sufficient variants of the experiments have not yet been made to confirm this hypothesis but the results obtained to date show that S-sulpho subunits of antibody to the dinitrophenyl group are very suitable

for further study and we hope that we shall succeed in detecting a series of further properties of antibody subunits on this model.

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Legendes to figures

Fig.1. Starch gel electrophoresis of bovine and pig antibody and gamma-globulins and their derivatives.
Composition of buffer: 0.05 M formic acid, 6 M urea. 1 - bovine gamma globulin, 2 - S-sulpho bovine anti-DNP antibody, 3 - S-sulpho bovine gamma globulin, 4 - S-sulpho pig gamma globulin, 5 - S-sulpho pig anti-DNP antibodies, 6 - pig gamma-globulin.

Fig.2. Starch gel electrophoresis of bovine gamma-globulin and anti-DNP antibodies S-sulphonated at pH 8.6.
Composition of buffer: 0.05 M formic acid, 6 M urea, 1 - gamma-globulin, 2 - antibody, 3 - antibody S-sulphonated in excess dinitrophenol, 4 - antibody S-sulphonated in excess ε-DNP-lysine.

Fig.3. Starch gel electrophoresis of bovine gamma-globulin and anti-DNP antibodies S-sulphonated at pH 5.7.
Composition of buffer: 0.05 M formic acid, 6 M urea. 1 - gamma-globulin, 2 - antibody, 3 - antibody S-sulphonated in excess dinitrophenol, 4 - antibody S-sulphonated in excess ε-DNP-lysine.

Fig.4. Starch gel electrophoresis of L chains of gamma-globulin and antibody. Composition of buffer: 0.035 M glycine buffer pH 8.8, 8 M urea. 1 - chains of nonspecific bovine gamma globulin, 2, 3, 4 - chains of isolated anti-DNP antibody from three individual bulls.

Fig.5. Chromatography of S-sulpho bovine gamma globulin and anti-DNP antibody on Sephadex G-100.
Medium: 0.05 M formic acid, 6 M urea. Abscissa: volume of eluate

Ordinate: optical density at 2537 Å /registered by Uvicord LKB/. Crosshatched area denotes fractions pooled. Different subunits denoted by letters.

Fig.6. Activity of subunits of bovine anti-DNP antibody S-sulphonated at different pH.
Medium for separating subunits: 0.05 M formic acid, 6 urea. Abscissa: volume of eluate. Ordinate left: optical density at 2537 Å. Ordinate right: value of r. Upper part - Preparation S-sulphonated at pH 8.6, lower part - Preparation S-sulphonated at pH 5.7. Activity expressed by quantities of r are depicted as crosshatched columns and given in numerical values appended to the separate subunits.

Fig.7. Activity of different mixtures of H and L subunits of bovine anti-DNP antibody. Abscissa: percentage of subunit in mixture. Ordinate: activity expressed as quantity r. Upper part - hypothetical curves corresponding to ideal case of very firmly bound H and L chains in stoichiometric complex. Lower part - experimental curve.

Fig.8. Polarographic registration of reaction of ε-DNP-lysine with complex of H and L chains of bovine anti-DNP antibody. Abscissa: time interval from mixing of protein with hapten. Ordinate: concentration of free ε-DNP-lysine. Total amount of protein in experiment 2.5 mg. 1 - hapten added immediately after mixing H and L chains, 2 - hapten added 20 hours after mixing H and L chains.

Table 1
Activity of bovine anti-DNP antibodies and their derivatives

No.	Method of modification	r+	Relative activity %
1	Native	1.77	100
2	S-sulphonated /pH 8.6, 20 hour./		
	a/ dissolved in borate buffer	1.40	79
	b/ dissolved in 0.05 M formic acid with 6 M urea and transferred after 20 hrs. in borate buffer	0.86	49

+After reaching equilibrium with 1 x 10^{-5} M ε-DNP-lysine

Table 2

Activity of mixtures of bovine anti-DNP antibody and bovine gamma-globulin chains

Type of + chain	Concentration in mixture mg/ml	Total concentration of protein mg/ml	r^{++}	r_H^{+++}	r_L^{+++}
H_{sp}	0.50	0.50	0.00	0.00	-
L_{sp}	0.50	0.50	0.09	-	0.09
H_{sp}+	0.33				
+ L_{sp}	0.17	0.50	0.58	0.58	0.58
H_{sp}+	0.33				
+L_g	0.17	0.50	0.34	0.34	-
H_{sp}+	0.33				
+L_g	0.85	1.18	0.36	0.86	-
H_g+	0.33				
+L_{sp}	0.17	0.50	0.11	-	0.11
H_g+	1.00				
+L_{sp}	0.17	1.17	0.06	-	0.14

+ H_{sp}, L_{sp} - chains of antibody H_gL_g - chains of nonspecific gamma-globulin

++ corrected for non-specific adsorption

+++ r_H and r_L - represent moles of ε-DNP-lysine bound to 107,000 g of H_{sp} chain and to 53,000 g of L_{sp} chain respectively.

The Allotypic Determinants of Rabbit Gamma-Globulin, Its Fragments and Antibodies

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Abstract

The allotypic determinants /As/ of rabbit γ-globulin are controlled by two gene loci a /As 1, 2 and 3/ and b /As 4, 5 and 6/.

1. Work already published /1/ has indicated that i/ the Light /B/ chains appear to contain only the determinants controlled by the b locus, ii/ the Heavy /A/ chains appear, within the limitations of the technical methods available, to contain, in most cases, both the determinants controlled by the a and those controlled by the b locus. As 5 formed a partial exception to this in that we could only demonstrate it in the A chain from one homozygous /As 3/5/ animal, and failed to find it in four animals heterozygous at this locus, although As 4 was demonstrable in these preparations. Similarly, in one preparation from as As 1/4/6 animal, As 6 was demonstrable but not As 4.

We have also recently /2/ been able to confirm by gel- diffusion Todd's /3/ observations, made using the interface /ring/ test, that in rabbit γ-macroglobulins, As 1 may be present as well as the determinants /As 4, 5 or 6/ present in the B chain : we were able to demonstrate As 2 and As 3 in Addition. These macroglobulin preparations were made by a combination of SG 200 gel filtration and starch block electrophoresis: they reacted with an antisorum containing antibody against γ-macroglobulin, but not with one against S 7 γ-globulin exclusively.

Theoretically one might forecast that the allotypes controlled by the a locus would occur on the A chain only, and therefore not in macroglobulins, which possess a distinct kind of A chain : and that those controlled by the b locus would appear on the B chain only, and therefore should not be demonstrable in A chain preparations. That neither of these forecasts is fulfilled may reflect either upon the techniques currently available for fractionation of proteins and their fragments, which may still be inadequate to effect really complete separations, or alternatively upon the oversimplicity of the genetic theory upon which the forecast was based.

2. Antisera to allotypes may be raised /4/ by immunizing a donor rabbit with bacteria, coating a suspension of these bacteria with the antiserum so raised, and injecting the γ-globulin /antibody/ - coated cells into a recipient rabbit. In an attempt to identify new allotypes, we carried out this process using As 1/4 rabbits only : and have recently published /5/ some preliminary results of this study, which are essentially similar to those of Oudin and Michel /6/.

Antibodies were indeed produced in four out of five As 1/4 rabbits immunized from an As 1/4 donor /D/, itself immunized with Proteus vulgaris, but these antibodies were found to react only with the immune /D1/ serum, not with a pre-immunization sample from D, nor with the blood of about 80 relatives or progeny of D. The positive reactions cannot therefore be due to the presence of a new allotype determinant occuring in D but not i R, but must be contigent upon the immunization of rabbit D. The substance present in D1 and antigenic in R1 could be shown to have the physico-chemical characteristics of a S 7 γ-globulin : it would

react as antibody with Proteus vulgaris extracts and as antigen /γ-globulin/ with anti-As 1 antisera : moreover by gel diffusion tests it appeared that all the R1-reacting substance is anti-Proteus antibody and all the anti-Proteus antibody was capable of reacting with R1. Full absorbtion of R1 with Proteus extract or cells did not affect the R1/D1 reaction, which was however abolished by absorbtion of D1 with proteus extract.

By immunoelectrophoresis and agar block electrophoresis followed by extraction, the reacting D1 substance was found to have a sharply restricted mobility within the γ-globulin range and in this respect resembled a myeloma protein.

R1 was not an anti-/anti-Proteus/-antibody in the strict sense, since it did not react with several strong anti-Proteus antisera and indeed had itself appreciable anti-Proteus activity.

These and other data les us to postulate that the D1 substance was a 'clone product' selected by immunization and present if at all only at undetectible concentration in the original pre-immunization sample. Further experiments along these Imes are being made and will be reported.

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Heterogeneity of Antibodies in Alergic Sera+

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Introduction

As in acquired immunity, the characteristic feature of allergic reactions in man, of both the immediate and delayed type, is their specificity with respect to the particular allergen. Since the introduction of the passive transfer test of Prausnitz and Kustner in 1921 /1/, it has been generally accepted that hypersensitivity states of the immediate type /such as hay fever, asthma, food allergies/ are associated with the presence of humoral skin-sensitizing antibodies, produced "spontaneously" by the allergic individual in response to the casual exposure of a given allergen by either inhalation or ingestion. Moreover, since 1935 /2/, it has been recognized that treatment of allergic individuals++ with a series of injections of the offending allergen leads to the formation of

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++Allergic individuals who have received at least one series of injections of the allergen are denoted as treated individuals; prior to such treatment the allergic patients will be referred to as non-treated individuals. For the sake of brevity, sera of allergic individuals will be also designated as allergic sera.

of an additional humoral antibody, termed blocking antibody, which was shown to be capable of neutralizing the allergen. In addition, to skin-sensitizing and blocking antibodies, which are demonstrated by the in vivo P-K test and by the blocking of this test, respectively, the presence of agglutinating antibodies has been regularly demonstrated within the last decade in the sera of both non-treated and treated allergic individuals by passive hemagglutination methods /3,4/.

By contrast, so far, delayed hypersensitivity has not been passively transferred with serum, but only with the sensitized donor's lymphoid cells or, in the case of tuberculin sensitivity in man, with the corresponding cellsextracts /5/. Consequently, it has been postulated that delayed hypersensitivity is mediated by a cellular antibody-like factor/s/, referred to as the transfer factor/s/. The presence of hemagglutinating antibodies had been demonstrated a long time ago /6/ in sera of individuals with tuberculin sensitivity to PPD /purified protein derivative of tubercle bacillus/, and recently it has been shown that these sera contain at least two physico-chemically distinct hemagglutinins /7/.

The distinguishing features of skin-sensitizing and blocking antibodies, were reviewed at the First Symposium held in Prague five years ago /8/ and were summarized in a table, which is reproduced here /see Table 1/, since the data listed in this table have been confirmed by many workers and since it represents a convenient starting point for the present discussion. This paper will be, therefore, confined primarily to a presentation of additional physico-chemical and immunochemical properties of skin-sensitizing and blocking antibodies, which have been unravelled in recent years, and to an analysis of the possible

relationships between these antibodies and the hemagglutinins invariably found in the sera of allergic individuals. In spite of the great variety of allergens, most of the systematic studies of the properties of allergic antibodies have been done with sera of grass- /9, 10, 11/ and ragweed-sensitive /8, 11, 12, 13/ individuals. As will become apparent, the exact nature of these antibodies and the mechanism of their in vivo and in vitro interactions with the homologous allergens remains still undetermined, partly because of the extremely small concentration of these antibodies in allergic sera, partly because of the failure to isolate them in a pure state, and partly because of the unavailability of pure allergens. Nevertheless, it has become evident that 1. the older differentiation of sera of non-treated allergic individuals from sera of treated patients is purely arbitrary and outmoded, 2. both types of sera contain multiple /and probably the same type of/ antibodies, and 3. these sera differ mainly in the relative amounts of the various antibodies rather than in the quality of the antibodies produced /14/.

Skin-sensitizing antibodies /REAGIN/

Physicochemical behaviour

As was previously established /15/, all observations to date indicate that skin-sensitizing antibodies are much more labile than "conventional" antibodies produced on immunization. Even, seemingly harmless procedures, such as dilution of sera and reconcentration by ultrafiltration, or by pervaporation combined with dialysis, leads to serious losses or reaginic activity of the order of 30-50% /12, 16/. Like immune antibodies, skin-sensitizing antibodies are degraded with papain.

However, whilst immune antibodies are split very rapidly, i.e. within the first 30 minutes of digestion 99% of these antibodies are destroyed /17/, skin-sensitizing activity remains practically unchanged within the first 30-60 minutes of digestion and decrease gradually requiring a 24-hour period of digestion for complete inactivation of reaginic antibodies /17/.

Unlike immune antibodies, skin-sensitizing antibodies are inactivated in the absence of papain by treatment with mercaptoethylamine at a concentration of 0.1M /17/. This inactivation appears to be the result of the reductive cleavage of skin-sensitizing antibodies since treatment of allergic sera with lower concentrations of the reducing agent or with iodoacetate alone /which is used for the stabilization of the SH groups liberated during the reductive cleavage/ does not lead to a measureable decrease of skin-sensitizing activity. The results obtained in the studies on the enzymatic and chemical degradation of skin-sensitizing antibodies indicate that during these reactions the portion of the skin-sensitizing antibody molecule responsible for its attachment to the skin becomes destroyed or dissociated from the rest of the molecule /17/.

Serum fractions enriched in skin-sensitizing activity were isolated by chromatography of allergic serum on 1. the anion-exchange resin diethylaminoethylcellulose /DEAE-cellulose/ /10, 12/ and 2. by gel filtration /18/ on the cross-linked dextran, Sephadex G-200. Thus, whilst blocking antibodies were almost quantitatively recovered from DEAE-cellulose in the first chromatographic fraction /eluted with 0.01M phosphate buffer at pH 7.5/, skin-sensitizing antibodies were eluted later in fract-

ions with buffers of higher molarity /0-02 and 0.05M/ amd of lower pH /pH 6.2 and 4.5/. These results confirmed the previous findings /19/ that skin-sensitizing antibodies were physicochemically heterogeneous and additional electrophoretic /10, 20/, ultracentrifugal /8, 21/ and immunochemical /10, 18, 20/ analyses revealed that the fractions possessing skin-sensitizing activity were complex mixtures of proteins.

Fractionation of sera on Sephadex G-200 yielded three fractions which were not resolved completely from one another and which were composed of 1. macroglobulins / γ_{1M}- and α_{2M}-globulins/, 2. other globulins / γ_2-, γ_1-, β- and α-globulins/ of lower molecular weight, and 3.albumin. Using this method for the fractionation of sera of ragweed-allergic individuals /18, 22/, skin-sensitizing activity was found primarily in the early eluates of the second fraction, i.e. in the eluates corresponding to the region of overlap between the first and second chromatographic fractions, the bulk of skin-sensitizing activity emerging before the peak concentration of the second fraction. By immunochemical analysis, the eluates containing skin-sensitizing activity were found to be composed mainly of γ_{1A}-globulins and 7 S γ-globulins, and contained also a small amount of γ_{1M}-globulins and possibly other components. These results were interpreted /18/ to indicate that the distribution of skin-sensitizing activity parallelled best that of γ_{1A}-globulins, a conclusion which was in agreement with the earlier suggestions of Heremans /23/ and of Augustin and Hayward /10/.

The interpretation of the experimental data concerning the ultracentrifugal properties of skin-sensitizing antibodies has led to much controversy. The reason for this controversy might

be due to the fact that in these investigations sera of individuals with different allergies /i.e. to ragweed and grass pollens, horse dandruff, rabbit and cattle hair, molds, glucagon/ were used and, in most of these studies, no systematic discrimination was made as to whether the sera had been obtained from non-treated or treated allergic individuals; moreover, usually no special mention was made of the length and method of hyposensitization treatment administered. The discrepancies in the observations may, therefore, merely reflect the heterogeneity of skin-sensitizing antibodies produced to different allergens and under different conditions of exposure. Furthermore, the techniques employed in practically all these studies were different /i.e. fractionation of whole serum by preparative ultracentrifugation, ultracentrifugation in a density gradient, ultracentrifugation in partition cells and ultracentrifugal analysis of serum fractions containing skin-sensitizing antibody/, rendering the correlation of the results difficult.

As was stated at the First Symposium /8/, the analysis of the distribution of antibody activities in serum fractions isolated by ultracentrifugation in partition cells indicated that reaginic activity was associated with serum components having sedimentation coefficients larger than 7 S, the actual values measured in terms of P-K titers being 12.4, 14.1 and 22.5 S /21/. However, because of the inherent inaccuracies of the titration procedures used, all these values were considered accurate only within 2.5 S /21/. These conclusions were challenged by a number of workers /10, 20/, all of whom favoured the value of 7 S for the sedimentation coefficient of reagins.

Since none of these conclusions was based on an unequivocal evidence, the suggestion was made that skin-sensitizing activity was not associated with either 7 S or 19 S serum components, but with other moieties having sedimentation coefficient/s/ intermediate between these values /24/. Obviously, such components would have been detected either with a light or with a heavy serum component depending on the experimental conditions used. Indeed, recently, using an improved method of density gradient ultracentrifugation /25/, skin-sensitizing antibodies to glucagon were found to sediment with serum components having sedimentation coefficients of the order of 8-11 S. Similar conclusions were reached also by Terr and Bentz /22/ from an ultracentrifugal study with four sera of untreated ragweed sensitive patients and an average sedimentation coefficient of 7.8 S was calculated for reagins to ragweed /27/. As already stated, in a number of recent studies it has been suggested that skin-sensitizing antibodies may be identified with γ_{1A}-globulins. However, it is worth pointing out that although the major portion of this globulin fraction has a sedimentation coefficient of 7 S, minor components with sedimentation coefficients of 10.5 S and 13 S are also present in this serum fraction /28/.

Immunochemical properties

Specific precipitation of serum components present in chromatographic fractions possesssing highest skin-sensitizing activity with a rabbit antiserum to human γ-globulins resulted in the removal of all skin-sensitizing antibodies /29/. However, since γ_{1A}-globulins, as well as 7 S and 19 S γ-globulins share common antigonic determinants, these results simply indicated that skin-sensitizing antibodies belonged to the class of immuno-

globulins but did not specify more precisely their antigenic nature. In a more recent study it was demonstrated that removal of 7 S γ-globulins from a skin-active serum fraction by precipitation with an antiserum, rendered specific to 7 S γ-globulins by prior absorption with γ_{1A}-globulins, did not result in a decrease of its reaginic activity /18/. However, complete removal of skin-sensitizing activity from three ragweed allergic sera was achieved by absorption with an antiserum rendered specific for γ_{1A}-globulins by the precipitation of antibodies directed to the determinants of 7 S γ-globulins. Thus, one can conclude that at least some of the antigenic groups of skin-sensitizing antibodies in these three sera were identical to those of γ_{1A}-globulins- However, since γ_{1A}-globulins have a tendency to complex with other proteins /28/, and since recently three sera of ragweed-sensitive individuals with high P-K titers were found to be devoid of γ_{1A}-globulins /26, 30/, it is felt that additional evidence is necessary before skin-sensitizing antibodies may be unequivocably identified with γ_{1A}-globulins. It is conceivable that skin-sensiting antibodies are complex molecular species, one of their building blocks being γ_{1A}-globulins. This latter component might be also responsible for the fixation of skin-sensitizing antibodies to tissues and for their retention by the placenta and choroid plexus. In this connection, it ought to be pointed out that Ishizaka et al. /31/ demonstrated that passive sensitization of human skin with ragweed reagin could be blocked with purified normal human β_{2A}-globulin fraction as well as with the A chain /and not with B chain/ of γ_{1A}-globulins /32/. In the light of all the experimental results derived from ultracentrifugal, chemical,

chromatographic and immunochemical studies, one cannot deny the fact that there is increasing circumstantial evidence in favour of reagins belonging to the group of γ_{1A}-globulins with sedimentation coefficients higher than 7.

The very recent investigation of Yagi et al. with the help of radioimmunoelectrophoresis, revealed that ragweed-binding capacity of allergic sera was not limited to a single protein /33/, but was associated with the γ-globulins of all the 8 sera tested and also with the γ_{1A}-and/or γ_{1M}-globulins. Of course, these results do not prove that skin-sensitizing antibodies are directly implicated in this reaction and, as will be shown later, it was actually demonstrated that sera of non-treated allergic individuals possess, in addition to reagins, antibodies devoid of skin-sensitizing activity but with binding capacity for allergen/s/. Thus, these results lend further support to the evidence presented above that there exists a spectrum of antibodies in allergic serum differing in their physico-chemical immunochemical properties.

Blocking antibodies

Formation of blocking antibodies can be induced in both allergic individuals and non-allergic volunteers and, as has been shown /2/, the various properties of blocking antibodies elicited in these two types of individuals are identical. Moreover, blocking ability is not confined only to antibodies produced in man, but is also possessed by antibodies formed by rabbit, goat and dog on immunization with appropriate allergen.

The chemical nature of blocking antibodies resembles that of precipitating antibodies and differs significantly from that of skin-sensitizing antibodies. Thus, blocking antibodies

are not inactivated by heating at 56°C, are stable under various conditions of storage and handling and are not degraded by reduction with 0.1M mercaptoethylamine /17/. Moreover, digestion of blocking antibodies with pepsin, with papain, or with pepsin followed by reduction with mercaptoethylamine - reactions known to lead to the breakdown of precipitating rabbit antibodies to smaller divalent or univalent antibody fragments - resulted in the degradation of blocking antibodies to fragments which were shown to retain to ability of combining with the allergen/s/ and of blocking the P-K reaction to the same extent as intact blocking antibodies /17/. Similarly, fragments prepared from precipitating rabbit anti-ragweed antibodies behaved in the same way, i.e. the blocking titers of the fragments were identical to those of the intact antibodies /17/.

The bulk of blocking activity was invariably found associated with the electrophoretically slowest migrating serum components, i.e. namely with γ_2-globulins /4/, but occasionally in sera containing large amounts of blocking antibodies, a small portion of the blocking activity was detected also in the faster migration γ_1-globulin reaction. This behaviour is similar to that of conventional precipitating antibodies, which have been demonstrated to be physico-chemically heterogeneous and to extend electrophoretically from the region of the slowest γ-globulins to that of α_2-globulins /34/. In the light of these findings, it seems reasonable to ascribe the ragweed binding activity of γ_2-globulins, as demonstrated by hemagglutination and by radio-immunoelectrophoresis, to blocking antibodies. Moroover, the binding of I^{131}-labelled ragweed pollen constituonts by γ_{1M}- and γ_{1A}-globulins /33/ reflects, in all probability, the electrophoretic and ultracentrifugal heterogeneity of blocking antibodies, though

skin-sensitizing antibodies may participate also in this reaction.

As demonstrated previously /4, 8/, the sedimentation coefficient of blocking antibodies was close to 7 S which would also support the interference that blocking antibodies have properties identical to those of the conventional antibodies produced on active immunization. Similarly, the chromatographic behaviour of blocking antibodies on DEAE-cellulose was analogous to that of precipitating rabbit antibodies /9, 12/, i.e. the major portion of both types of antibodies was eluted in the first fraction with 0.01M phosphate buffer at pH 7.5. Moreover, in the first few eluates, i.e. in the first subfraction /Fraction 1A/, which were rich in antibodies, there was no detectable skin activity /12/. It outght to be also pointed out that small amounts of blocking activity were found in subsequent chromatographic fractions eluted with buffers of higher concentration and of lower pH /12/. Essentially identical results were obtained with sera of non-allergic volunteers or rabbits, immunized with ragweed pollen extract, and with sera of non-treated or treated allergic individuals, except that the first chromatographic fraction /Fraction 1A/ of sera of non-treated allergic individuals had to be first concentrated - to a hemagglutination titer of about 2,500 - before the presence of blocking antibodies could be demonstrated /12/. Moreover, after appropriate concentration of subfractions 1A to a hemagglutination titer of the order 40,000 precipitating antibodies were readily demonstrable by the micro-Ouchterlony procedure /35/. On the basis of these findings, it was interred that these various immunological manifestations were attributable to the same type of antibodies and, consequently, it was proposed that blocking antibodies had the properties of normal immune antibodies /14/. On the

basis of this argument, it must be concluded that even non-treated allergic individuals, i.e. patients who have not received any hyposensitization treatment, form blocking antibodies and, therefore, the older classification of sera of allergic individuals into two categories, depending on whether they were obtained from patients prior to or after hyposensitization treatment, can be regarded as purely arbitrary. It ought to be stressed, however, that during hyposensitization treatment, the patient may produce additional antibodies to antigenic constituents in ragweed, which may have no relation to the allergenic determinant groups. These antibodies would then excape detection by the in vivo procedure for the demonstration of blocking antibodies, although their presence could be manifested by in vitro methods, i.e. by hemagglutination and precipitation. Of course, similar considerations apply also to blocking antibodies produced "spontaneously" by the non-treated allergic individual, as well as by non-allergic volunteers and rabbits on immunization.

The relationship of hemagglutinins to skin-sensitizing and blocking antibodies

From an analysis of the hemagglutination and P-K titers of sera of non-treated allergic individuals, it appears that, although all sera tested were never devoid of hemagglutinins, there exists no simple relationship between these two titers, i.e. sera with high P-K titers may possess low or high hemagglutination titers, and sera with low P.K titer may possess high or low hemagglutination titers. This lack of correlation seems to suggest that the hemagglutinating ability of sera of non-treated allergic individuals is due to multiple factors, the proportion of which differs from one serum to another, and

represents the individual variation in antibody response amongst a group of allergic individuals. The possibility that multiple hemagglutinins may be present in allergic sera is also supported by the findings that the hemagglutination titers obtained with erythrocytes senstized via tolylene-2,4-diisocyanate were substantially lower than the BDB-titers and that there was no obvious relationship between these two sets of titers /36/.

There seems to exist some correlation between the hemagglutination and the blocking titers of the sera of treated allergic individuals /as well as of immunized non-allergic volunteers and rabbits/, in as much as, in general, a detectable blocking reaction could not be obtained unless the serum /or serum fraction/ had a BDB-hemagglutination titer of about 2,500; correspondingly, sera with higher hemagglutination titers had also proportionately higher blocking titers. The fact that serum fractions possessing blocking antibodies were found to have also the other properties of "conventional" antibodies produced on active immunization, i.e. precipitation with the homologous antigens or allergens, passive cutaneous sensitization of guinea pigs /17/, could be interpreted as indicating that either 1. there are multiple types of antibodies present in these sera, each type being responsible for one of the immunological manifestations, or 2. in accordance with the "unitarian concept", one and the same type of antibodies is capable of participating in different immunological reactions. Obviously, neither of these two possibilities can be ruled out as long as the serum factors responsible for the different immunological manifestations are not isolated in a pure form and no dose-response relations can be established for these reactions.

In order to settle unequivocally the question as to whether or not there exists any relation between hemagglutinins and skin-sensitizing and/or blocking antibodies, it would be necessary to establish if 1. the hemagglutinating ability of sera of non-treated allergic individuals as a property of skin-sensitizing antibodies or, exclusively, of a type of blocking antibodies produced "spontaneously", i.e. elicited simply by the casual exposure to the allergen/s/ and not in response to hyposensitization treatment; 2. the hemagglutinating ability of sera of treated allergic individuals is a property of blocking antibodies or of a different type of antibodies, directed against some antigenic determinants of ragweed pollen constituents which are unrelated to the allergenic groups; 3. blocking antibodies produced on active immunization /in allergic individuals, in non-allergic volunteers or in experimental animals/ are identical to the blocking antibodies produced "spontaneously" by the allergic individual or exposure to the allergen/s/.

The similarity between the physico-chemical and chemical properties of blocking antibodies and of hemagglutinins strongly suggest that the major portion of the hemagglutinins of sera of treated allergic individuals may be identified with blocking antibodies. On similar grounds and also in view of the reactions of identity given by antibodies of sera of treated and non-treated individuals in agar gel diffusion experiments /35/, one may postulate that blocking antibodies produced "spontaneously" by the non-treated allergic individual are indistinguishable from those produced on hyposensitization treatment. On the other hand, the lack of correlation between the hemagglutination and P-K titers of sera of non-treated and treated allergic individuals, and more importantly the findings that the hemagglutination titers

of chromatographic fractions possessing the bulk of skin-sensitizing activity represented only a small fraction of the hemagglutination titer of the whole serum /12/, indicates that even if skin-sensitizing antibodies had hemagglutinating capacity their contribution to the overall hemagglutination titers would be minimal. Obviously, in order to establish if these two different immunological reactions are due to the same serum factor/s/, it is necessary to isolate the corresponding antibodies in a pure state, or at least to separate them from other antibodies reacting with the same antigen.

With this in view a number of studies have been conducted using different physico-chemical and immunochemical fractionation procedures. Suffice it to say here that hemagglutinating capacity was associated with fractions containing skin-sensitizing and/or blocking antibodies and never with any fraction devoid of either of these antibodies. From a careful analysis of the distribution of the hemagglutinating activity in fractions isolated by ultracentrifugation in partition cells, it was concluded that at least two physico-chemically distinct hemagglutinins were present in allergic sera and it was suggested that one of these hemagglutinins had a sedimentation coefficient of the order of 19 S and that the other had a sedimentation coefficient of 7 S.

On the basis of the similarity of the sedimenting properties of the former class of hemagglutinins and of skin-sensitizing antibodies, it was suggested that sera of non-treated allergic individuals might contain two types of antibodies: 1. antibodies with sedimentation coefficients higher than 7 S which may possess both skin-sensitizing and hemagglutinating abilities, and 2. antibodies of the conventional 7 S type, possessing only hemagglutinating activity. These latter hemagglutinins may be identi-

fiable with the bulk of the blocking antibodies.

The problem of identifying hemagglutinins with skin-sensitizing and/or blocking antibodies is, however, complicated since the sensitivity of the hemagglutination reaction is lower than that of the P-K test and, furthermore, the sensitivity of the P-K test is not a constant value, i.e. the amount of antibody detectable by this technique depends both on the antigenantibody system as well as on the individual antiserum used. In general, the BDB-hemagglutination titers of sera of ragweed-sensitive individuals were higher than the titer of grass-sensitive individuals, both for a group of non-treated and treated allergic patients /11/. Correspondingly, the hemagglutination titers of the chromatographic fractions containing the bulk of reagins to ragweed were strikingly higher than the hemagglutination titers of the corresponding fractions of sera of grass-sensitive individuals. Thus, hemagglutination titers of these fractions obtained with sera of ragweed-sensitive individuals were of the order of 10 to 40 /12/, and not higher than 4 with sera of grass-sensitive individuals /10/. This difference in the hemagglutination titers of the reaginic fractions of these two types of sera is difficult to interpret, particularly since it might be merely due to the difference in the sensitivity of the hemagglutination technique for the two systems.

The fact that skin-sensitizing activity can be destroyed by heating without impairing the hemagglutinating ability does not necessarily imply that these two manifestations are associated with two distinct types of antibodies. It is conceivable that the same antibody molecule may possess the two antibody combining sites required for the hemagglutination reaction /and which are complementary to the determinants of ragweed pollen constituents/,

in addition to the site which has the ability to become fixed to skin and tissues. It can be also visualized that the portion of the molecule responsible for skin-sensitizing can be destroyed or modified irreversibly by heating, without impairing the ability of the antibody combining sites to react with ragweed allergens /19/.

Theoretically, one may visualize that some of the hemagglutinating antibodies may be directed against some antigenic determinants which are not involved in the reaction with reagins and which, nevertheless, may be situated on the same molecule in the vicinity of the allergenic determinants, or on antigenic constituents of ragweed pollen which are unrelated to the allergens. From all these considerations one is led to conclude that the contribution of skin-sensitizing antibodies to the hemagglutination titer of allergic sera is only minimal. Nevertheless, the results of all physico-chemical fractionation studies /i.e. fractional precipitation, electrophoresis, ultracentrifugation, chromatography/ do not rule out the possiblity that skin-sensitizing antibodies possess also hemagglutinating properties.

The comparison of the behaviour of skin-sensitizing and blocking antibodies on degradation with papain /17/ add further support to this interference. In these experiments the rate of inactivation of reagins was measured in terms of the decrease of skin-sensitizing titer, whilst the rate of degradation of blocking antibodies was measured in terms of the decrease of the hemagglutination titer; the lattor procddure had to be resorted to since it had been shown that degradation of blocking antibodies to univalent fragments was not associated with any loss of blocking capacity. Thus, the kinetics of the degradation

of the hemagglutinins in sera of non-treated allergic individuals with papain appeared to be represented by two rate processes: an initial rapid destruction of about 95 percent of the hemagglutinins within the first 30-60 minutes of digestion, followed by a much slower inactivation of the residual hemagglutinating activity. The first process, by analogy with the rate of degradation of blocking antibodies elicited on active immunization, was considered to represent the degradation of blocking antibodies produced "spontaneously" by the non-treated allergic individual. On the other hand, the second process, by analogy with the slow rate of inactivation of skin-sensitizing antibodies, was considered to represent the degradation of antibody molecules possessing both skin-sensitizing and hemagglutinating properties. It ought to be stressed that, because of the inherent inaccuracies of the methods used for the quantitative determination of reagins and hemagglutinins, these kinetic data could not be subjected to a mathematically rigorous treatment to test this interpretation. It is, therefore, not possible at this stage, to dismiss the alternate explanation, namely that the apparent similarity between the rates of enzymatic degradation of skin-sensitizing antibodies and of a small portion of the hemagglutinins of sera of non-treated allergic individuals might be purely fortuitous and might reflect simply the presence of multiple and chemically different hemagglutinins in these sera.

Additional evidence that each serum contained multiple hemagglutinins was derived from studies in which increasing volumes of allergic sera were absorbed with a constant amount of a specific immunosorbent /13/. The results of these experiments demonstrated that the finer details of each of the absorption curves representing the absorption of hemagglutinins and/or

reagins from serum fractions obtained by chromatography on DEAE-cellulose were peculiar to the serum used. In addition, the removal of skin-sensitizing activity from Fraction 7 obtained by chromatography on DEAE-cellulose /i.e. the fraction containing the major portion of reagins/, of each of these two sera was almost parallelled by the removal of the hemagglutinins. These results were interpreted as indicating that the hemagglutinating capacity of Fraction 7 was an inherent property of skin-sensitizing antibodies, namely that reagins were either antibody molecule or molecular complexes possessing /at least/ two sites capable of combining with the allergen, in addition to the site which was capable of attaching itself to the skin. Furthermore, it was shown that considerable amounts of skin-sensitizing antibodies could be displaced by blocking antibodies from an immunosorbent which had been previously saturated with respect to reagins. These results indicate clearly that allergic sera contain a spectrum of antibodies with different physico-chemical and biological properties and which are directed against antigenic groups on the same molecule/s/ of the ragweed constituents.

In summary, it can be stated that in spite of the accumulation of a large amount of information on the physico-chemical and immunochemical properties of skin-sensitizing, blocking and hemagglutinating antibodies, the nature of the serological factors involved in the appropriate immunological manifestations has not been unequivocally established and no simple relationships have been derived amongst them. However, it is obvious that the antibodies in allergic sera are heterogeneous with respect to their size, shape, composition, charge, affinity for the

antigen/s/ and ability to become fixed to tissues. Nevertheless, it seems plausible to conclude that blocking antibodies may be regarded as immunoglobulins of the conventional γ_2-type with a sedimentation coefficient of 7 S, which are detectable by classical immunological techniques. On the other hand, skin-sensitizing antibodies are electrophoretically faster-moving globulins belonging possibly to the class of γ_{1A}-globulins and/or γ_{1M}-globulins, they have sedimentation coefficient/s/ higher than 7 S, and may contribute to a small extent to the hemagglutination titer of allergic sera.

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Table 1

Properties of antibodies in allergic sera /as summarized in 1959 /8/.

Reagins	Blocking antibodies
does fix to skin	does not fix to skin
does not pass through placenta	passes through placenta
heat labile with respect to skin-sensitization	heat stable
combines with Ag in vitro	combines with Ag in vitro
does not precipitate at 30% ammonium sulfate	precipitates at 30% ammonium sulfate
γ_1-/or β-/globulins by electrophoresis	γ_2-globulins by electrophoresis
fraction III by Cohn 's method	fraction II by Cohn 's method
sedimentation constant 7 S	sedimentation constant = 7 S

Both sera of non-treated and treated individuals, containing skin-sensitizing and/or blocking antibodies, possess hemagglutinating ability.

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For discussion in the topic II c.

A Method for The Isolation of γ_{1A}-globulin from Human Serum Using Salting-out and Chromatography

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Abstract

Gamma-1A-globulin /β_{2A}-globulin, IgA/ may be isolated from normal or pathological human sera by a series of steps consisting in the removal of euglobulins at pH 6.5 and pH 5.4, salting-out of the globulin fraction by means of 2 M ammonium sulfate at pH 6.8, chromatography of carboxymethylcellulose, and chromatography on triethylaminoethylcellulose. The end-product shows no traces of contamination with the other immune-globulins /7 S γ and 19 S γ/.

A method for the isolation of γ_{1A}-globulin from human serum using salting-out and chromatography.

The immune globulin called γ_{1A}-globulin /β_{2A}-globulin, IgA/ was initially prepared by means of a procedure involving precipitation with zinc sulfate, salting-out with ammonium sulfate, and preparative elctrophoresis /1/. In spite of different improvements /2, 3/, this method has proved to be poorly reproducible and to yield products whose purity was often unsatisfactory. Contamination with trace amounts of 7 S γ-globulin and 19 S γ-globulin has been particularly inconvenient, as the isolation of γ_{1A}-globulin from human serum is usually undertaken with the aim of assessing its antibody activity spectrum. Chromatographic methods have given excellent results when applied to the isolation

of γ_{1A}-type myeloma proteins /4/ or to the purification of the γ_{1A}-globulin present in milk /5/. However these methods have proved much less suitable for the recovery of γ_{1A}-globulin from normal serum, in spite of some claims to the contrary /6/. The difficulties, in obtaining purified γ_{1A}-globulin from such a complex a mixture of proteins as human serum have been stressed by Davis, West and Hong /7/, who investigated several fractionation procedures.

The method to be described here, permits the isolation of highly purified γ_{1A}-globulin from normal human serum. However, yields of purified protein are still low.

Step 1. Removal of euglobulins

The aim of this step is to remove two proteins which would be difficult to eliminate at later stages of the fractionation. One is γ_1-macroglobulin /19 S γ/, the larger part of which is removed by dialysis against dilute buffer of pH 6.5. The other euglobulin is β_{1A}- / and β_{1C}-/ globulin, which precipitates during dialysis against dilute buffer of pH 5.4.

One volume of serum is diluted by addition of 9 volumes of 0.02 M NaH_2PO_4 /Na_2HPO_4 buffer of pH 6.5 and dialyzed against 500 volumes of the same buffer, with continuous stirring. The dialysis is performed at 4° and the outer buffer solution is renewed twice over a period of 24 hours. The euglobulin precipitate is removed by centrifugation at 3 000 x g during 10 minutes, at room temperature.

The supernatant is now dialyzed against 50 times its volume of 0.02 M NaH_2PO_4 /Na_2HPO_4 buffer of pH 5.4, with continuous stirring, at 4°. The outer buffer is renewed twice over a period of 24 hours, and the precipitate is eliminated by centrifugation

at 3000 g during 10 minutes, at room temperature.

Step 2. Salting-out of the crude immune globulin fraction by means of 2 M ammonium sulfate.

This step is aimed at the removal of the bulk of the albumin and other irrelevant proteins.

The supernatant from the second centrifugation is mixed with an equal volume of a 4 M solution of ammonium sulfate. This solution has its pH adjusted to 6.8, before use, by means of the required amount of a saturated NaOH solution. After mixing the protein solution with the ammonium sulfate, the pH is checked and, if necessary, adjusted to 6.8. After 1 hour incubation at room temperature /20°/, the precipitated globulins are recovered by centrifugation at 13,000 x g for 10 minutes, at room temperature. The precipitate is redissolved in distilled water, employed in an amount equivalent to 10 times the original serum volume. The protein solution thus obtained is precipitated again by adding its own volume of the above described 4 M ammonium sulfate solution. The new precipitate is left to stand for 1 hour, at room temperature, and centrifuged off at 13,000 x g, for 10 minutes, at room temperature. The final sediment is redissolved in 10 times the original serum volume of distilled water.

Step 3. Chromatography on cyrboxymethylcellulose /CM-cellulose/.

The aim of this step is to free the preparation from all but traces of 7 S γ-globulin.

The chromatography column is packed with an amount of carboxymethylcellulose /Serva, Heidelberg, 0.68 mEq/g/ calculated on the basis of 0.6 g of CM-cellulose per ml of the original serum sample. Prior to use the CM-cellulose is washed for several days by stirring in approximately 10 times its own volume of 0.25

N NaOH solution, then with portions of distilled water until the pH of the washing fluid ceases to be alkaline, and finally with several portions of McIlvaine phosphate-citrate buffer of pH 5.0. This buffer is made by mixing 48.5 volumes of 0.01 M citric acid and 51.5 volumes of 0.02 M $Na_2HPO_4 \cdot 2H_2O$. The final suspension is poured into the column and allowed to sediment, after which the column is washed with 50 ml of the same buffer per gram of CM-cellulose employed.

The sample to be chromatographed is dialyzed against 50 times its own volume of the same buffer, at 4°. The outer fluid is renewed twice during the 24-hour period of dialysis. Some precipitate will form and is removed by centrifugation, after which the sample is concentrated to one-quarter its original volume, by means of ultrafiltration in vacuo. The concentrated sample is now applied to the chromatographic column and recoved in the effluent by washing the column with the same buffer, at room temperature, until the extinction at 280 mμ of the effluent drops to the baseline value.

Step 4. Chromatography on triethylaminoethylcellulose /TEAE-cellulose/.

This step is intended to separate the γ_{1A}-globulin from the accompanying proteins, mainly α-globulins and albumin, which are also present in the breakthrough volume from the CM-cellulose column.

The chromatography column is packed with an amount of tri-ethylaminoethylcellulose /Serva, Heidelberg; 0.68 mEq/g/ calculated on the basis of 2.0 g of TEAE-cellulose per ml. of the original serum sample. Prior to use the TEAE-cellulose is washed for several days by stirring in approximately its own volume of 0.5 N NaOH, and then with several portions of distilled water u ti

until the pH of the washing fluid ceases to be alkaline. After decantation of the final bath of distilled water, the slurry is resuspended in 10 times its volume of 0.2 M Na_2HPO_4. After one hour the buffer is removed and replaced by several portions of distilled water until the pH of the washing fluid ceases to be alkaline. The wet slurry is resuspended in 10 times its volume of 0.2 M NaH_2PO_4 and, after one hour, washed again repeatedly with distilled water until the pH ceases to be acid. The paste is now suspended in 10 times its volume of 0.01 M disodium phosphate buffer of pH 8.0. This buffer is obtained by mixing 50 volumes of 0.01 M Na_2HPO_4 and 3 volumes of 0.01 M NaH_2PO_4. The final suspension of TEAE-cellulose is poured into the column and allowed to settle, after which the column is washed with 50 ml. of equilibrating buffer per gram of TEAE-cellulose employed.

The sample to be chromatographed is dialyzed against 50 times its own volume of starting buffer /0.01 M; pH 8.0/, at 4°. The outer fluid is renewed twice during the 24-hour period of dialysis. The sample is then concentrated to one-fifth its volume by ultrafiltration in vacuo, and run into the column. Five times the column volume of starting buffer is now passed and the effluent discarded. No protein is recovered at this step. Elution is now performed with 0.02 M disodium phosphate buffer of pH 8.0, and the effluent is collected in 3 ml. aliquots. Extinction readings are made at 280 mμ and elution is continued until baseline values are again reached. This eluate will contain a mixture of γ_{1A}-globulin and 7 S γ-globulin in the proportion of 2 : 1. No attempt has been made to purify this preparation any further. Elution is continued with 0.03 M disodium phosphate buffer of pH 8.0 and the effluent is again collected in 3 ml. aliquots until

the extinction at 280 m/u has returned to baseline values. This product has proved to be γ_{1A}-globulin free from any contaminants detectable by immunoelectrophoresis and immunodiffusion against polyvalent, anti-7 S γ, or anti-19 S γ antisera.

Elution is resumed with 0.05 M disodium phosphate buffer of pH 8.0 and continued until the effluent no longer contains any protein. This product still contains much γ_{1A}-globulin together with approximately equivalent amounts of α-globulins. Separation can be achieved by means of preparative electtrophoresis, which will thus yield an additional amount of pure γ_{1A}-globulin.

Results and discussion

Steps 1 and 2 may cause losses of γ_{1A}-globulin amounting to as much as 30% of the quantity initially present in the serum sample. About 60% of the amount of γ_{1A} introduced into the CM-cellulose column are collected with the effluent. From this recovered amount about 28% is eluted with the 0.02 M buffer, 16% with the 0.03 M buffer, and 5% with the 0.05 M buffer. The yield of the method is thus about 7% if only the product eluted at 0.03 M is recovered, and this can be raised to 8.8% if the γ_{1A} from the 0.05 M step is included.

One advantage of the presently described method in comparison with the earlier zinc sulfate fractionation procedures /1, 2, 3/ is its excellent reproducibility. Another considerable advantage resides in the purity of the γ_{1A} fractions thus obtained. Such products should prove most suitable for investigations on the antibody activities of γ_{1A}-globulin.

Native γ_{1A}-globulin, as also the 7 S γ- and 19 S γ components of the immune globulin system, consists of a hetero-

geneous population of molecules which are distributed over a rather broad electrophoretic spectrum of mobilities. One of the disadvantages of the presently described method is that the purest γ_{1A} fractions here obtained appear to contain a predominant proportion of the faster component of γ_{1A} family. The slower portions are eluted with the 0.02 M fraction, which, as already stated, is heavily contaminated with 7 S γ-globulin.

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The Formation of Specific 7 S and Macroglobulin Type Antibodies in Chickens

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The heterogeneity of antibodies has now been demonstrated in different characteristics of the antibody molecule, i.e. in serological properties, physico-chemical character and in the antigenic structure /6/. Considerable data have been collected on the formation of different types of antibodies. For example, the immunization of rabbits with foreign erythrocytes led to the formation of antibodies which were mainly of the macroglobulin type at the beginning of immunization, while after repeated immunization a large proportion of antibodies was formed with an electrophoretic mobility of $gamma_2$ and a sedimentation constant of 7 S /15, 14, 8/. This time course in the formation of antibodies of the $gamma_{1M}$ and $gamma_2$ type was demonstrated in further types of corpuscular and soluble protein antigens /2/.

These facts, pointing to the interrelationship between the formation of macroglobulin and 7 S antibodies, were further confirmed by the finding that both types of antibody are also formed together in the lower vertebrates /16, 17/ and bytthe fact that a similar time course in the formation of the two types of antibodies was found in the period of immunological immaturity /13, 11/.

Little is yet known of the serological properties of different types of antibodies. It has been shown that macroglobulin antibodies have higher haemolytic activity and bind less complement

than 7 S antibodies /14, 8/. Macroglobulin antibodies are also essentially more effective in the haemagglutination reaction in which 700 times less macroglobulin than 7 S antibodies are necessary to produce the same effect /9/. These results, however, touch onlonly a small part of antibody activity and it would be most important to carry out a comparison and different serological manifestations on reaction with antigen.

We have attempted to make a comparison of the activity and specificity of antibodies of the macroglobulin and 7 S type. Chickens were used as the source of antibodies since they are very good producers of antibodies to soluble protein antigens and produce macroglobulin antibodies in relatively high amounts /1, 4/. In addition antibody formation in chicken is very rapid, a high level appearing even after primary immunization.

A group of chickens /Leghorn, weight 1.5 kg/ were immunized with one dose of 50 mg. of human serum albumin p-azobenzoic acid /p-ABA-HSA /9/.

Antibodies to protein carrier determined by the haemagglutination of HSA sensitized erythrocytes appeared in all chickens as soon as on the third day after immunization, whereas antibodies to hapten, also determined by haemagglutination, did not appear until the seventh day after immunization and then only in low titres similarly as described by Gold and Benedict /7/. The birds were exsanguinated on the seventh day and the types of antibodies present in the serum determined by ultracentrifugation in a sucrose gradient and by haemagglutination of the separate fractions. In accord with the data in the literature /1, 4/ antibodies to HSA were of both the macroglobulin and 7 S type. On the other hand, antibodies to hapten were exclusively of the macroglobulin type /Fig.1/.

Since in the first experiment we found that, after primary immunization, antihapten antibodies were of the 19 S type, in a further experiment we attempted to determine whether antibodies of two physico-chemical types can be formed to one determinant group, i.e. haptenic group bound to the protein molecule. We therefore investigated whether antibodies of the 7 S type were not also formed against hapten on repeated immunization. A further group of chickens was immunized repeatedly with 40 mg. of p-ABA-HSA given intravenously at weekly intervals. Blood was collected on the seventh day after immunization before giving the next immunization dose. As evident from Fig.2, antibodies to hapten in the first two collections were of the macroglobulin type and after the third immunization dose antihapten antibodies of the 7 S type appeared, so that after further immunization antibodies of both types were present in the serum. These antibodies to hapten were demonstrated by haemagglutination of p-ABA, bound by azo-linkage to erythrocytes. In this system, in addition to the actual hapten, the amino acids, with which diazotized p-ABA reacted, could form part of the determinant group, whereas the other carriers, i.e. HSA on immunization and erythrocytes on detection, are quite different. This denotes that both types of antibodies, macroglobulin and 7 S, are formed against the same determinant group and that hapten alone or possibly a hapten-azo-amino acid residue /12/ are sufficient in both cases to produce a positive reaction. Bauer /3/ demonstrated 19 S and 7 S antibodies to the same hapten in rabbits, similarly.

That the two types of antibodies have the same specificity does not, of course, denote that both must have the same immunochemical properties, i.e. an equally large combining site, the same space configuration of the combining site and therefore an equally

firm binding with antigen. Unfortunately, we could not used antihapten antibodies to obtain more details about macroglobulin combining site because these antibodies occured in too small amounts in the serum and attempts to concentrate them were unsuccessful. In further experiments, therefore, we used antibodies to BSA which were formed after immunization in sufficient amounts for serological study.

The anti-BSA antibodies were separated into macroglobulin and 7 S types by filtration on Sephadex G-200. Antibodies from the corresponding fractions were concentrated by precipitating the globulins with 30% saturated sodium sulphate, and dissolved in a small volume of saline. The sodium sulphate was removed by filtration on Sephadex G 25.

We worked with pooled sera from three groups of chickens, i.e. taken after primary immunization, after secondary immunization and finally with hyperimmune serum. The birds were immunized intravenously with 40 mg BSA at monthly intervals, hyperimmune serum was collected after the fifth immunization.

In accord with the data in the literature, in all three groups we found haemagglutinating antibodies in both the macroglobulin and 7 S fractions. Fig.3 shows the eluates of the primary and hyperimmune sera from the Sephadex column and haemagglutinating titres of the separate fractions. It is evident that in the primary sera most haemagglutinating activity was present in the fraction containing macroglubulin and in the fraction containing antibodies of the 7 S type anti-BSA antibodies were demonstrated only after concentration with sodium sulphate. In hyperimmune sera haemagglutinating antibodies were present in both peaks. A most interesting fact was discovered on determining the presence of cross-reacting antibodies. If a comparison is made of the titres of haemagglu-

tination of erythrocytes sensitized to BSA and cross-reacting with HSA in the separate fractions of the eluate, it can be seen that antibodies cross-reacting with HSA are mainly present in the fractions containing the macroglobulin antibodies, whereas in the 7 S fraction their titre is incomparably lower as compared with that of anti-BSA antibodies.

Antibodies were further determined by quantitative precipitation. In concentrated fractions of antibodies of 7 S or macroglobulin type the precipitin reaction was made in 0.15 M NaCl and in 1.5 M NaCl to determine both types of antibodies /4/. In concentrated antibodies of the macroglobulin type we were not successful in demonstrating the presence of detectable amounts of precipitating antibodies in any of the pooled sera tested. However, in the case of macroglobulin antibodies the sensitivity of the reaction was greatly decreased since these antibodies precipitate spontaneously so that the control values were so great that they did not permit the determination of amounts of antibody of less than 20-30 /ug Ab/ml.

The precipitation of antibodies of the 7 S type was positive in 1.5 M NaCl in all three pools of sera investigated. In 0.15 M NaCl the reaction was positive after the secondary immunization and in hyperimmune serum, but not in the primary serum. The amount of antibodies precipitating in 0.15 M NaCl or in 1.5 M NaCl varied so that the relatively highest amount of antibodies precipitating BSA at 0,15 M NaCl in the mixture of hyperimmune sera similarly as was described by Benedict et al. /4/. This also explains the increase in haemagglutinating antibodies in fractions corresponding to antibodies of the 7 S type during immunization, since only antibodies precipitating in 0.15 M NaCl

/4/ are effective in haemagglutination.

A comparison of the amount of type 7 S antibody precipitating in 0.15 M NaCl with haemagglutination titre of that fraction showed that the sensitivity of the haemagglutination reaction is within the usual range of sensitivity for passive haemagglutination. If we compare the high haemagglutination titres of antibodies of the macroglobulin type with the negative results of quantitative precipitation /what means that the amount of these antibodies was less than 30 /ug Ab/ml/, it is evident that the macroglobulin antibodies in chickens are much more active in haemagglutination than the 7 S type antibodies. Evidently in passive haemagglutination chicken macroglobulin antibodies react similarly as rabbit antierythrocyte γ_{1M} antibodies, where Greenbury et al. /8/ found that for producing the same haematolytic effect an incomparably smaller amount of macroglobulin antibodies is neededthan in haemagglutination with 7 S antibodies.

We next attemted to determine the specificity and binding power of haemagglutinating antibodies of the two types. For this purpose we used cross reactions with HSA and inhibition of haemagglutination. Inhibition was studied by adding 5 /ug of BSA or HSA to each tube of serial dilution of the sera tested and by comparing the result of haemagglutination with that of the control.

Fig.4 gives a comparison of the reactions of all three test sera. It is evident that in cross reactions the antibodies of the two types differ considerably. Antibodies of the anti-BSA macroglobulin type give cross reactions with HSA sensitized erythrocytes to a high degree in all three cases. In the case of antibodies of the 7 S type cross reactions were essentially less, i.e. these antibodies showed a far higher specificity to BSA. Similar differences were displayed in the haemagglutination in-

hibition reaction. Macroglobulin antibodies were only inhibited by homologous antigen to a small extent, whereas the reaction of the 7 S type antibodies was almost completely inhibited by homologous antigen. On comparing the results of cross reactions of macroglobulin antibodies from the samples tested no essential differences could be found; 7 S type haemagglutinating antibodies on the other hand cross react in hyperimmune and secondary sera less than do antibodies in the primary sera.

The high degree of cross reactivity of macroglobulin antibodies can be explained in two ways. First, by a different mechanism of formation of these antibodies and thereby a different degree of response to separate determinant groups of antigen used for immunization, which could lead to a higher formation of antibodies to determinant groups common for related antigens. The second possibility explanation is that the combining site of macroglobulin antibodies is less complementary to the determinant group of antigen which would permit a higher degree of cross reactivity. The lower degree of complementarity could be due to the combining site being smaller in macroglobulin antibodies than in 7 S antibodies or to its being less exactly delimited in space /less rigidity of the structure of the site of linkage/. If the second explanation of the high cross reactivity of macroglobulin sera, i.e. difference in the combining site of antibody were true, this property would necessarily manifest itself in greater dissociation of the antigen-antibody complex. This corresponds to the results of the inhibition reactions which show that the system macroglobulin antibody-BSA-erythrocyte is only very little inhibited in the presence of free antigen which is evidently due to the high dissociation of the antigen-antibody complex.

Summarizing our results we can say that macroglobulin antibodies have different properties from 7 S antibodies. Both types of antibodies can arise to the same determinant group but the specificity and firmness of the binding of macroglobulin antibodies is lower than that of 7 S antibodies.

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Formation of 19 S and 7 S Type Viral Antibodies. The Role and Nature of "Antibody Cofactor".

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In recent years data have been published on the formation of 19 S and 7 S type antibodies in response to various viruses /6, 7, 1 et al./. In our contribution, findings will be presented mainly concerning the nature of antibodies to influenza viruses. A further difference in the character of anti-influenza antibodies will be described in addition to the 19 S - 7 S type, i.e. the change in their ability to be potentiated by the so-called "antibody cofactor". We will show that misleading results in the detection of 19 S, and especially of 7 S influenza antibodies can be obtained if the additive effect of cofactor is disregarded. Some characteristics of this serum component will be therefore presented first.

We found /4, 5/ that the titres of specific influenza antihaemagglutinins decreased after heating of antisera /56 °C/30 mins./, if a "non-avid" A2 influenza strain /i.e. a strain of low antibody sensitivity, also designated A2- /cf 2/ was used as antigen in the haemagglutination inhibition test /HI/. The decrease was much more marked in sera taken after first virus administrations than in hyperimmune sera /Fig.1/. It was possible to restore the titre of HIT antibodies to their original, or even to a higher, level /Fig.1/ by adding normal unheated serum containing no inhibitors to A2-virus.

The fact that the activity of heated or stored immune sera increases after the addition of normal unheated serum, is not a new finding in virology /Gordon, Mueller, Meyer, Morgan, Whitman, Leymaster and Ward, Chanock, Casals and others/. Our experiments, however, offered evidence that, at least with influenza viruses, the potentiating factor is not complement, but an entirely different thermolabile serum component. We proposed the name "serum cofactor" or "antibody cofactor" for this component.

Several approaches were used to prove that the cofactor is not identical with complement or its components /C'1 - C'4 /. Clear evidence was obtained when comparing the titres of cofactor and C' components in sera freed of complement by binding on the antigen-antibody complex. Further, substances with anticomplementary activity, such as dextran sulphates, heparin, pelentan and trypsin, or the removal of the C'3 component of complement by zymozan treatment, do not substantially affect the cofactor titre. The presence of EDTA, which blocks the activity of C'1 component of complement, doeshot inhibit the cofactor activity in human, mouse, pig, piglet and horse sera.

With some animal sera we were successful in separating the cofactor from the components of complements, using gel filtration. Separation of guinea pig serum on Sephadex G-200 is illustrated in Fig.2. Cofactor activity occurred mostly in the first peak containing the macroglobulins, whereas the individual complement components were mainly present in the 7 S peak. It must be added that with many other sera the first C' component was also detected in the macroglobulin peak. /C' components were detected using reagents R1, R2, R3 and R4, deficient in the respective C' component/.

On paper electrophoresis, the cofactor from mouse serum moved along with β-globulins. On immunoelectrophoresis, the cofactor activity of bovine serum was bound to the α_2-macroglobulins. The macroglobulin character of cofactor from various animal sera was also demonstrated by density gradient zonal centrifugation /3/. In Fig.3 the macroglobulin nature of mouse, bovine and pig cofactor is evident. However, the cofactor from serum of newborn unsuckled piglets is of lower molecular weight showing an $s_{20,w}$ value of 3.0 to 4.0 S. The smaller molecular size of piglet cofactor was also confirmed by chromatography of piglet serum on Sephadex G-200 /Fig.4/.

Now some remarks on the 19 S and 7 S influenza antibody formation. The character of antibodies in rabbit serum taken 10 days after administration of non-avid A2-influenza virus is illustrated in Fig.5. Both 19 S and 7 S antibodies can be detected in unheated samples against the homologous antigen. The titres of these anti-haemagglutinins decrease after heating /56°C/30 mins/ but can be restored by adding cofactor in the form of normal mouse serum. - Using the A2+ virus as antigen in the HI test, the 7 S globulins can also be detected in this antiserum, though in a low titre, which is rather surprising. We cannot say, however, in what quantity 19 S anti-A2+ antibodies are present in this antiserum. The samples from the macroglobulin peak show quite high HI activity, but we found that the nonspecific γ inhibitor /against A2 + strains/ is present in this peak after Sephadex chromatography of normal sera.

The Sephadex separation of another rabbit antiserum is illustrated in Fig.6. The serum was taken 10 days after A2+ the

administration of influenza virus. The formation of 7 S antibodies against the homologous antigen is obvious. The 19 S antibodies are also probably present, but - as in the previous case - they cannot be differentiated from the non-specific γ inhibitor. Both 19 S /in a small quantity/ and 7 S antibodies can be detected against a non-avid /-/A 2 strain, but only after addition of the serum cofactor, the importance of which thus becomes clearly evident.

Extensive immunisation experiments on white mice revealed great variability as far as 19 S and 7 S antibody response is concerned. There were cases where 19 S antibody formation preceded that of 7 S antibody, as expected. However, in other experiments, 7 S antibody appeared simultaneously with, and in equal or even higher amounts than 19 S antibody. This occured on the 3rd - 5th day. Detection of antibodies - or their titre - depended on whether serum cofactor was present in the respective fractions. This is especially true of 7 S antibody, because owing to the macroglobulin character of cofactor, the later was not present in fractions from the 7 S globulin peak. A similar case is shown in Fig.7 which illustrates the separation of mouse serum taken 5 days after virus administration. Judging from results which samples tested in saline, a small amount of 19 S and 7 S antibody should be present. However, if the same samples are tested with cofactor added /i.e. using normal mouse serum 1: 15 as diluent/, the detected amount of 7 S antibody increases considerably. This is also true for virus neutralizing /VN/ antibodies. /VN activity was tested only in fractions from the top of the first and second protein peaks/. In many cases the activity of 7 S antibody was much more enhanced by the cofactor than that of 19 S antibody.

On summary, we would like to point out that in addition to the replacement of 19 S by 7 S antibodies, a further change in the character of antibodies from "early" and "late" influenza antisera can be observed, i.e. their ability to be potentiated by the "antibody cofactor". The former change /19 S - 7 S/ is usually more rapid and does not coincide with the latter because antibody which is and that which is not potentiated by the cofactor can be both of the 7 S type.

Serum cofactor does not play any role in the activity of antibody /either 19 S or 7 S/ to bacteriophage Ø X 174. Our results of 19 S and 7 S antibody formation were similar to those in experiments of Uhr and Finkelstein /7/. However, in addition to the expected sensitivity and insensitivity of 19 S and of 7 S antibody, respectively, to mercaptoethanol, we observed the formation in rabbit of 7 S type anti-phage antibody which was sensitive to 2-mercaptoethanol treatment.

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The Occurence and Significance of Natural Antibodies

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The means by which the antibody-forming system discriminates between material to which it is naturally responsive and that to which it is naturally unresponsive is unknown. The point is a very crucial one in immunological theory /20, 19/ and knowledge of the mechanism of the discrimination would undoubtebly help towards an understanding of the whole process of antibody formation.

A similar problem faces those who are interested in phagocytosis, for the phagocytic system of multicellular animals also possesses a remarkable discriminative capacity. The resemblance of these two problems is indeed close, for in each instance the cells appear to "recongize" and respond positively to the same category of substances: in both the antibody-forming system and the phagocytic system, the response appears to be elicited by macromolecular groups which are not normally present in the body fluids + /6, 7/- A difference between the two problems is that recognition by the antibody-forming system seems only to apply to

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+For want of a better term, the word "foreign" will be used to denote material, whether autochotonous or of extrinsic origin, which is not normally present or exposed in the body fluids.

vertebrates, whereas recognition of foreign matter is a property of phagocytes throughout the metazoa.

The suggestion has been put forward that the means by which phagocytes, in vertebrates and invertebrates, identify foreign matter is the same, or at least very similar, to that which operates in the immune response /6, 7/. It seems reasonable to postulate that, since a very efficient mechanism of discrimination between foreign and not-foreign matter was already present in the invertebrate ancestors of the vertebrates, the same mechanism may have been utilised during evolution to form the basis of discrimination by the cells responsible for the production of antibodies. It must be admitted that this concept is not compatible with the clonal selection theory /19/, according to which immune discrimination is due to the presence in the body of a vast number of genetically different cells, each responsive only to one or a few foreign antigens, and to the absence of mutants which would be capable of responding to normally exposed self-components. Obviously such a mechanism could not account for discrimination by phagocytes, which are multipontential with regard to the macromoleculas which they appear to recognize as foreign. However, we feel that a more complete examination of the problem of discrimination by phagocytes might help towards an understanding of the first essential step in antibody formation- the recognition of foreign antigens.

A number of theories of antibody formation require the existence in the body fluids, or on the surfaces of cells, of what may be termed "recognition factors" /24, 68, 39, 58, 5/. According to these theories, recognition of the antigen depends on the presence of special substances which combine with foreign determinant groups in the initial stage of the process of anti-

body formation, serving initially to link or associate the foreign molecule with certain cells. Only recognition factors reactive with foreign or unfamiliar determinant groups are present.

These introductory remarks provide the background to our interest in "natural antibodies". The question we ask is whether "recognition factors" can be detected in non-immunised animals, and whether natural antibodies, or certain types of natural antibodies, might be the postulated recognition factors. At the present state of our knowledge the best we can do is to consider whether the information available about natural antibodies is compatible with this notion, and we will do so bearing in mind the concept that recognition by phagocytes and by the antibody-forming system may be due to essentially the same mechanism.

At this point something must be said about the term "natural antibody", in order to avoid, as far as possible, unnecessary semantic debate. Difficulties arise if we follow rigidly any of the usual definitions of antibody. Some definitions, for example, insist that an antibody must be a globulin. But if we find substances in the blood of, for example, an invertebrate with the same sort of biological activity and function as the gamma-globulin antibodies of vertebrates, but with different electrophoretic mobility - are we then precluded from calling them antibodies? The requirement that antibody should react specifically with antigen raises difficulties which will be mentioned below. Biological activity does not provide a good basis for a definition. Many virologists use specific neutralisation of virus infectivity as a test for antibodies; nevertheless gamma-globulins are sometimes detectable in sera of immunised animals which react specifi-

cally with virus, but which enhance its infectivity /30/. Nor can phenomena such agglutination, complement fixation, opsonisation etc. be used as a basis for definition, for not all antibodies give positive reactions in these tests even when the antigen is suitable.

Further discussion on this point is not worthwhile. In this paper, the term natural antibody will be used in a very broad sense for substances /probably always protein/ which are present in the body fluids of normal animals and which can be shown to combine with foreign matter but not with normally exposed "self-components"; it is assumed that natural antibodies have some role to play in keeping the tissues free of extraneous and unwanted matter although this function is not, of course, always demonstrable.

Natural Serum Antibodies

Occurrence

The fact that normal serum from many different species of vertebrates contains substances with physico-chemical and biological properties similar to those of the antibodies which appear, usually to much higher levels, following immunisation has been known since the end of the last centrury. The references in the literature to such natural antibodies are innumbrable; it is almost impossible to pick up a number of a current immunological journal which does not contain some reference, direct or indirect, to natural antibodies. The subject has frequently been discussed in textbooks and review articles /88, 73, 47, 50, 71/.

Natural antibodies have been described in vertebrate sera reactive in one test or another with very many different kinds of bacteria /see reviews mentioned above/, with bacteriophage /40, 80/,

zymosan /61/, starch particles /62, 81/, protozoa /72/, fungi /15/, metazoal parasites /70/, heterologous and isologous erythrocytes /56, 31, 2, 47/ and mammalian tissue cells /48, 77/. Sera of normal animals have also been shown to contain antibodies against components of the host not normally exposed in the circulation, including spermatozoa /53/ and extracts of various tissues /43, 9/.

Natural antibodies appear to have been demonstrated in sera of all vertebrate species examined, including reptiles and fishes /see 72/. Some authors have attempted to rank different species according to the levels of natural antibodies in their sera /18, 41, 28/, but the usefulness of this procedure is doubtful, especially since the authors differ in the order in which they place the different species.

There are also many reports of substances in the body fluids of invertebrates with in vitro biological activities similar to those of antibodies in mammals. Bactericidins, haemagglutinins for various animal cells etc. have been described /91, 35, 21, 84/.

It is thus clear that normal sera from all vertebrate animals and many invertebbates possess antibody-like activity detectable by one procedure or another against a vast array of antigens. In connection with the present topic the important question arises whether or not natural antibodies detectable in the sera of vertebrates have been produced in response to an antigenic stimulus, perhaps from a foreign macromolecule which shares determinant groups with the test antigen. Many authors /e.g.87/ believe that this to be the case in the vertebrates, and there can be little doubt that some of the antibody activity of normal serum is due to this cause. There is plenty of evidonce that many antigenic determinant groups are shared among microorganisms and animal tissues;

the Forssman antigen is the most well-known example /26, 16, 4/. The fact that titres of many natural antibodies increase with age is compatible with this view /22, 88/.

If all natural antibody activity were the consequence of previous antigenic stimulation, then the idea that the postulated recognition factors are detectable as natural antibodies would obviously not be tenable. However, although stimulation by cross-reacting antigens is responsible for some of the antibody activity of normal serum, particularly when high titres are demonstrable, dogmatic statements that all antibodies detectable in normal serum are the consequence of antigenic stimulation are not justified at the present time. Some authors, in fact, take the view that both the level and the specificity of natural antibodies are genetically determinad /33, 42/, and there are several studies indicating that genetic factors control the level in the serum of certain natural antibodies against heterologous antigens /52, 74, 75, 44, 36/. It is well know, of course, that the production of certain isoantibodies is under genetic control.

The question can be put another way; are natural antibodies detectable against all the antigens against which the host is capable of responding immunologically? An affirmative answer to this question would be strong support for the recognition factor hypothesis, but unfortunately, available evidence does not justify a definite answer one way or the other. The problem does not seem to have been attacked systematically, perhaps because the task would seem to be too stupendous. However, a single clear demonstration that "natural" antibodies do not exist against any single antigen to which a given animal is immunologically responsive would be sufficient evidence to eliminate the hypothesis that natural anti-

bodies are the recognition factors for acquired immunity. Even in such a case, one could always argue that the test used was not sufficiently sensitive. Certainly a negative result in one of the classical techniques, depending on agglutination, passive haemagglutination, bacterial activity and so forth would not warrant the conclusion that natural antibodies are not present. It is sometimes stated, for instance, that normal guineapig serum contains no natural antibodies reactive with sheep erythrocytes /e.g. 54/. The resuls given in Table 1 show this statement to be incorrect. It is true that normal guineapig serum does not agglutinate sheep cells, although it has fairly high agglutination titres against possum and rabbit red cells. However, if sheep cells, after exposure to dilutions of normal guineapig serum, are washed and resuspended in rabbit anti-guineapig serum, they agglutinate to a titre of about 1/2560 of the guineapig serum. The factor in the guineapig serum which renders the sheep cells agglutinable by rabbit anti-guineapig serum is specific, in that absorption of the serum with sheep cells completely removes it, but leaves the titre against rabbit and possum red cells unchanged.

Another source of error is the possibility that the test mixture may contain substances which interfere with the combination of an antibody with its antigen or with some observeable effect of this reaction. It has been found that certain antibacterial antibodies interfere with the bactericidal activity of other antibodies reactive with the same organism /1/. Thus, negative results of a test which depends on some secondary manifestation of antibody-antigen interaction certainly cannot be taken as evidence of the absence of antibody.

Although an answer to the question whether natural antibodies to all potential antigens are present in vertebrate sera is not yet permissible, we cannot but be impressed by the frequency with which natural antibodies are found when we look for them. It is relevant here to recall that it is a common complaint of very sensitive serological tests that "non-specific" reactions are obtained with normal sera.

Heat stability

It is sometimes stated that natural antibodies in mammals are heat labile /56°C/, whereas "immune" antibodies are stable at this temperature /73/. However, the literature on this point is extremely confusing, and it would not be worthwhile to deal with the question fully here. Some of the contradictions may be due to the fact that heat treatment has been carried out at different hydrogen-ion concentrations or at different dilutions, factors which can influence the heat lability of proteins /73, 55/. There are many other factors which may contribute to the confusion, including the fact, often forgotten, that heating does not merely destroy the chemical activities of certain labile substances; it also results in the production of new activities which may interfere in some way with serological reactions /11/. One view that has been expressed is that, among the bactericidal substances in normal serum, those active against gram-negative bacteria are relatively heat stable and require complement for their activity, while those active against gram-positive bacteria are relatively less resistant to heat and do not require complement /e.g. stable at 57.5°C, but labile at 60°C - /55/ /, but there are many contradictory reports on this point /23, 81, 32, 25/. The significance of such differences is not clear, but the work of Kleckovski /45/

and of Bawden and Kleckovski /3/ suggests that apparent differences in heat stability may in fact be due not to basic differences in the antibodies themselves, but rather to differences in the antigens.

It would not be useful here to attempt to compare in much detail the results of different workers using so many different techniques to study different reactions of different antigens with normal sera from different species.

Specificity

The general statement is often made that true natural antibodies are less specific than "immune" antibodies, and are less easily absorbed out of serum /88/. On the other hand, there are many reports of highly specific antibodies in normal serum /e.g. 60, 59, 1, 81, 36, 31, 17/. Two factors need to be taken into account in this connection. First, the difficulty in completely absorbing antibody activity out of normal serum as compared with immune serum does not mean that there is any fundamental physicochemical difference between the antibodies in the two cases; it may mean only that the proportion of antibodies with high avidity for the antigen is greater in the serum of an immunised animal than in normal serum, a fact which may be anticipated from any selection theory of antibody formation /76/. Second, when comparing antibody activity against different antigens, the demonstration that the antibodies against one antigen are more specific than those against another does not necessarily mean, as is often implied, that one is dealing with two different kinds of antibody. In many such cases it is just as likely that it is the antigens which differ, one being more common in nature than the other. Thus, relative non-specificity can be anticipated in anti-

bodies reactive with polysaccharides, since there is much more sharing of determinant groups among the polysaccharides in different species of plants and animals than among the proteins.

Some recent experiments in our laboratory have confirmed the suspicion that there is an apparent trend towards a decrease in specificity of natural antibodies as we go backwards in the evolutionary scale.

Tyler and Metz /84, 83/ have suggested that the body fluids of certain invertebrates contain relatively few antibodies reactive with animal antigens, each specific for an antigen characteristic of whole groups of animals. There are some difficulties in accepting this view and further work needs to be done on the subject.

Recognition by phagocytes

It has been known for 70 years that phagocytosis by leucocytes of mammals is promoted by components of normal serum which were called "opsonins" by Wright and Douglas /89/. Opsonins became adsorbed onto the surfaces of foreign particles which are thereby rendered attractive to the phagocytic cells. Much work since that time has shown that in most cases two factors in normal serum are involved in this activity, the one heat stable /56°C/ and specific, the other heat labile and non-specific. The latter factor is often considered to be complement; the former is natural antibody. There is, however, no general agreement in this field /see 11/; it has recently been claimed, for example, that the opsonic activity of normal rabbit serum against certain bacteria is due to a single non-specific heat labile substance, which is not complement /32/.

While there is still some confusion as to the exact nature of all the opsonic factors in normal serum, there is no doubt that

serum factors which combine with the surfaces of foreign particles are of immense importance in phagocytic recognition. In most cases no interaction occurs between phagocytes and particles if the latter are not coated with host gamma-globulin.

There is evidence that natural antibodies also play an essential role as recognition factors not only in the actual process of phagocytosis, but also in the first phase of the leucocytic response to foreign particles - chemotaxis. For instance, the chemotactic effect of cellulose, and foreign red cells on rabbit polymorphs depends on the adsorption onto the surface of the particles of serum components. The results summarised in Table 3 indicate that cellulose is not chemotactic for polymorphs in medium adsorbed with cellulose in the cold, although sheep red cells are chemotactic in this medium. Conversely, in medium absorbed with sheep red cells, cellulose elicits a response, while sheep red cells do not /not shown in the Table/. As in the case of complexes between soluble antigens and their antibodies, the chemotactic response seems to depend on the interaction of antigen-antibody complexes with non-specific heat labile components of serum.

Information bearing on the mechanism of recognition by the phagocytes of invertebrates is almost entirely lacking. However, in some recent experiments in vitro phagocytes of the snail /Helix aspersa/ were found to phagocytose formalinised sheep red cells in balanced salt solution only if the latter had been pretreated with snail serum /North and Boyden, unpublished/. Earlier Ruediger and Davis /67/ demonstrated "opsonins" in the blood of a number of invertebrates active against a number of bacteria. Unfortunately these authors were studying the uptake of the

treated bacteria by mammalian phagocytes in medium containing mammalian serum. Thus, while they certainly showed that the insect blood modified the surface of the bacteria, they did not eliminate the possibility that the opsonic activity migh have been due to the adsorption of mammalian serum components onto the modified bacteria, perhaps by reacting directly with adsorbed insect proteins.

Cell-bound recognition factors

It has been suggested that the specific host substance which combines with the antigen in the intial phase of the immune response is present on the surface of the potential antibody-producing cells /24, 58, 5, 6, 7/. In this connection it is pertinent to mention that there exists in rabbits a kind of antibody which, while present in the serum, also has a strong affinity for spleen cells /13/. The cells, when coated with this antibody, are capable of specifically adsorbing antigen. In a recent study a similar "cytophilic" antibody has been demonstrated in guineapigs immunised with sheep red cells mixed with complete Freund's adjuvant, and in these experiments its presence was found to be correlated with the existence of a state of delayed-type hypersensitivity /8, 10/. Guineapig macrophages which have been treated with this antibody have a strong affinity for sheep red cells, and cytophilic antibody can therefore be said to play a role in the recognition by these cells of the foreign particles. There is also some evidence suggesting that an antibody of this type, present in normal guineapigs, is important in the recognition by macrophages of effete autochtonous erythrocytes /85, 86/. The significance of natural autoantibodies has benn discussed elsewhere /Boyden, 1964a; Grabar, 1958/.

Further work is necessary to show whether cytophilic or cell-bound antibodies are always present in small amount in normal animals; but on the face of it, it would seem that natural cytophilic antibodies are good candidates for the role of recognition factors in antibody formation.

Comment

In spite of the large ammount of work done on natural antibodies, it is not possible at present to draw any definite conclusions as to their nature and origin. We may safely state, however, that the body fluids of both vertebrates and invertebrates have a very broad range of reactivity towards different foreign antigens, and that the natural antibodies responsible for this reactivity are the main means by which the phagocytes of vertebrates and possibly those of invertebrates discriminate between foreign and non-foreign matter. The natural antibodies have been separated into groups by various workers on the basis of differences in heat stability, range of specificity, electrophoretic mobility /66/, weight /34, 46, 27, 82/, cytophilic properties and so forth. While the significance of the various differences is by no means clear at present, it is not impossible that one of the groups represent the postulated "recognition factors" of the immune response.

Of possible relevance in this connection is the report that the capacity of 3 week old baby pigs to produce antibodies against certain antigens is decreased if they are deprived of maternal colostrum. Antibody production in the colostrum-deprived pigs is said to be stimulated by mixing small amounts of horse antibody with the antigen at immunisation /69/. It has also been shown that antibody production in mice /78/ and in rabbits /51/ is

greater if antigen-antibody complexes are injected /in antigen excess/ than if the antigen is injected alone. Perhaps experimental use could be made of the interesting observations that the general level of natural antibodies /unrelated serologically to the stimulus/ increases markedly in various illnesses /37/ and also following the injection of certain bacterial products /50, 64, 65, 38, 57/.

The time is now ripe for a new onslaught on natural antibodies, using new physico-chemical techniques, and preferably very sensitive serological methods which detect antibody-antigen combination directly and which do not depend on some unreliable manifestations of the interaction.

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Table 1

	Serum dilutions												
	1/5	1/10	1/20	1/40	1/80	1/100	1/300	1/640	1/1280	1/2560	1/5120	1/10240	Saline Control
Direct test													
Sheep red cells	-	-	-	-	-	-	-	-	-	-	-	-	-
Rabbit red cells	+	+	+	+	+	+	-	-	-	-	-	-	-
Possum red cells	+	+	+	+	-	-	-	-	-	-	-	-	-
Anti-globulin test													
Sheep red cells	+	+	+	+	+	+	+	+	+	+	±	-	-
Rabbit red cells	+	+	+	+	+	+	+	+	-	-	-		-
Possum red cells	+	+	+	+	+	+	+	+	+	-	+	-	-

Twofold dilutions of normal guineapig serum /heated 56°30'/ were made in 0.4 cc. quantities of saline. 0.1 cc. of a 1% suspension of red cells were added to each tube. The tubes were left overnight on the bench and the test was read /Direct test/. The cells were then washed once in saline and resuspended in 0.5 cc. 1/50 rabbit anti-guineapig serum and a second reading made after 4 hours /Anti-globulin test/.

Table 3

Effect of absorption of serum with cellulose

	Migrating cells
Medium	3
Absorbed Medium	0
Medium and Cellulose	143
Absorbed Medium + Cellulose	16
Medium + Sheep Red Cells	132
Absorbed Medium + Sheep Red Cells	155

Medium, consisting of 20% Normal Rabbit Serum, was absorbed with cellulose at 0°C /100 mg. cellulose per 0.1 cc serum/. The figures refer to the number of polymorphs /per field, 400 x magnification/ which have migrated through a Millipore Filter Membrane /ave.pore size 3 micron/ towards the test mixture /see 6/.

Carbohydrate Heterogeneity in Rabbit 7 S Gamma Globulin

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Gamma-Globulins from several species have been shown to contain a small amount of carbohydrate which is covalently bound to the protein. 7 S Y-globulins contain about 2 - 2,5% of carbohydrate consisting of mannose, galactose, hexosamine, sialic acid, and fucose, though the content of fucose is less than a mole/mole for rabbit Y-globulin. Although the biological function of the carbohydrate is not known, it is an essential feature of the structure, and chemical studies have been carried out to determine whether there are one or more oligosaccharide units on each molecule of Y-globulin.

Emil Smith and co-workers isolated glycopeptides from papain digests of heat denatured Y-globulin and concluded, from amino acid sequence studies on the glycopeptides, that human, bovine, and rabbit Y-globulin contained only one oligosaccharide unit /13,8,9/. However, Porter /11/ found that when native rabbit Y-globulin was split into three pieces by digestion with papain, about three-quarters of the hexosamine was present on one of the pieces, III, and the other quarter was associated with piece I. Also, the ratio of hexose to hexosamine of III was about 1,0, whereas for piece I it was 1.35 /4/. This suggested that there were two oligosaccharide units on the rabbit Y-globulin molecule and that they were of differing composition. Similar results were reported by Franklin /6/ for human Y-globulin.

It was shown by Edelman /2/, by Edelman and Poulik /3/, and by Franěk /1961/ that when Y-globulins are reduced in 6M urea, their molecular weight falls from about 150,000 to 50,000, indicating that Y-globulin of all species studied contained several peptide chains joined together by disulphide bonds. As a result of further studies on the products of reduction and their relationship to the products of papain digestion, the four-chain structure shown in Fig. 1 was postulated /12,4/. The two A chains have molecular weights of 50,000 and the B chains 20,000 /10/. Analyses of the isolated chains revealed that 95% of the carbohydrate was covalently linked to the A chains /4/ and, since there are two A chains per molecule, it is assumed that there is one oligosaccharide unit on each A chain or two to each molecule of Y-globulin. The carbohydrate composition of rabbit Y-globulin and the isolated chains is given in Table 1.

The value of four hexosamine residues A chain is in agreement with the analyses of the glycopeptides isolated by Nolan and Smith /8/ from a papain digest of heat denatured rabbit Y-globulin /Table 2/. They also obtained, in small yield, a glycopeptide containing only aspartic acid and carbohydrate and, by digesting the other glycopeptides with leucine amino peptidease and also by the Edman degradation technique, they concluded that the carbohydrate was linked to an aspartic acid residue which was in the C-terminal position in these glycopeptides. We have digested fully reduced rabbit Y-globulin with pronase and separated the glycopeptides into two fractions which account for the whole of the carbohydrate of the Y-globulin. Analyses of the two glycopeptide fractions /Table 3/ show that there are four

hexosamine residues/mole aspartic acid. Fraction 1 is probably pure, and carboxypeptidase A released free serine and threonine but no aspartic acid, which supports the conclusion of Nolan and Smith that the carbohydrate is linked to the aspartic acid residue. The hexose-hexosamine and sialic acid/hexosamine ratios of the two fractions differ /Table 3/ but fraction 1 accounts for only 16% of the total hexosamine. However, it is apparent that there is some heterogeneity in carbohydrate composition and, since the relative yields of these two glycopeptide fractions is the same for both fast and slow mobility Y-globulins, the heterogeneity is unlikely to be due to contamination with other serum components. Glycopeptide fraction 2 is presumably a mixture, but both fractions could be part of the same amino acid sequence. On the other hand, comparison with the glycopeptides isolated by Nolan and Smith /8/ /Table 2/ shows striking differences in amino acid composition, in particular, the absence of phenylalanine from the pronase glycopeptides. From sequence studies on their three major glycopeptides, Nolan and Smith deduced that they were all part of a single amino acid sequence:

$$\text{Glu} - \text{Glu}\ /NH_2/ - \text{Glu}\ /NH_2/ - \text{Phe} - \text{Asp}$$
$$\text{Carbohydrate}$$

the aspartic acid being C-terminal and linked to carbohydrate. From this they concluded that there was only one oligosaccharide unit attached to Y-globulin. As our glycopeptides contained no phenylalanine /Table 3/and in Noland and Smith's glycopeptides there was no serine or threonine /though glycopeptide 4 did contain traces of these amino acids/ we assume

that in the glycopeptides obtained by pronase digestion the aspartic acid residue is N-terminal, and the sequence around the carbohydrate deduced from our work and that of Nolan and Smith is:

Pronase Papain

Glu - Glu /NH_2/ - Glu /NH_2/ - Phe - Asp - /Ser, Thr/ /Pro, Glu, Lys/

Carbohydrate

Presumably the pronase splits the peptide bond between phenylalanine and aspertic acid, and papain cleaves the bond involving the alpha - carboxyl group of aspartic acid.

Although 95% of the carbohydrate or rabbit γ-globulin is attached to the A chains, it was found, as stated above, that carbohydrate was associated with two of the three pieces resulting from papain digestion of native rabbit γ-globulin, three-quarters with piece III, and one quarter with piece I. As can be seen, from Fig. 1, piece I consists of the B chain and part of the A chain /A piece/ and these can be isolated by reduction of piece I and fractionation on a Sephadex G-75 column in N-propionic acid. When A piece and B were analysed for carbohydrate, only trace amounts were found. It was then discovered that the carbohydrate associated with piece I was not covalently linked to it but could be dissociated from it, without prior reduction, at low pH and it has been isolated as a glycopeptide fraction by running papain piece I down a Sephadex G-75 column with N-propionic acid /Fig. 2/. The yield of the glycopeptide fraction varies, for different preparations of γ-globulin, from 20 - 30% of the hexosamine content of the γ-globulin, but there is no

apparent relationship between the yield and the mobility of the Y-globulin. As shown in Table 4, with a Y-globulin preparation of slow electrophoretic mobility which on papain digestion gave predominantly piece II, 30% of the hexosamine was recovered as glycopeptides, whereas for another Y-globulin preparation which yielded equal amounts of I and II on digestion, the glycopeptides accounted for 27% of the total hexosamine. The association of this glycopeptide fraction with piece I is fortuitous and results from the method used to separate the products of papain digestion. We have found that pieces I and II do contain a small amount of covalently bound carbohydrate, 0.2-0.4 mol. hexosamine/mol. I or II, which is of the same order as that found in the B-chain. Mandy et al. /1963/ reported 2.5 mols. hexosamine/mol. I or II, though there was no evidence as to whether this carbohydrate was covalently bound. However, as these authors used the very short time of 5 mins. for papain digestion of Y-globulin /in contrast to the 16 hr. digestion routinely used in this laboratory/ this raised the question of whether pieces I and II isolated after only a 5 min. papain digestion did contain covalently bound carbohydrate. We have analysed the products of a 5 min. papain digest of Y-globulin and found only 0.3 mol. hexosamine covalently linked to a mole of I or II, which is of the same order as found routinely after 16 hr. papain digestion. We also recovered 20% of the hexosamine as the glycopeptide fraction dissociable from piece I, after the 5 min. digestion. We conclude, therefore, that the glycopeptide fraction is not essentially the product of prolonged digestion by papain, but is released during the first few

minutes.

The carbohydrate associated with III is covalently bound and cannot be dissociated by lowering the pH. The carbohydrate composition of III and the glycopeptide fraction is given in Table 5 in terms of residues per mole of γ-globulin as determined on one sample of γ-globulin. Since there are four hexosamine residues/oligosaccharide unit, we presume that some of the molecules of piece III have two oligosaccharide units and some have none or one. The heterogeneity of the carbohydrate moiety of γ-globulin is again evident from the different ratios of hexose/hexosamine and of hexosamine/sialic acid for the two fractions in Table 5. The glycopeptide fraction dissociated from piece I is a mixture of small glycopeptides which have been separated into two fractions, alpha and beta /Table 6/. Their amino acid composition shows similarities with those isolated by Nolan and Smith /Table 2/. There is some phenylalanine, though not in molar quantity, and on digestion with pronase the phenylalanine content is reduced, as would be expected from the specificity of pronase for the phenylalanine-aspartic acid bond suggested above.

Dische and Franklin /1/ obtained a glycopeptide containing fraction from the S fragment of human γ-globulin by precipitation of the protein with trichloracetic acid and found that the carbohydrate composition was different from that of the F fragment.

In conclusion, the composition of the carbohydrate moiety of rabbit 7 S γ-globulin is not the same for all the molecules, as evidenced by the differing values for the ratio of hexose-hexosamine found for the glycopeptides. On the other hand, the

amino acid sequence immediately adjacent to the aspartic acid linked to the carbohydrate is probably the same in all molecules, though there may be sequence differences, particularly on the C-terminal side, beyond the serine and threonine residues.

It is reasonable to conclude that there is an average of one oligosaccharide unit/molecule of A chain and that papain digests some molecules of the native protein on the N-terminal side of the carbohydrate and the III piece produced has carbohydrate attached /Fig. 3a/, whereas about 20 - 30% of the molecules are split by papain on both sides of the carbohydrate, thus giving rise to a glycopeptide /Fig.3b/. This second enzymic cleavage may be due to an amino acid sequence difference in these molecules, but there is no direct evidence for this and the amino acid composition of the mixture of glycopeptides produced shows that the second cleava cleavage does not always occur exactly in the same place. In the structure suggested in Fig. 3, we have made the assumption that the digestion by papain midway along the A chain, as shown in Fig. 1, which results in production of pieces I, II and III, also gives rise to the glycopeptide fraction from some molecules. If, on the other hand, the oligosaccharide unit is near the C-terminal end, it is necessary to postulate that papain also digests about 20-30% of molecules near the C-terminal end of the A chain, thus releasing some of the carbohydrate as a glycopeptide.

The heterogeneity of γ-globulin is well known and it is apparent that this heterogeneity extends to the carbohydrate moiety, though it should be stressed that the oligo-

saccharide units cannot be involved in antibody activity since pieces I and II produced by papain digestion of antibody and devoid of carbohydrate are still able to bind their specific antigen.

TABLE 1

CARBOHYDRATE COMPOSITION OF RABBIT Y-GLOBULIN AND ITS CONSTITUENT PEPTIDE CHAINS

	Moles/mole		
	Y-globulin	A chain	B chain
Hexosamine	8.2	4.0	0.16
Hexose/mannose and galactose/	9.6	4.5	0.27
Sialic acid	0.9	0.4	< 0.01

The assumed molecular weights of A and B chains and Y-globulin are 50,000, 20,000 and 140,000, respectively.

Ref: Fleischman et al. /1963/.

TABLE 2

COMPOSITION OF GLYCOPEPTIDES ISOLATED FROM PAPAIN DIGEST OF HEAT DENATURED RABBIT γ-GLOBULIN

	Glycopeptide 2 residues	Glycopeptide 3 residues	Glycopeptide 4 residues
Hexosamine	4	4	4
Hexose	3	3	4
Fucose	1	1	1
Sialic acid	0	0	0
Aspartic acid	1	1	1
Phenylalanine	1	1	1
Glutamic acid	2	1	3

Cf Nolan and Smith /8/

TABLE 3

COMPOSITION OF GLYCOPEPTIDE FRACTIONS ISOLATED FROM PRONASE DIGEST OF FULLY REDUCED CARBOXYMETHYLATED RABBIT γ-GLOBULIN

	Fraction 1 residues	Fraction 2 residues
Hexosamine	4.0	4.0
Hexose	6.5	4.1
Sialic acid	1.1	0.3
Aspartic acid	1.0	1.0
Serine	1.0	1.0
Threonine	1.0	1.0
Glutamic acid	0.0	0.25
Proline	0.0	0.60
Lysine	0.0	0.20

TABLE 4

PROTEIN AND HEXOSAMINE RECOVERED FROM PAPAIN DIGEST OF RABBIT γ-GLOBULINS OF DIFFERENT MOBILITY

Electrophoretic mobility	Fragment	Yield of protein	Recovery of hexosamine
		%	%
Slow	I	8	0
	II	48	0
	III	33	70
	Glycopeptides	-	30
Fast and slow	I	28	0
	II	28	0
	III	33	73
	Glycopeptides	-	27

TABLE 5

DISTRIBUTION OF CARBOHYDRATE BETWEEN PIECE III and THE GLYCOPEPTIDE FRACTION DISSOCIATED FROM PIECE I FROM A PAPAIN DIGEST OF RABBIT γ-GLOBULIN

	I	III
	Moles/mole γ-globulin	
Hexosamine	2.00	6.00
Hexose	2.70	6.20
Sialic acid	0.55	0.35
Fucose	0.20	0.45

TABLE 6

COMPOSITION OF GLYCOPEPTIDE FRACTIONS DISSOCIATED FROM RABBIT PAPAIN PIECE I

	Fraction alfa residues	Fraction beta residues
Hexosamine	4.0	4.0
Hexose	6.4	4.1
Sialic acid	1.1	1.0
Aspartic acid	1.0	1.0
Phenylalanine	0.5	0.6
Glutamic acid	1.0	2.2
Serine	0.25	0.6
Threonine	0.25	0.6
Proline	0.0	2.1
Lysine	0.0	0.5

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Legends to figures

Fig.1. Diagrammatic structure of rabbit Y-globulin /Porter 1962/.

Fig.2. Separation of a glycopeptide fraction from papain-digestion piece I on a Sephadex G-75 column /61 x 2.8 cm/ in N-propionic acid, O extinction at 280 m/u. ● hexose concentration /Fleischman et al. 1963/.

Fig.3. Sites of papain digestion of A chain relative to the carbohydrate, in native rabbit Y-globulin, resulting in: a - carbohydrate attached to piece III, b - carbohydrate as glycopeptide fraction.

The Onset and Nature of the Immune Response to Heterologous Erythrocytes in Embryos and Young Chicks.

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The onset of immunological competence of the young chick to perform certain transplantation reactions occurs two days after hatching /12, 14/. The onset of antibody formation to homologous erythrocytes has also been detected at this age by measuring the rate of clearance of antigen /15/. However, although it has been possible to sensitise members of the small population of lymphoid cells of the embryo as early as fifteen days of incubation to transplantation antigens /13, 16/ similar attempts to sensitise embryos of various ages with homologous erythrocytes have only been partially successful /Solomon and Tucker, unpublished results/. In these latter experiments, embryos were injected between eleven and nineteen days of incubation with rather large doses of chicken erythrocytes; the type of clearance of a second injection of chicken cells labelled with Cr^{51} at four days after hatching was then determined. In the controls, which had received no injections of erythrocytes during embryonic life, there was no evidence for circulating antibody or for secondary-type responses /rapid elimination after a short lag phase/. In the chicks which had been previously injected, only 7 out of 31 chicks showed a secondary-type response or circulating antibody /Table 1/. Unfortunately these results are only qualitative and the doses were probably too high to provoke immunity except when the donor erythrocytes were

strongly antigenic to the recipients. The persistence of chicken erythrocytes in the embryo /$t_{1/2}$ = 7 days/ makes it cirtually impossible to find the age of the embryo when sensitisation actually occured because a small amount of antigen could persist in the circulation until the time of challenge. In order to avoid this latter difficulty, heterologous erythrocytes from a goat were used as antigen because these erythrocytes are rapidly removed from the embryonic circulation by phagocytosis. The use of mammalian cells as antigen provides a more sensitive method which can also be made quantitative. In this way the measurement of trace amounts of maternal antibody and naturally acquired antibody as well as the sensitisation of the embryo to heterologous erythrocytes has been made possible at a period in development when serological techniques are too insensitive.

Quantitative determination of antibody to goat erythrocytes in embryos and young chicks

Whole goat's blood in Alsever 's solution was irradiated with 40,000 or to destroy the lymphoid cells. A suspension of saline-washed goat erythrocytes was injected intravenously into embryos or young chicks and blood samples were taken from another vein two minutes later and thereafter at increasing intervals until goat erythrocytes were no longer detectable in smears of the blood. Goat erythrocytes are readily differentiated from chicken blood cells, not only because of their much smaller size, but also because they are enucleated. Blood smears may be examined qualitatively for the presence of goat erythrocytes immediately after withdrawal of blood; the smears are then stained with N.R.G. stain and the number of goat erythrocytes per 500 -

1,000 chicken erythrocytes counted. The percentage of goat erythrocytes cleared from the circulation is plotted against time on semi-log paper. From this line the phagocytic index

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

/where C = % concentration of goat cells at time T in hours/ is determined.

One advantage of this method over Cr^{51}-labelling of the donor erythrocytes is that there is no possibility of irradiation effects on the recipient. Another advantage is that only about 0.001 ml of blood is required for each bleeding, so that only an insignificant proportion of the blood volume of even a small animal is taken at each bleeding. Also, no fixed amount of blood need be taken thus avoiding errors in measuring small volumes ov blood. Unfortunately, sufficient goat erythrocytes must be injected to ensure that 10 - 15% of the resulting erythrocyte chimaera at two minutes after injection consists of goat cells.

a/ The direct method

The first injection of goat cells assays maternal antibody, naturally acquired antibody /in chicken/ and any other opsonising proteins /non-specific/; this injection also supplies the first antigenic stimulus. A second injection some days later assays the same factor as the first injection together with antibody produced in the primary response and also supplies the second antigenic stimulus. A third injection assays the samo factors as the first, together with any residual antibody remaining from the primary response and antibody produced during the secondary response.

Clearance curves of goat erythrocytes in embryos and chicks which are not actively immune are straight line plots on semi-log

per or, but when the chicks are actively immune /or when antibody is added to embryos - see b/ The indirect method/ the clearance curves are initially very steep during the first few minutes after injection. After this there is a much slower rate of clearance for an hour or so which, inturn, is followed by a slightly accelerated clearance rate which only occurs after second injections of antigen. The initial rapid clearance shows that antibody is present in the circulation and the rate of clearance of goat erythrocytes during this period is a measure of the amount of circulating antibody. The second phase of slower clearance is probably due to small amounts of residual antibody on the remaining goat erythrocytes /10/, and the accelerated clearance which follows may be due to active synthesis of antibody to the erythrocytes just injected.

A hazard of measuring very rapid rates of clearance in highly immune birds is that all the cells may be cleared within the first two minutes following the injection. In birds with slightly less antibody, smaller doses of erythrocytes will be cleared so rapidly that at two minutes after injection a considerable proportion of the dose will have already been cleared.

In humans, mixing of red cells is complete within two minutes /10/ and this is the earliest practicable time after injection at which bleedings may commence. This dosage effect illustrates the importance of using the same dosage for each bird within one experiment. Birds found to be highly immune by the direct method are obviously unsuitable for quantitative assay. These birds are then unsuitable for serological studies as their antibody has been depleted by the large dose of antigen. However, serum can be obtained from other members of the same group not so treated and used for serology or for testing by the indirect

method in embryos.

b/ The indirect method

The indirect method is essentially the use of an embryo as a titrating medium for the action of antibody on erythrocytes. Serum is injected into 16 day-old embryos which have just received 1.10^9 goat erythrocytes. The antibody titre can then be determined from the phagocytic index of the accelerated clearance of the goat cells. This method is about five times more sensitive than a haemagglutination test, even though about one hundred times more goat cells must be used for the test in the embryo. So far we have not used this method sufficiently to report more than a few small experiments. For instance, it is possible to "titrate" goat erythrocytes against adult chicken serum containing antibody to goat cells before and after treatment with 2-mercaptoethanol /for one hour at 37°C/. This treatment destroyed 94 % of the antibody /as by the reduced phagocytic index/ which indicates that the major part of the antibody in the primary response of the adult is almost entirely 19 S.

It has been shown that the amount of antibody in the circulation determines the rate of phagocytosis for a particulate antigen /2/. This also holds true when different amounts of serum containing antibody to goat cells was added to embryos in the indirect assay /Fig.1/.

In the direct method of assay in chicks the phagocytic index may be depressed by the injection of large doses of antigen; for example in 7 day-old chicks K = 0.5 for 15.10^9 erythrocytes injected and was depressed to 0.28 when 46.10^9 were injected. In embryos no variation occured in the value of K within a dose range 1.10^9 to 9.10^9. Benacerraf et al. /2/ have shown that only large

doses of bacteria affect the phagocytic index in mice.

Antibodies of maternal origin

Phagocytosis of colloidal particles begins in the liver and spleen of the chick embryo as soon as these organs differentiate at four to five days of incubation /9/. The phagocytic index for goat erythrocytes rises from 0.03 at nine days of incubation and remains constant at 0.05 from eleven to seventeen days of incubation. The rate of clearance of goat cells during these mid-stages of development of the embryo is as rapid as the clearance rate of the "rough strains" of certain bacteria such as Escherictria coli by the chick embryo /8/. The possiblity that from nine to seventeen days of incubation the embryonic serum contains a small amount of opsonins for goat erythrocytes is indicated by the considerably lower phagocytic index /K = 0.009/ for carbon clearance in 12 day-old embryos; this could only be increased to a K value of 0.025 when 17 day-old embryos were injected with smaller doses of carbon /8/. The nature of the these opsonins for goat erythrocytes in the embryo is not yet established, it is likely that they are antibodies of maternal origin. Such antibodies have not generally been detectable /even in embryos hatched from immunised hens/ by serological methods until the seventeenth day of incubation /e.g. 3, 5/. However, antibodies of maternal origin to diphtheria toxoid and infectious bronchitis have been detected as early as ten days of incubation /6, 7/. The greater sensitivity of the clearance method used in this work is capable of detecting trace amounts of antibody which may well have been passed from the yolk sac to the embryonic circulation as early as nine days of incubation. After seventeen days of incubation the clearance rate increases until soon after hatching; this is probably

due to the absorption of the majority of the maternal antibody during ingestion of the yolk sac contents /4/. The decline in the amount of maternal antibody, after this time cannot be detected because it is obscured by an exponential increase in clearance rate until at least seventeen days after hatching. This increase is probably due to naturally acquired antibody.

Naturally acquired antibody to goat erythrocytes

At two days of age the chick has completed the absorption of the yolk sac and has begun to feed. It is possible that antibody which will combine with certain antigonic determinants on goat erythrocytes can be produced by antigenic stimulation from common antigenic determinants in the diet /17, 18/. Now that it is known that active synthesis of antibody to homologous erythrocytes begins at two days after hatching /15, 16/ the exponential increase in phagocytic index after this period of development may indicate sythesis of natural antibody. Only traces of natural antibody to goat erythrocytes are detectable in normal adult serum /1/.

This increase in phagocytic index is not due to dilution of the dose by the greater blood volume, as doses were adjusted in proportion to the blood volume of the recipient; this should also have allowed for the increased phagocytic ability of the chick which is due to the increased size of the liver and spleen. The natural antibody concept was tested by an irradiation experiment. Newly hatched chicks were whole-body irradiated with 200 r, 1,000 r and 2,000 r and two weeks later the effect of these doses of γ-irradiation of the clearance rate of goat cells was determined. The greater the dose of irradiation the lower was the phagocytic index /Fig. 3/. This provides evidence

in favour of the early synthesis of natural antibody as lymphoid cells are more susceptible to irradiation damage than are the liver cells which would be concerned in the production of non-specific opsonins or complement. When large doses of goat erythrocytes are injected into 15 or 17 day-old embryos there is a depression of formation of antibody for some time after hatching /Table 2/. This is probably because partial tolerance to certain of the antigenic determinants has been induced in the embryo and subsequent exposure to common determinants in the diet has not produced the normal amount of natural antibody in the partially tolerant chicks.

The possibility that some of the natural antibody might have been produced in early post-natal life by antigenic stimulation from bacterial contamination of the intestinal flora was examined by comparing the phagocytic indices of normal and germ-free 10 day-old chicks. The mean index /K/ for the germ-free chicks was 2.9 and for the controls 6.1. This suggested that half the natural antibody was produced in response to infection during the first 10 days of life.

Complement

The results of Sherman /11/ suggest that complement appears in chick embryo just before hatching. In order to test for the possible participation of complement in the clearance of goat erythrocytes, both heat-treated /50°C for 30 minutes/ and untreated adult serum /titre 1/256/ were injected into 16 day-old embryos which had received 1 x 10^9 goat erythrocytes 5 minutes earlier. There was no marked decrease in the phagocytic index /corrected by subtraction of the embryo's contribution/ for the heat treated serum /K = 0.24/ compared to that for the untreated

serum /K = 0.33/. This indicates that heat-labile factors in the serum play no major part in the clearance of goat erythrocytes.

The primary response in embryos and young chicks

Embryos and young chicks were injected at various ages with 1×10^4, 1×10^6 and 1×10^8 goat erythrocytes /Table 2/. Any production of antibody is shown by a phagocytic index higher than the previously uninjected controls. At the lowest dose /1×10^4/ 15 day-old embryos showed some production of antibody two weeks later, whereas 13 and 14 day-old embryos were made slightly tolerant; antibody was produced to a greater extent when 19 day-old embryos and newly hatched chicks were immunised with this dose. A dose of one million goat erythrocytes failed to provoke antibody production in 15 day-old embryos. The similarity in antibody levels at five and three days after injection of 1×10^6 cells into 19 day-old embryos, newly hatched and 2 day-old chicks respectively, is probably because the antibody response in the latter two age-groups had not reached maximal level. The course of antibody production in embryos has not yet been followed. In month-old chicks antibody production is maximal at seven days after first injection, so the low levels of antibody thirteen to fourteen days after injecting 15 or 17 day-old embryos may be due to assaying these chicks too long after injection.

The injection of one hundred million cells or more, induced a state of partial tolerance in 15 and 17 day-old embryos as shown by decreased antibody production in older embryos and young chicks.

Antibody response to further injections of goat erythrocytes

So far, secondary responses have only been measured by serology, because they are too vigorous to measure by the direct

method of phagocytosis in chicks older than a few days of age. All sera were incubated at 56°C for thirty minutes prior to titration with goat erythrocytes. Agglutinating antibody was titrated at both 4°C and 37°C. Agglutinins /37°C/ formed during both the primary and secondary response of both adult fowls and 17 day-old chicks were exclusively of the 19 S type /dissociable by pre-incubation at 37°C for one hour with 2-mercaptoethanol/. The cold agglutinin titres resembled those measured at 37°C but a small proportion of the cold agglutinins of adults sera were not dissociable with 2-mercaptoethanol. Our rather fragmentary evidence to date indicates that birds given an injection during embryonic life can manufacture more antibody after a second and third injection during the first three weeks of life than chicks injected with only two doses after hatching. The titres in the former cases can be as high /e.g. I^{128}/ as those following one injection in adult bidrs. One month-old chicken also appear to produce more antibody after one injection of goat erythrocytes than adult birds /J.F.Delhanty, unpublished results/.

The above experiments have shown that sensitisation can occur during embryonic life. They have not yet shown exactly how soon antibody can be detected after sensitising the embryo or how vigorous the response is at a given time after injection. Clearance of the small sensitising doses of heterologous erythrocytes used in this work would have been very rapid in the embryo. If the erythrocyte must be intact to pass on all, or most of, its antigenic information to the few lymphocytes present in the embryo, we have obtained evidence that the embryos may be sensitised to erythrocytes as early as fifteen days of incubation.

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Table 1

Sensitisation of young chicks to homologous chicken erythrocytes before four days of age.

Age of embryo at injection /days of incubation/	Sensitising dose	Type of antigen clearance /approx. $t_{1/2}$ in days/ circulating antibody	secondary response
11	$6 \cdot 10^6$	1.0/1/5/	-
13	$5 \cdot 10^5$	0.3/1/3/	-
13	$3 \cdot 10^6$	1.0/1/5/	-
15	$1.5 \cdot 10^6$	-	1.2/1/5/
17	$3 \cdot 10^6$	-	1.0/1/5/
19	$6 \cdot 10^8$	0.5/1/4/	-

Antigen clearance measured by challenge at four days of age.
In parentheses - number of birds sensitised/number injected

Table 2

Antibody production during the primary response

Age at first injection /days of incubation/	Age at testing /days after hatching/	Time from first injection /days/	Changes in phagocytic indices from normal values /1.0/ for various doses of goat cells at first injection			
			0	1.10^4	1.10^6	1.10^8
13	5	13	1.0	0.84	-	-
14	5	12	1.0	0.77	-	-
15	8	14	1.0	1.6	1.0	0.91
17	9	13	1.0	1.1	1.3	0.84
17	3	7	1.0	-	-	0.25
19	5	7	1.0	2.9	7.2	14.1
21	5	5	1.0	15.2	8.1	12.8
Two days after hatching	5	3	1.0	-	7.1	22.2

Figures greater than 1.0 = antibody produced.

Figures less than 1.0 = natural antibody depressed /partial tolerance/.

Legends to figures

Fig.1. Correlation of phagocytic index with antibody titre.

Fig.2. Natural and maternal antibody during embryonic and early post-natal development.

Fig.3. Effect of whole-body irradiation at hatching on the formation of natural antibody 2 weeks later measured by phagocytosis of a large dose /166.10^9/ of goat erythrocytes.

Studies on the Inductive Phase of Antibody Formation: Effects of Corticosteroids and Lymphoid Cells

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Among inhibitors of antibody formation 17-OH-corticosteroids and X-ray are unique in their effect on the early part of the inductive phase. The limitation of this effect to the initiation of the immune response suggests that these injurious agents strike one or more processes that are never repeated during the rest of the inductive phase or during the productive phase.

It has recently been found that administration of nuclei from lymphoid cells in some experimental situations has a stimulating effect on the primary hemolysin response /1/. This effect is also limited to the early part of the inductive phase.

The following studies are attempts to elucidate the mechanism of action of two modifying agents with opposite effects on the inductive phase: 17-OH-corticosteroids and lymphoid nuclei.

The effect of 17-OH-corticosteroids on the primary hemolysin response and on lymphoid tissue

Studies in mice. The use of prednisolone-21-phosphate+ in water solution instead of slowly absorbed crystalline preparations has enabled more accurate time studies to be made on the effect of

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+For generous gifts of this hormone thanks are due to Erik Lindblom and Co. /Merck Sharp and Dohme/.

17-OH-corticosteroids on the primary hemolysin response. A series of studies was first performed in white male mice /2/. The following experiment will illustrate the main results.

Seventy mice weighing 20-24 g were divided into 15 groups of four and one group of ten. The last group received intramuscular injections of 0.2 ml. saline every hour from hour -10 /ten hours before the antigen injection/ to hour -6 and served as a control group. The other groups were given intramuscular injections of 1 mg prednisolone /dissolved in 0.2 ml. saline/ every hour for a period of four hours. The beginning of the treatment period was varied among the groups as follows: Group 1 received the first injection at hour -24, group 2 at hour -22 and so on with two-hour intervals. Thus, group 15 was given the first injection at hour +4. All of the animals were immunized by a single intraperitoneal injection of an 0.8 per cent suspension of sheep erythrocytes. They were bled on day +5.

The group-means of hemolysin titers have been plotted against the hour of the first prednisolone injection in the respective groups /Fig. 1/. Statistical analysis confirms the impression that the four-hour prednisolone treatment reduces the antibody response when the administration is begun within a few hours before and after hour -10. For an approximation of the time limits of this "sensitive period" the regression lines of the falling and rising parts of the curve of Fig. 1 have been calculated. These lines and their lower 95 per cent confidence limits are indicated in Fig. 2. The horizontal broken line in this figure represents the limit below which group-means of hemolysin titers are significantly reduced in comparison with the control group. This line and the lower 95 per cent confidence limits of

the regression lines intersect at hour -16 and hour -3. These times are approximations of the limits outside which the four-hour prednisolone treatment should probably not be initiated if a significant reduction of the early hemolysin response is desired.

What do these results mean in terms of the prednisolone-induced damage to the immunogenic tissue [++] and the sensitivity of the inductive phase to this damage. Our ignorance of the two following important factors seriously impedes attempts to make such interpretations:

1. The time after initiation of prednisolone treatment when the damage to the immunogenic tissue is first manifest. This experiment suggests, however, that this period is shorter than 16 hours.

2. The length of time that the damage to the immunogenic tissue must be manifest during the inductive phase in order to interfere significantly with the antibody formation process.

At the moment we can only suggest the following interpretations:

1. In order to influence the antibody response the damage to the immunogenic tissue must be fully developed before hour +13. The inefficacy of prednisolone treatment begun at the hour of antigen injection is in agreement with the results obtained with cortisone in rats /3/.

2. The normal function of the immunogenic tissue is restored much more rapidly after the four-hour prednisolone treatment than after X-irradiation.

It may be added that the general condition of the animals is very little affected by this type of prednisolone treatment.

It is of interest to compare these observations with the

++ The expression "damage to the immunogenic tissue" is here used in a restricted meaning denoting the injury which influences the course of the inductive phase

results of recent experiments on the effect of prednisolone on lymphoid tissue in mice /4, 5/. Inbred CBA male mice were given the same four-hour prednisolone treatment as used in the above-mentioned experiment. Groups of five mice were killed at different times up to 21 hours after the beginning of prednisolone treatment. The right inguinal lymph node, spleen and thymus were weighed and examined histologically. Some of the animals were given tritiated thymidine intraperitoneally one hour before killing. Autoradiograms of Feulgen stained sections of the three organs were prepared. The detailed study of the material is still in progress but some preliminary results may be mentioned:

1. The weight of the spleen and lymph node dropped rapidly during the first 9 and 13 hours /after beginning of treatment/ respectively and then remained fairly constant up to 21 hours, that of the spleen at a level of 60 - 70 per cent of the initial weight, and that of the lymph node at a level of about 50 per cent. The weight loss of the thymus was more protracted /Fig.3/.

2. Histological examination showed destruction of lymphocytes in all three organs. This was moderate in the lymph node and spleen, almost complete in the cortex of the thymus and almost absent in the medulla of the thymus. In the lymph node and spleen small islands of destroyed cells were spread over the organs. Most of these islands were found in the lymphoid follicles. At 13 hours most of the cell debris had disappeared and at 17 hours none was left. In the thymus this clearance was not completed until 21 hours.

3. Autoradiograms of the lymph node and spleen showed a markedly reduced uptake of H^3- thymidine up to 9 hours, a little more labeling at 13 hours, and a considerable increase at 17 hours.

The labelling was still more pronounced at 21 hours. In the thymus the recovery of H^3-thymidine uptake was much slower.

With regard to the lymph node it is striking that weight drop, signs of cell destruction, and H^3-thymidine uptake cease at about the same time, i.e. between 13 and 17 hours. That weight loss and cell destruction are correlated is expected. The recovery of the H^3-thymidine uptake between 13 and 17 hours may be an independent expression of the termination of one phase of the prednisolone-induced damage. Another possibility is that the depressed H^3-thymidine uptake before 17 hours is secondary to the cell destruction. Nuclear break-down products may compete with the injected H^3-thymidine. From the experiment on the action of prednisolone on the hemolysin response it was concluded that the hormone-induced damage to the immunogenic tissue was probably established before 16 hours after initiation of treatment. It is then theoretically possible that this damage is not manifest until the end of the destructive phase. If, on the other hand, the definite increase of H^3-thymidine uptake between 13 and 17 hours means that the immunogenic tissue has recovered functionally, this leads to the following conclusion: The antibody response becomes impaired even if the damage to the immunogenic tissue is manifest during a very short period of the inductive phase, may be one hour or less.

Studies in rats. It has been shown that prednisolone in solution has to be given in a larger dose per gram body-weight and for a longer time in order to reduce the primary hemolysin in the rat. Seven hourly injections of 20 mg. prednisolone begun at 12 hours before the antigen injection /hour -12/ significantly inhibited

the early hemolysin response. When the same treatment was started at hour -28 no effect was noted. In another experiment as much as 11 hourly injections of 20 mg. begun at hour -35 produced no significant reduction of hemolysin levels. This indicates that the immunogenic tissue of the rat as well as that of the mouse recovers rapidly from the functional impairment induced by prednisolone.

The above mentioned experiments in mice suggested that the re-established function of immunogenic tissue was evident before its anatomical reconstruction was quantitatively completed. If so, this offers the possibility of studying a normal induction phase in a lymphoid tissue with a reduced cellular set-up. If an altered cellular response to the antigen is found, this may point to tissue reactions which are not essential to the inductive phase. A comparison between observations of this sutuation and of the course of the inductive phase in lymphoid tissue that has not yet recovered functionally after prednisolone treatment, may give important information. Such an experiment has recently been made:

Thirty-two white male rats /180 - 200 g/ were divided into three groups: I. "Early steroid" group /11 rats/, which received 11 intramuscular injections hourly of 20 mg. of prednisolone /dissolved in saline/ begun 36 hours before the antigen injection; II. "Late steroid" group /11 rats/, which was given the same treatment as group I with the exception that the administration was started 12 hours before the antigen inejction; and III. "Control" group /10 rats/, which received saline according to the same schedule as usdd for group II /Fig.4/.

Eight rats from each group were immunized by one intra-

peritoneal injection of 2 ml. of a 0.2 per cent suspension of sheep erythrocytes. At the same time /hour 0/ the remaining animals in each group were killed. Of the immunized animals four from each group were killed at 6 hours after antigen injection /hour +6/ and the remaining four in each group at hour +20. Immediately after killing the peritoneal cavity of each animal was washed with 20 ml. of 0.25 M sucrose and the concentration of cells in the washing fluid was determined. Mediastinal lymph nodes, spleen and thymus were weighed and processed for histology. One and a half hours before killing at hour 0 and at hour +6 one animal from each group was given an intraperitoneal injection of H^3-thymidine /1 /ucC per gram/.

The histological and autoradiographic examinations are not yet completed. At the moment the following conclusions seem to be possible:

1. At hour 0 the left middle mediastinal lymph node had decreased in weight in both prednisolone-treated groups. As judged by histology and weight curves /Fig.4/, the cell destruction in the lymph node had finished in the "early steroid" rats but not in the "late steroid" animals. The number of cells in the peritoneal cavity had not been significantly changed by the hormone treatment /Fig. 5/.

2. At hour +6 histology showed that cell destruction had now ceased in the "late period" group. In other respects there was little change from hour 0.

3. At hour +20 the lymph node of the "early steroid" group showed a definite increase of cell density, which equaled that of the normal non-stimulated node. There might also be some increase in the "late steroid" group of rats, but this lymph node still seemed to be subnormally populated. There was no dif-

ference in the weights of lymph nodes between the two prednisolone-treated groups. The peritoneal washing fluids of the control group showed the expected rise in the number of cells. This rise failed to appear in both hormone-treated groups.

It is apparent that the difference in effect on the inductive phase between early and late hormone treatment is not expressed as measureable differences in lymph-node weight or inflammatory response to the antigen as estimated by the total number of cells in the peritoneal fluid. The impression that the lymph nodes of the "early steroid" group showed a livelier response to the antigen than did those of the "late steroid" group will have to be checked by cell counts.

This experiment has been presented not because it has solved any problems but as a possible model for further investigations into the processes of the induction period, and as an invitation to discussion on methods of exploration of this phase.

The effect of lymphoid-cell nuclei on the primary hemolysin response in the rat

When not otherwise stated, the lymphoid-cell material was tested by intraperitoneal injection into rats immunized by a single intraperitoneal injection of a small amount of sheep erythrocytes . This effect was measured by estimation of hemolysin titers on day +5.

It was first found that a homogenate of homologous spleen cells increased the hemolysin titers if given a few hours before or simultaneously with the antigen but had no effect when injected 2 or 6 hours later. Homologous whole spleen cells or thymocytes were equally effective. There was no significant difference in stimulating capacity between homologous and autologous spleen cells. About 4×10^7 cells were required for a

a significant effect. With 2 x 10^8 cells or more a considerable amount of antibody formation took place in the spleen. When fewer cells were used most of the hemolysin was produced in the mediastinal lymph nodes /determined by a modified agar-plaque technique, see below/. This was also the case when antigen was given alone. In peritoneal fluid, omentum and mesenteric lymph nodes, only occasional hemolysin-producing cells were found.

In cooperation with Fagraues and Sköld /1/ some studies have been devoted to the subcellular localization of the material that has an enhancing effect on the hemolysin response /enhancing factor/. Cell fractions were produced by differential centrifugation of disintegrated spleen cells or thymocytes. Most of the activity seemed to be present in the nuclei. Mitochondria, microsomes, and the 105,000 g supernatant /60 min./ had no significant effect. Incubation of thymocyte nuclei with DNAase deprived them of the stimulating effect. In one experiment a significant amount of activity was released from the nuclei and could be demonstrated in the supernatant after centrifugation.

The possibility that the enhancing factor is DNA has been investigated in two ways: 1. A purified DNA from rat spleens was injected together with the antigen. No effect was found. 2. Attempts at tracing isotopically labled nuclear material injected together with the antigen to hemolysin-producing cells. Thymocytes from 2-week-old rats were labeled with H^3-thymidine in vivo. Nuclei were prepared and injected with sheep erythrocytes intraperitoneally into homologous rats. On day +5 the mediastinal lymph nodes were excised. The cells were suspended in Eagle's medium. Hemolysin-producing cells were identified /7/ by a microslide modification of the agar-plaque technique described by Jerne et al. /6/. The glasses were dried, fixed in methanol, and

covered with auboradiographic liquid emulsion. So far, labeled nuclei have not been found in hemolysin-producing cells. Longer time for exposure of films will be tried.

Finally, we have not been able to demonstrate any effect of lymphoid nuclei on the cellular reaction to the antigen as estimated by 1. weight and total cell count in the local draining lymph node /weight studies were made on the popliteal lymph node after injection of antigen and nuclei in the footpad/, and 2. the number of free cells in the peritoneal cavity.

The possible relation between these results and those of some other investigators will be briefly discussed.

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Immunological Competence of Different Stages of the Lymphoid Cell

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The ontogenetic scheme of the development of mammal lymphoid cells which are the carriers of immunological reactivity, is now regarded to be probably as follows: The first lymphocytes develop from the epithelial base of the thymus or analogous organs under the humoral influence of the mesenchyme /2, 19, 1/. These lymphocytes disseminate into the circulation and secondary lymphatic tissues: There, they are either reutilized and provide the stimulus for the proliferation of reticulum cells and lymphocytopoiesis /21/ or themselves become full value lymphoid cells /22/. In any case, at the end of the neonatal period there is a supply of small lymphocytes at the periphery, and in the secondary lymphatic organs. Small lymphocytes here are capable of transition to large lymphoid cells /4/. These possibly develop also from reserve of primitive cells /of the character of reticulum cells/ in which lymphocytopoiesis can also be stimulated by the humoral effect of the thymic stroma /12, 15, 7/.

The lymphocytopoiesis enhancing function of the thymic stroma and the medium of the thymus seems to be contradictory to the immunological reactivity of the lymphoid cells /13/.

Small lymphocytes in the circulation and in the secondary organs, like most lymphoid cells, have the power of differentiation and modulation, into histiocytes, and also possess

immunological competence /6/.

In this work we have attempted to compare the immunological competence of lymphoid cells on their route from the thymus via secondary lymphatic tissue to the lymph and during their transformation from large and small lymphocytes to histiocytes.

Material and Methods

1. Source of cells: All cells were obtained from young adult Chincilla rabbits. Thymus cells and mesenteric or mediastinal lymph node cells were isolated from the organs by teasing into Hank's or Earle's solution. Lymph cells were obtained by direct puncture of the cisterna chyli of donors immediately after they had been sacrificed and were resuspended in Hank's or Earle's solution with heparin. Histiocytes were washed out of the lungs in Hank's or Earle's solution according to the method of Myrwick et al. /14/.

2. Fractionation of cell suspensions. The lymph node, thymus and lymph cell suspensions were separated into a fraction with a complete predominance of small lymphocytes and fractions with a higher content of larger cells /other than small lymphocytes/ by gradient centrifugation on layers of sucrose at 25, 30 and 35% concentration in Hank's or Earle's solution with 20% autologous rabbit serum. Centrifugation was continued for 6 - 9 min. at 25 - 30 g. and sometimes repeated, particularly in nodes. After centrifuging, the cells were washed twice with Hank's or Earle's solution, resuspended in the same medium and their absolute number and differential count determined.

3. Antigen. With the exception of the control samples, BSA antigen was added to the cell suspension in amounts of 0.01 mg. per 10-20 million cells.

4. Cultivation. Suspensions of 10-100 million cells were placed in diffusion chambers of 0.8 ml. volume, with filters of Czech production /VUFS, 0.1 - 0.3 /u porosity, exceptionally HUFS 0.3 - 0.5 /u porosity; in these filters the great majority of pores are smaller than 0.45 /u and as distinct from the filters Millipore HA they were found to be impermeable for cells/ and in most cases with Membranfilter-Gesellschaft Göttingen filters with a porosity of 0.15 - 0.275 /u. Carefully sealed chambers were introduced into the peritoneal cavity of 3-6 day-old Chinchilla rabbits treated with antibiotics. 1 - 3 chambers were placed in each infant rabbit. The rabbits are not themselves capable of an antibody reaction to the amount of antigen used. The chambers were removed at various time intervals from 7 - 13 days, exceptionally after longer cultivation.

5. Morphological analysis. Smears of the initial suspension and smears and imprints of the inner surface of the chamber filter after cultivation were fixed wet with Carnoy and stained with methylene green-pyronine Y and also examined without treatment in phase contrast. In some cultivations filter imprints were also rapidly dried, fixed with ethanol and stained by the one-stage method for antigen and the two-stage method for antibody using conjugates of anti-BSA serum with fluorescein-isothiocyanate according to a modification of the method of Marshall et al. /11/. Differential counts were made from 10,000 cells of the initial suspension and from 1000-5000 cells after cultivation.

6. Serological determinations: The fluid from the chambers was used to determine the anti-BSA antibody content by the method of passive microhaemagglutination with tanned and benzidine-treated

sheep erythrocytes.

7. Types of experiments. Antibody production and the number of antibody-containing cells in the different cell suspensions were determined after cultivation in the separate chambers according to the method described previously /5/.

The effect of thymus stroma on the immunological properties of cultivated lymphoid cells was determined in two further modified cultivation methods: a. In chambers where thymus stroma, obtained by washing out the small cells from 3 - 6 small fragments of the organ /2 x 2 mm./, was cultivated for 12 and 43 days. Chambers filled with Earle's solution were placed into the recipients at the same time. After 12 and 43 days all the chambers were taken out and the fluid inside replaced through a hole in the wall of the plexiglass ring with a suspension of lymphoid cells freshly isolated from lymph nodes or lymph with antigen /BSA/.

b. Double chambers were made of two rings of plexiglass and closed by three filters, the centre one /of 0.25 - 0.4 /u porosity/ being common to both assembled chambers. The outer filters had a porosity of 0.15 - 0.275 /u. Fragments of thymus /3 - 6 fragments 2 x 2 mm/ were enclosed in one of the assembled chambers, or as a control, only Earle's solution or fragments of lymph nodes. In the second of the assembled chambers lymphoid cells were cultivated together with antigen /BSA/.

The total number of chambers used in the experiments can be seen from the tables. Recipients from one litter were always used for the cultivation of different types of cells so that the influence of individual factors of the recipient on the cell suspensions should be minimal. In some experiments the chambers from each recipient were removed successively at different time

intervals.

Results

From tables 1 and 2 it is seen that all cultures containing more than $10 \cdot 10^6$ lymph or lymph node cells produce antibodies inside the chambers in titres up to 1 : 256. The suspensions of pure small lymphocytes from these sources are somewhat inferior in this respect to suspensions which include larger cells. The production is both number and time dependent: higher numbers of cells in starting suspensions in most cases give higher titres of antibodies; on the 7th day of cultivation the production is negligible, the highest titres appear between the 8th and 10th day of cultivation.

Lung histiocytes /table 4/ seem at least to contribute to antibody formation, because the admixed amount of lymphoid cells gave demonstrable antibodies if cultivated alone.

On the other hand, thymus cells /table 3/ do not give any relaible titres with the exception of separated small thymus lymphocytes which produce demonstrable titres in more than 80% of cultivations. Their production is again time and number dependent. In comparison with small lymphocytes from secondary lymphoid tissues their production is lower and a little delayed.

The same picture is provided by the count of antibody containing cells traced by the immunofluorescent method /Fig.1/. 1.5 - 2% of cells from lymph node and lymph are stained specifically on the 9th day of cultivation, thymus small lymphocytes are a little inferior and thymus cell suspensions including larger cells range from zero to 0.7%.

In lymph node and lymph cultivations plasma or immature plasma cells could be identified among the antibody containing

cells, whereas in small lymph and thymus lymphocytes cultures there was a remarkable prevalence of reactive forms of small lymphocytes among the positive cells. Large lymphoid cells and even histiocytic forms were occasionally found to contain antibodies both in the cytoplasm and sometimes /on day 8 or 9/ even inside the nucleus. In some histiocyte cultivations only histiocytes were found to contain antibodies, indicating that they might have the capacity to produce antibodies on their own.

In routine cytological analysis, the thymus cell cultivations differed from secondary lymphoid organ cell cultures in their tendency to histiocytic and epithelial outgrowth which was pronounced especially in the samples including higher proportions of large cells in starting suspensions.

In histiocyte cultures there was a development of either heavily phagocytosing cells or large pyroninophil histiocytes with activated nucleoli. The proportion of macrophages to pyroninophil histiocytes is about 4 : 1.

The morphological details of cellular differentiation during cultivation and antibody formation have been described in previous work /5, 6/.

The idea that thymic stroma and thymic reticulum cells might exercise some depressive action on the immunological capacities of lymphocytes was tested in cultivations of thymic stroma plus lymphocytes from secondary lymphoid organs. As shown by tables 5 and 6, both in double chambers and in chambers containing precultivated thymic stroma, which forms a typical epithelial sheet after 10-12 days, lymph or lymph node lymphocytes cultivated together with thymus tissue produced lower titres of antibodies if any. On the other hand, in such joined cultivation of thymus reticulum plus lymphoid cells from secondary organs, a good survival and prevalence of small lymphocytes was found: after

8 days of cultivation in double chambers, 24 . 10^6 cells have been counted in chambers containing Earle solution and target lymphoid cells, and 35 - 51 . 10^6 cells were found in chamber containing thymus fragments and target cells. Over 90% from these surviving cells were small lymphocytes.

Discussion

Both antibody formation inside the diffusion chambers and counts of antibody containing cells show that immunological capacity decreases in the sequence: 1. lymph node and lymph cells with higher proportions of larger cells /large lymphocytes, blasts, recticulum cells/; 2. small lymph node and lymph lymphocytes which enter the immunologically competent stage either by differentiation towards larger lymphoid cells /4/ or by direct modulation /6/; 3. small thymic lymphocytes /with the same morphological changes accompanying their immunological function/; 4. thymus cells indlucing large lymphoid cells and thymus reticulum, which appears to depress the immunological reactivity of lymphocytes in enhancing their proliferation. Even lung histiocytes /which might at least in part be descendants of lymphoid cells, see ref.16/ appear to have the capacity of differentiating into heavily pyroninophil forms and elaborating antibodies.

It is likely that the lymphoid cell from the blast stage on is immunologically competent and preserves this quality throughout its life up to the stage of the small lymphocyte and even nonphagocytic histiocyte. In the passage of the cell from primary to secondary lymphatic organs and into the circulation this quality might be influenced by local environmental factors among which the proliferation stimulating factors - such as the

thymic Metcalf /12/ factor - are at variance with intracellular events connected with adaptive proteosynthesis. As to the immunological competence, thymic lymphocytes appear not to be different from lymphocytes resident in secondary lymphatic tissues and in circulation. Thymus might ensure the nonspecific differentiation and multiplication of lymphoid cells not because it is antigen proof /10/, but because steadily proliferating lymphoid cells do not enter the inductive phase of antibody formation. The immunological competence of thymus lymphoid cells /chiefly small lymphocytes/ can be detected even by transfer of cellular suspensions into whole recipient where thy might be separated from the depressive action of the reticulum /20,19/.

The term "immunologically competent cell" can thus hardly be attributed to some morphological character of the lymphoid cells: as a morphological phenomenon, the lymphoid cell seems to be nonrestricted in its functional capacities in either stage of differentiation. A speculative attempt /e.g.3/, to correlate the small lymphocyte with the functional concept of incompetence cannot be justified. There might be only quantitative differences in the ability of the lymphoid cell to undergo the intracellular changes required and to multiply as a committed dell.

A high proportion of cells has been found to contain antibodies. The percentage of positive cells is comparable to the results of Makinodan and Albright /9/. It is in marked variance with the results obtained by the plaque technique /18/. This difference might be due to absorpiton of antibodies in diffusion chembers. It is, however, hard to believe that this could be the case even in cells which do contain intranuclear antibodies. In the first days of antibody formation these cells form considerable part of all antibody containing cells. On the other hand, not

antibody containing but antibody secreting or liberating cells are revealed by the plaque technique, and this might be the main source of discrepancy between the two methods.

Summary

Lymphoid cells from normal rabbits were cultivated in diffusion chambers with BSA antigen, the chambers being implanted into newborn rabbits. Antibody titres from the chamber fluids and counts of antibody containing cells /detected by immunofluorescence/ show that lymph node and lymph cellular systems including higher proportions of larger cells are better producers than lymph node and lymph pure small lymphotic suspensions; in thymus the relation is the reverse: only small thymic lymphocytes give reliable antibody production. The idea that thymic reticulum might have an enhancing effect on lymphocytopoiesis and in the same way a depressive action on the antibody forming capacity of lymphoid cells has been proved by cultivations of thymic stroma with lymphoid cells from secondary lymphatic organs. Antibody formation was heavily depressed by thymic tissue in the chambers. It is likely that even lung histiocytic cells are antibody producers.

It is concluded that a lymphoid cell, from its blast stage to the small lymphocyte or histiocyte, preserves its antibody forming capacity and only its localization and local effects /such as the effect of thymic stroma/ might influence its specific changes towards antibody containing types.

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Table 1.

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lymph node cells with different proportions of small lymphocytes and larger cells, cultivated with BSA antigen

Total number of cells	Days of cultivation	% of small lymphocytes in the starting duspension: 99-100	mean	95-99	mean	90-95	mean	85-90	mean	85	mean
100											
75-100	7					0;3;0;0;0;0	0,5				
	8					8;8	8				
	9			6	6	5;5;4	4,7				
	11			5	5	6;0	3				
50-75	9	5;5	5	6;7;6;6	6,3	8;8;4;5;5	6				
	10			6;7	6,5						
	11					7;7;6;2;4	5,2				
25-50	8	4;3	3,5	4;3;5;4	4			8;8	8	8;8	8
	10	8	8	6;6	6						
10-25	10							5;6	5,5		
10											

Table 2.

Total number of cells	Days of cultivation	% of small lymphocytes in the starting suspensions: 99-100	mean	95-99	mean	90-95	mean	85-90	mean	<85	mean
100	10							7	7	7	7
75-100	7							0	0		
	9	6;3;5	4,7					4;6	5		
50-75	7	5;0	2,5			0	0				
	8	7	7								
	9									5	5
	10	6	6	6	6	7;8;7;7;8;6	7,2			7	7
	11	2	2			4	4				
25-50	8	4	4	4;4	4	3;4;5	4	5	5	6	6
	9	4;4;5	4,3	3;5	4			6	6	7;6	6,5
	10	4;4	4	6	6	5	5	7	7	5	5
	11					4	4	5	5	5	5
10-25	8	1;2;2	1,7								
	9	3;3	3	3;3	3			4;5;3	4	4;6;4;4	4,5
	10	3;4	3,5	3	3	4	4				
10	8									0	0
	10			0	0			0;2	1		

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lymph cells with different proportions of the small lymphocytes and larger cells, cultivated with BSA antigen.

Table 3.

Log 2 titers of anti BSA antibodies formed in diffusion chambers by thymus cells with different proportions of the small lymphocytes and larger cells, cultivated with BSA antigen

Total number of cells	Days of culti-vation	% of small lymphocytes in the starting suspensions									
		99-100	mean	95-99	mean	90-95	mean	85-90	mean	85	mean
100	10					1	1	0;1;0	0,3		
	13			6	6	2;2	2				
75-100	7							0;0;0	0		
	8			0	0			0	0		
	9	6;5;5	5,3					1;1	1		
	10			2	2			2	2		
	11			2	2			1	1	0	0
	13	3	3								
50-75	8	3;0	1,5	1;1;0;0	0,5	0;2	1				
	9	3;3;4;4;6	4	2;2;0;0	1					0	0
	10	3;6;1;4;3;3;3	3,3	4;4;4;2;2;2;1;1;1	2,3	0;0;0;1;1;1;0	0,4	0;1;0;0	0,3		
	11	3;2;6;4;6	4,2	2;2	2	0;0;0;0;0;0	0			1	1
25-50	8					1	1				
	9							0;2	1		
	10	3	3			1	1	2;2;1;0	1,3		
	13	8;3	7,5	4	4						
10-25	7							0	0		
	10	3;2;4;1	2,5								

Table 4.

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lung histiocytes with 1-10 % admixture of lymphoid cells, cultivated with BSA antigen.

Total number of cells	Days of cultivation	% of histiocytes in the starting suspension					
		95-99	mean	90-95	mean	85-90	mean
100							
75-100							
50-75	8			2	2		
	13			4	4		
	15	5;5;8	6				
25-50	9	5;5	5				
	10			0;2	1		
10-25	8					0	0
	9					2	2
	10					1	1
	11					4	4
	22	1;2	1,5				

Table 5.

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lymphoid cells cultivated with BSA antigen; experiments with double chambers: in one of them the target lymphoid cells were grown, the other was filled either with Earle solution, with lymph node fragments or with thymus fragments.

Lymphoid cells +Earle sol.	Lymphoid cells +lymph node	Lymphoid cells + thymic stroma	Source of lymphoid cells, % of small lymphocytes	No of lymphoid cells $.10^6$	Time of cultivation of lymphoid cells in days
4;4	3;3	0;0;0; 0;0;1	l. node, 94%	50-55	11

Electron Micrography of Antibody Producing Cells Individualized by the Technique of Local Hemolysis in Gel

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Identification and study of antibody producing cells by microdrops technique /1, 2/ provide a very fruitful way to attack many problems of antibody formation.

These methods, nevertheless, do not provide an easy means for the cytological examination of the immunocytes /antibody producing cells/.

The introduction of the method of localized hemolysis in gel /L. H. G./ by Ingraham and Bussard /3, 4/ and by Jerne and Nordin /5/ has given us a tool by which immunocytes can be identified without any doubt. Furthermore, these immunocytes can be individualized /even if the proportion of active cells is very low, for instance 1 for 10^5 cells/ and their morphology studies either by optical or by electronic microscopy.

The production of antisheep erythrocyte /SE/ hemolysin by lymph node cells of immunized rabbits, in a physiological gel, has been studied by the method described by Ingraham and Bussard. Minor modifications of the gel method have been introduced permitting the immunocyte to be studied with the electron microscope.

Preparation of the cell suspensions: Popliteal lymph node cells of adult rabbits in a stage of secondary reaction /4 days after booster injection of SE into the footpad/ were prepared and counted for viability by the trypan blue exclusion method /see 4/.

Preparations for electron micrography were made only when the population of the lymph node cells had an activity greater than 200 active cells/million viable cells. Suspensions of the active lymph node cells /10^7/ml/ were added to carboxy-methoxy-cellulose gel, containing SE /5 x 10^9/ml/ and guinea-pig complement as previously described /4/. When the activity of the lymph node cells suspension was very high a concentration of 10^6 lymph node cells/ml was used. This measure increased the probability of finding only one lymph node cell in each zone of hemolysis. A small amount of the gel containing cells /I.1 ml/ was pipetted onto the bottom of the glass tube /10 mm diameter/ and incubated at 37°C for twou hours. When an aliquot preparation, on a microscope slide, showed that the number of plaques of hemolysis had reached its maximum, Palade fixative ways layered onto the bottom of the tube on the top of the gel. After 30 minutes of fixation, the osmic acid was removed and the pellet washed, dehydrated and finally embedded in Epon.

The pellet of dehydrated gel was removed by breaking the tube and was then fixed on a Porter-Blum microtome. The whole pellet was cut in serial sections - thick ones at first /1 /u/ for observation with the optical microscope, until the boundary of a zone of hemolysis was reached - then thin sections /200 to 300 Anström units/ were made for electron micrography. After cutting through the lymph node cells, thick sections were made again as long as no leucocyte was found. As soon as thick cut showed the presence of a leucocyte as seen by phase contrast miscroscopic examination, thin cuts were resumed through the cutting of the leukocyte. By such a technique a whole series of hemolysis zones were completely examined without losing a section of the preparat-

ion and we were able to observe and photograph all the cells present in each zone of hemolysis.

Results

In our attempts to obtain serial sections through zones of hemolysis containing only one or two white cells, we were successful in twelve cases. Only hemolytic zones in which all sections could be examined have been included in our results.

1. The hemolytic zones in which all of the red blood cells have lost their hemoglobin and have become ghosts are as easy to recognize with the phase contrast as with the electron microscope. These zones have a characteristic appearance which is constant from one experiment to another. Although the size of the hemolytic zone may vary from one experiment to the next, in any one experiment their size is the same. In cross-section the zones are round with a diameter of 70 to 120 /u /see 4/. The volume of the zone varies with the activity of the cells and the duration of the incubation.

All of the dimensions and the shape of the zones can be determined from the known shape and thickness of the sections and the total number of sections in the zone. The hemolytic zones are not spherical but are elliptical with the large axis lying along the length of the tube. This flattening occurs not only in the zone as a whole but also in the individual cells in the zone. Thus, the plasma cells are 15 to 20 /u long and are not more than 3 /u wide.

2. The type of white cells found in the hemolytic zone have been determined. In all cases plasma cells have been found. These are readily identified with the phase contrast microscope /Fig.1/ and by their large content of ergastoplasm as seen in the

electron microscope /Fig.2b/. In seven out of twelve cases plasma cells were isolated in the centre of the hemolytic zone, in a single case two plasma cells were found. In each of two other zones a lysed white cell was found which resembled plasma cells under phase contrast but which could not be identified in the electron microscope. Finally, in each of two other zones a lymphocyte /Fig.3c/ in addition to a plasma cell /Fig.3b/ was found. One of the lymphocytes had a very well developed centriole.

3. The appearance of the plasma cells in the electron microscope was not unusual. Inclusions could not be discerned in their nuclei. Many dilated ergatoplasmaic sacs were seen in the cytoplasm /Fig.2B/. A dense electron-opaque material close to the Russel bodies could be seen between the surface of the ergatoplasmic sacs. Frequently, hemolysed red blood cells were found adhering to the cytoplasmic membrane. Occasionally, openings in the cytoplasmic membrane were seen at the site of attachment of the red blood cells; it is however difficult to ascribe a function to the openings. Most of the hemolysed red blood cells were not in contact with the plasma cell and sometimes red blood cells which were not hemolysed were found adhering to the plasma cell.

4. It was found by sucrose gradient ultracentrifugation /6/ that most of the hemolytic antibody in rabbit serum was of the 19 S type. However, some samples of serum contained appreciable amounts of 7 S hemolysin /as much as 20%/. Thus, not all hemolysin is of the 19 S form, at least in the rabbit in a secondary phase of immune response.

In cases of hyperimmunization /local injections/ it was found that all circulating hemolysins were of the 7 S type. In these cases no immunocytes could be found among the 2.2×10^6 cells screened for each rabbit.

The antibody titers of the serum were 2^{10} /final dilution for total hemolysis/ in each case, which is a reasonably high titer for animals locally injected.

It could thus be concluded that 7 S hemolytic antibodies may be formed while no immunocytes are detectable in the lymph node draining the site of injection. One must conclude that this type of antibody is synthetized by other reticulo-endothelial tissues, such as the spleen, in such cases.

This result favors the conclusion that the hemolytic antibodies formed in the gel by lymph node immunocytes are 19 S antibodies.

A direct proof for this idea would be given by local inhibition of hemolytic plaques if a really specific inhibitor of 19 S could be found. In fact, mercaptoethanol /0.1 M 3 min. at 37°C/ completely inhibited the hemolytic activity of all samples of serum including those containing 7 S antibody. Thus mercaptoethanol inhibition of an antibody activity cannot be used as a specific proof that the antibody involved is of the 19 S type.

Discussion

1. In the preparation devised for work with the electron microscope the shape of the lytic zone is different from that obtained under a cover-slip on a glass-slide. The placing of a thin layer of gel near the bottom of a test tube for electron microscopy studies results in a hemolytic zone of ellipsoidal shape whose long axis is parallel to the long axis of the tube. The ellipsoid is flattened by the process of dehydration, necessary for viewing in the electron microscope. A lymph node cell is always practically all of the red blood cells are lysed with the occasional exception of some red blood cells which are

adherent to the lymph node cell.

Hemolytic zones formed by a white cell are easy to distinguish from clear areas that are artefacts. In the latter there are no red blood cells or ghosts or there may be extensive agglutination with only slight partial hemolysis of red cells. Further, white cells are not found in the centres of these areas.

2. Earlier work /3, 4/ support the conclusion that the lymph node cell is responsible for the localized hemolysis. Briefly, the evidence is: only the cells from immunized animals give rise to the zones - the number of hemolytic plaques is proportional to the number of lymph node cells in the preparation, - at the centre of every plaque there is only one lymph node cell. There is good evidence that the hemolysis is due to antibody newly synthetized by the cells in the gel and is not simply due to the diffusion of performed antibody since the formation of the hemolytic zone is markedly reduced by dinitrophenol, actinomycin and puromycin /4/. The cells in the hemolytic zones have a well developed endoplasmic reticulum which is known to be associated with protein sythesis.

3. Since in almost all cases, a single plasma cell was found at the centre of the hemolytic plaque, one can conclude that it is, at least, one of the types of cell which produces hemolysin. In the plaque with one plasma cell and one lymphocyte it is not possible to say which cell was the hemolysin procedure. Since we did not find plaques with only a lymphocyte present we cannot say whether they are capable of hemolysin synthesis.

4. The hemolytic antibody found in the serum of the immunized rabbits from which the lymph node cells were derived, was of the 19 S type. This was shown by ultracentrifugation is sucrose gradient /6/. If we assume that in our cases the essential producers of

these 19 S antibodies were the lymph nodes, then we may conclude that the lymph node cells in the plaques observed with the electron microscope were 19 S producers. In the work of de Petris electron microscopy was applied to the study of cells producing anti-ferritin in hyperimmune animals /7/ and it is most probable that these cells were synthesizing 7 S type antibodies in contrast to the cells in our studies which formed mainly 19 S antibodies.

We wish to thank Dr Bessis for his interest and help. We also wish to thank Mr.J.C. Mazie and M^{lle} Vinzens for their able assistance.

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Legends to figures

Fig.1. Phase contrast micrography. Zone of hemolysis in a gel preparation. One immunocyte can be seen in the centre of the zone.

Fig.2. Phase contrast micrography on a thick section in Epon. One plasma cell in the centre.

Fig.2b. Electron micrography. Plasma cell from the zone shown in Fig.2a.

Fig.3a. Phase contrast micrography on a thick section in Epon.

Fig.3b. Electron micrography of the cell in the centre of the zone shown in Fig.3a.

Fig.3c. Electron micrography of a lymph node cell situated peripherally in the zone shown in Fig. 3a.

Is Antigen Associated with Macrophage RNA?

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It is a basic problem whether antigen as such stimulates immunologically competent cells to form and secrete antibody globulin or whether the process requires another cell as an intermediary. Macrophages have been implicated in this role. It has been suggested that the antigen may be altered or complexed with macrophage cellular components or that the macrophages are capable of transferring nucleic acid carrying information to globulin producing cells, or even that macrophages can turn into plasma cells. It is known that injected antigen is taken up by macrophages and that it persists for many weeks in phagocytic cells in the liver, spleen, and lymphoid organs /see reviews /1/, /2/, and /3/. After injection of I^{131} labeled bacterial flagellar antigen macrophages in lymph glands remain heavily labeled with I^{131} for more than 4 weeks /4/. Antigen remains immunogenic for 130 days in liver phagocytes /5/, and Garvey and Campbell suggest that it is present in a nucleic acid rich fraction. Franzl /6/ found spleen lysosomes to be immunogenic, and immunogenic fractions rich in nucleoprotein could be isolated from spleen 3 weeks after injection of Shigella protein or from liver 3 weeks after administration of BSA. /8/. It is still an open question as to whether macrophages actually store antigen in close proximity to immunologically reactive cells or whether they take an active and necessary part in antibody formation. Fishman and Adler /9/ found that phenol extracted RNA from macrophages after contact with bacteriophage

+J.M.Rhodes - on leave of absence from Statens Serum Ins., Copenhagen, supported by a grant from The Carlsberg Foundation, Copenhagen.

induced neutralizing antibodies against phage, suggesting that macrophages make information for antibody forming cells. This factor was species specific and ribonuclease sensitive and appeared to be localized in a small molecular weight RNA fraction /10/.

This paper presents some preliminary attempts to study this problem further. Haemocyanin /HCY/ was chosen as a reasonably good antigen and well defined protein with a molecular size /S_{20} of 26/ convenient for characterization on sucrose gradients. The HCY was labeled with I^{131} or I^{125}. This is open to the same criticisms as other work using radioiodinated protein antigen - namely, possible deiodination in the tissues. Nevertheless it has been very useful for some aspects of the work and gave a rough indication of the amounts of antigen taken up by macrophages and the quantities contaminating RNA extracts.

We investigated the fate of antigen in macrophages and the induction of antibody formation by RNA extracted from macrophages after a/ in vivo contact with antigen or b/ addition of antigen immediately before phenol treatment. Immunogenicity of the preparations was tested in C_3H/He inbred mice. Since soluble HCY does not give a good primary response in mice, mice primed at least 2 months previously were used as recipients. RNA preparations were preincubated with normal spleen cells before transfer of the cells into recipient mice. Such cell transfers appear to provide a more sensitive method for detecting small amounts of antigen than does simple injection into whole animals /7, 11/.

Both types of RNA extracts contained macromolecular I^{131} labeled material /although to different extents/ and proved to be immunogenic. Not enough work has been done as yet to decide

whether RNA-Ag complexes are more highly immunogenic than correspondingly small amounts of HCY.

General methods

Inbred mice /C_3H/He/ were used for this work to enable us to make cell transfers. The yield of peritoneal macrophages was increased by an i.p. injection of 10% peptone 3 days before the experiment. The peritoneal cells were collected in saline - heparin - 5% normal rabbit serum and contained about 80% macrophages /about 10^7/mouse/.

Haemocyanin /HCY/ was purified from Maia squinado blood /Humphrey, unpublished/, and labelled with carrier free I^{131} or I^{125} by the method of Hunter and Greenwood /12/ yielding specific activities between 5-30 /uc/ug. At higher levels of radioactivity, HCY appeared to deteriorate; it showed degradation on sucrose gradients and was only partly precipitated with specific anti-HCY.

Preparation of RNA from macrophages with phenol: Peritoneal cells were collected from 20-25 mice /yielding 2-3 x 10^8 cells/, washed twice with Gey's medium, homogenized in 5 ml 0.025 M Na phosphate pH 7 and extracted with an equal volume of phenol at $+4^{\circ}C$ for 15 min. with continuous vigorous shaking. The aqueous phase was twice re-extracted with phenol for 5 min. the phenol was removed by five extractions with 4 volumes of washed ether and the ether was removed by bubbling N_2 gas through the solution. The cells yielded about 700 /ug - 1 mg RNA /absorption density of 22 at 260 m/u was taken to represent 1 mg RNA/ml/. In some experiments the first phenol extraction was carried out at + $15^{\circ}C$ /15/ and all subsequent steps as above at $+4^{\circ}C$.

Test for immunogenicity of small amounts of HCY and macrophage-RNA extracts: Macrophage preparations and small amounts of antigen were incubated with normal mouse spleen cells in Gey's medium for 1 hour at 37°C and then the mixture was transferred intraperitoneally into primed mice /3 x 10^7 spleen cells/mouse/./7/. Recipient mice had been primed with 5-10 /ug alum precipitated HCY in the footpads at least 2 months previously. After two months the antigen binding capacity of their visa was low /.1 /ug antigen/ml/ - although occasionally experiments had to be discarded because control antibody levels were too high. Peak titers appeared 6 to 9 days after the cell transfers.

For the assay of small amounts of antibody in recipient mice mainly 2 methods were used:

1. Indirect antigen binding test: a microtest was exmployed /13/. 0.02 /ug of I^{131}-HCY /about 50,000 counts/min./ in 0.1 ml saline, plus 5 /ul of mouse antiserum and 5 /ul of a 1/3 dilution of the antiserum in normal mouse serum were incubated for 1/2 hour at 37°C. Slight excess of anti-mouse γ-globulin was added to precipitate the mouse γ-globulin - I^{131}-HCY complex. After another 30 mins. incubation at 37°C, saline was added to make the volume 1 ml. The samples were left in the cold overnight, centrifuged at +4°C and the I^{131}-HCY left in the supernatant was counted. Normal mouse serum served as control for 0% binding. The antigen capacity of the serum /ABC/ was calculated as /ug I^{131}-HCY bound by 1 ml serum.

2. Direct radioprecipitin test combined with radioautography /14/ was modified by Dr.Rhodes for very small quantities of antiserum. 1 /ul mouse sera was mixed with 10 /ul HCY at

concentrations varying from 0.02 - 8 /ug/ml, each HCY concentration containing the same amount of I^{131}-HCY to give 200-600 counts/ /ul. After incubation at 37°C and 24 hours at +4°C, the tubes were shaken. 1 /ul was spotted on cellulose acetate paper and the cellulose acetate strips were washed for 1 day in phosphate buffer pH 7. Free I^{131}-HCY is washed away, whereas any I^{131}-HCY bound by antigen/antibody complexes sticks to the cellulose acetate and shows up as a black spot on an X-ray film. Normal mouse serum showed no binding of radioactive HCY. The end point is given as the concentration of HCY /in /ug/ml/ at which the X-ray film detects binding of radioactive I^{131}-HCY by the antigen/ antibody complex.

Sucrose gradients: 4-20% sucrose gradients were prepared by careful layering of four concentrations of sucrose /4, 10, 15, 20%/ containing 0.15M-KCl, 0.01M Tris, and 0.00015M-$MgCl_2$. For analysis of RNA preparations bentonite was added to the samples to inhibit ribonuclease activity. The gradients were run in the cold at 3 - 3 1/2 hours at 34,000 RPM in the Spinco SW 39 swing out rotor.

Results

1. Fate of antigen in macrophages.

Uptake of I^{131}-HCY by peritoneal cells was studied to choose optimum conditions for the experiments. One /ug of I^{131} labelled HCY was injected i.p. into mice. The peritoneal cells were collected after varying time intervals, the cells were washed twice with Gey's solution and radioactivity due to I^{131} was counted in washed macrophages as well as the peritoneal fluid cell supernatant.

The radioactivity is eliminated rapidly from the peritoneal fluid. The cells initially take up I^{131}-HCY quickly with a peak at one hour; the uptake varies with the I^{131}-HCY preparation from 0.7-1.5% of the total injected dose. Radioactivity on the macrophages then falls and by 21/2-3 hours reaches a plateau which is maintained almost at a constant level for 24 hours /Fig.1/. Therefore a 21/2 hour interval was chosen for the preparation of RNA. The distribution of radioactivity was studied at this time interval and most of the I^{131} was found to be bound by subcellular particles. The homogenate was therefore treated with 0.1% Triton 100 at +4°C to lyse lysosomes, layered on a sucrose gradient and centrifuged for 4 hours at 34,000 RPM. Fractions were analysed for radioactivity due to I^{131} /see Fig.2a/. It can be seen that 2/3 of the I^{131} counts are of small molecular weight /< 8 S/ and a considerable amount appears to be still firmly bound to subcellular particles larger than ~50 S and which sediments in pellet form under these conditions. There is a small amount of radioactivity present in fractions of S values between 40-8 with the mobility of the original HCY. Figure 2b shows I^{131}-HCY added to mouse macrophages and treated in the same way. Antigenicity of the radioactive material was tested by adding specific rabbit anti-HCY to the gradient fractions and a slight excess of anti-rabbit γ-globulin to precipitate any I^{131}-HCY-Ab complex. It can be seen /arrows on Fig. 2a/ that in the region of HCY peaks a high percentage of the counts are specifically carried down by the antigen/antibody complex, but that 2/3 of the material is degraded /top 3 tubes/ and does not react with the antiserum.

2. RNA extracts of macrophages following contact with haemocyanin.

A series of different experiments were carried out. The last

two will be described in detail and the remained of the results will be summarized. About half of these RNA extracts proved to be immunogenic.

a/ RNA extract of macrophages 21/2 hours after the inejction of I^{131}-HCY in vivo /20 mice received i.p. injections of 4 /ug I^{131}-HCY/mouse /6 /uc/ /ug/. Two and a half hours later the peritoneal cells contained after washing 0.3% of the total injected I^{131} dose /i.e. 0.24 /ug/. They were extracted with phenol in an inebath as described in the Methods section /similarly to the procedure described by Fishman and Adler /9/.

This RNA extract /RNA-M-HCY/ contained about 750 /ug RNA and 3% of the total I^{131} radioactivity present in the washed macrophages. The aqueous phase selects the free counts but 5-10% of the radioactivity was in macromolecular form /precipitated with 10% TCA and with specific anti-HCY serum/. This pic ture is confirmed when RNA-M-HCY is analyse on a sucrose gradient /Fig. 3/. The major proportion of the radioactive counts stay on top of the gradient /small molecular weight/ but there is always some radioactivity associated with S values of the original HCY /tube 7/ and also small amounts of material both with lower and higher rates of sedimentation /tubes 3,4 and 9,10/ which may represent possibly degraded or complexed HCY. Fig.3 also shows the distribution of radioactivity when I^{131} is added to control RNA preparation. The gradient values check well with S_{20} values obtained by Dr.Charlwood for the same HCY preparation in the analytical ultracentrifuge. Main peak S_{20}= 26, and two more peaks were S_{20} of 15 and 36. The RNA thus contains in macromolecular form about 0.15-0.3% of the total HCY taken up by the macrophages or approximately .0035-0.007 /ug.

The absorption density of the nucleic acid at 260mμ/u is illustrated in Fig.3 and showw that the phenol RNA preparation contains the usual sized components, about 16 S, 30 S, and some smaller molecular weight RNA. This particular RNA was frozen immediately after preparation before analysis in the gradient. If RNA extracts are permitted to stand at +4°C over night or longer the RNA is partly degraded /Fig.4/ presumably by contaminating RNAase. However, this does not alter the distribution of radioactivity due to I^{131}.

This RNA preparation was immunogenic in our test mice. The RNA /immediately after preparation/ was incubated with normal mouse spleen cells and transferred intraperitoneally into 5-6 primed mice per group /see Methods/. Each mouse in the RNA-M-HCY group received approximately 50 /ug RNA containing macromolecular I^{131} of the order of 0.00004 /ug /calculated on the basis of radioactivity of the injected I^{131}-HCY/. The mouse sera were assayed for antigen binding capacity 9 days after i.p. injection. Table 1 shows that mice in the control group had titers averaging 1.25 /ug ABC/ml; RNA-M-HCY increased the Ab titers to an average of 6.4 /ug ABC/ml. This may not seem a very striking difference, but conditions of our assay are such that the second antiserum dilution binds only a small percentage of the I^{131}-HCY in the controls, whereas the higher titers bind most of the I^{131}-HCY present. Pretreatment of RNA-M-HCY with 20 /ug ribonuclease for 30 min. at 37°C inhibited the antibody response.

Seven similar experiments were carried out, extracting RNA from peritoneal cells 21/2 hours after in vivo injection of HCY or in some cases 4 hours after in vitro incubation of macrophages where similar amounts of I^{131}-HCY had been taken up. In most of

these cases the cells were trated with phenol at 15°C for 20 minutes for the first extraction since 15°C appeared to yield more messenger-type RNA in liver and plasma cell tumours /15/. Extraction at 0°C is not efficient for obtaining messenger RNA from mammalian cells. Only 50% of the RNA-M-HCY extracts proved to be at all immunogenic in the recipient mice. No visible differences in the molecular size of RNA or macromolecular I^{131} radioactivity could be correlated with any success or failure in antibody induction.

b/ RNA extracts prepared from macrophages immediately after addition of I^{131}-HCY.

It was important to ascertain whether or not an active process in the peritoneal cells is responsible for formation of the immunogenic material in RNA-M-HCY. I^{131}-HCY was therefore added to normal peritoneal cells prior to homogenization and phenol treatment.

The same number of cells were used for extracting RNA as in the in vivo experiments, and I^{131}-HCY, equivalent to the amount taken up in vivo /0.2 - 0.4 /ug/ was added to these cells. These RNA extracts were not immunogenic in primed mice. Far less I^{131} labelled macromolecular material appeared to be associated with this type of phenol extract. However, in the last experiment we added ten times the amount of I^{131}-HCY /i.e. 3.2 /ug/ to the normal macrophage homogenate before treatment with phenol. Then we obtained similar amounts of macromolecular radioactivity associated with the RNA as compared with the in vivo experiments. After 3 hours' dialysis about 0.1% of the added I^{131}-HCY was present in the RNA; 30% of this radioactivity was precipitable by TCA, and 25% with specific antiserum to HCY.

This RNA preparation was immunogenic /Table 2/. Each mouse received about 70 /ug of RNA containing radioactivity equivalent to approximately 0.00005 /ug of the original HCY. Table 2 shows that control mice have titers averaging 2 /ug ABC/ml, whereas the experimental group have higher titers with an average of 5.8 /ug/ml. Pretreatment of the RNA with ribonuclease inhibited the increase in titer. The distribution of I^{131} radioactivity on a gradient was similar to the one shown in Fig.3 for RNA-M-HCY /after in vivo injection of I^{131}-HCY/. Radioprecipitin tests were carried out as a confirmatory test and these also showed increased titers in the experimental group Table 3/.

Discussion

The work of Fishman and Adler /9/ raised the possibility that phenol extracted RNA from peritoneal cells contains information for immunologically reactive cells, since it induced neutralizing antibody to bacteriophage. Similarly, RNA from immune lymph glands was reported to transfer immunity to transplantation antigens /16/. One finding shows that RNA extracted with phenol from peritoneal cells 21/2 hours after contact with I^{131}-HCY in vivo, can be immunogenic in perviously primed mice. Furthermore, RNA prepared immediately after addition of I^{131}-HCY also proved to be immunogenic, although compared to the in vivo experiments, it was necessary to add a greater amount of HCY /about 10 times/ to obtain similar amounts of macromolecular I^{131}-HCY associated with the RNA. This macromolecular I^{131} radioactivity suggested the presence of antigen or fragments thereof in both types of RNA. The finding that small amounts of protein are present in phenol RNA preparations agrees

with the experience of other workers in the field.

Thus, at least in our system, the immunogenicity of the extracts cannot be due to the formation of a specific informational nucleic acid by the macrophages after contact with antigen, but it is conceivable that there is an association with macrophage RNA which is of physiological significance. Very low amounts of antigen /of the order of 5×10^{-5} /ug/ associated with RNA induced a secondary response in primed mice.

Pretreatment with ribonuclease inhibited this response /20 /ug enzyme/300 /ug RNA/. There may be several reasons for this phenomenon, and we are planning to carry out further experiments with lower concentrations of RNAase to reduce any possible injurious effect on the spleen cells. The RNA may enhance immunogenicity by facilitating the uptake of antigen by the relevant immunologically competent cells, and thereby ensuring that antigen by the relevant immunologically competent cells, and thereby ensuring that antigen reaches the vital control centre within the cell. Dutton and Harris /17/ also have evidence for a highly immunogenic factor in spleen cells. 15 minutes after incubation with HSA, irradiated, frozen and thawed spleen cells, specifically stimulated DNA synthesis in spleen cells from HSA immunized animals. Similar amounts of HSA had no effect.

We are in the process of testing the immunogenicity of small amounts of HCY /of the same order as those associated with the RNA preparations/. As yet, variable results make it impossible to decide whether we are dealing with a "super antigen" in our peritoneal cell extracts or not. In one experimental series levels down to 0.00005 /ug I^{131}-HCY stimulated antibody formation in our primed mice. In another series, mice receiving 0.0005 - 0.000005 /ug

HCY showed no increase in antibody titer.

Conclusions

Some phenol RNA preparations from mouse peritoneal cells, after contact with I^{131}-HCY in vivo, were immunogenic in primed mice.

The addition of I^{131}-HCY to macrophages immediately before phenol treatment also yielded immunogenic RNA extracts, provided that a sufficient quantity of HCY was added. With a given amount of I^{131}-HCY more macromolecular radioactivity was associated with RNA after contact with antigen in vivo than with an equivalent amount of I^{131}-HCY added just before phenol treatment. This fact may be of physiological significance.

Pretreatment of both types of RNA preparation with ribonuclease inhibited this induction of antibody in recipient mice.

Since the RNA preparations contained some I^{131} radioactivity in macromolecular form which reacted with antiserum to HCY, it cannot be excluded that their immunogenicity is due to the presence of small amounts of macromolecular HCY or its degradation products, possibly complexed with the RNA. Further work is required to decide whether the immunogenic fraction behaves as a "super antigen" as compared to equivalent amounts of HCY.

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Table 1

Immunogenicity of RNA-M-HCY.

Indirect I^{131}-Ag binding test.

I.p. injection		Individual titers					Average titer
Spleen cells	Addition	/ug HCY/ml AS					
+	-	3.7	0	0.5	1.6	2.0	1.3
		0	0	0.8	1.4	4	
+	RNA-M-HCY[a]	3	3.1	>9.2	>8.8	7.7	>6.4
+	RNA-M-HCY + RNAase	3.6	1.6	0	4.3	3.6	2.6

[a]RNA-M-HCY extracted from macrophages 21/2 hours after injection of I^{131}-HCY in vivo.

Spleen cells /3 x 10^7 cells/mouse/ were preincubated with RNA-M-HCY 1 hour at 37°C before injection into mice. The recipient mice had been primed 2 months previously with 5 /uf alum HCY in the footpads. In the last group RNA-M-HCY was treated with 20 /ug RNAase for 30 mins. at 37°C before incubation with the spleen cells. The mice were bled 9 days after the transfer of spleen cells.

Table 2

Indirect I^{131}- Ag binding test.

i.p.inejction		Individual titers[b]						Average titer
Spleen cells	Addition	/ug HCY bound/ml mouse serum						
+	-	0.6	3.0	2.9	1.2	0.9	4.0	2.2
+	RNA-M[a]	6.2	4	7.7	6.8	4		5.8
+	RNA-M + RNAase	1.2	2.7	2.7	2.3	0.8		2.0

[a]RNA-M extracted from macrophages immediately after addition of 3.2 /ug I^{131}-HCY.

[b]Antigen binding capacity of antisera of recipient mice 9 days after transfer of spleen cells.

Other conditions as in Table 1.

Table 3

Direct radioprecipitin test /autoradiography/.

I.p. injection		Individual titers /ug HCY/ml						Average titer
Spleen cells	Addition							
+	-	0.5	0.5	0.02	0.02	0.25	0.02	0.26
+	RNA-M[a]	1	1	2	1	0.5		1.1
+	RNA-M+ 20 /ug RNAase	0.5	0.5	0.5	0.5	0.5		.5

a RNA-M from macrophages to which I^{131}-HCY had been added before phenol extraction.

b Titers are expressed as concentration of HCY at which radioautographs show positive binding of I^{131}-HCY by antiserum /see Methods/.

Other conditions as in Table 1.

Legends to figures

Fig.1. Uptake of I^{131}-HCY by peritoneal cells in vivo. 1 /ug I^{131}-HCY was injected i.p. per mouse. Peritoneal cells were collected after varying time intervals. Each point represents an average of 3 mice. △——△, Radioactivity due to I^{131} in peritoneal fluid /cell supernatant/. O——O, Radioactivity due to I^{131} on washed peritoneal cells.

Fig.2. Sucrose gradient of macrophage homogenates.
a - Peritoneal cells collected 21/2 hours after I^{131}-HCY in vivo. Homogenate was treated with TRITON 100 and layered on a 4-20% sucrose gradient. Tube 14 represents the top of the gradient /4% sucrose/ after centrifugation for 31/2 hours at 34,000 RPM. O —— O, Counts due to I^{131}. Arrows /↓/ indicate % of I^{131} radioactivity precipitated by rabbit anti-HCY and anti-rabbit γ-globulin.
b - Sucrose gradient as on a. I^{131}-HCY /.005 /ug/ was added to an homogenate of normal macrophages and treated with TRITON 100. O——O, Counts due to I^{131} radioactivity. Arrows /↓/ as on a.

Fig.3. Phenol extracted macrophage RNA on a sucrose gradient.
O --- O, D_{260} of nucleic acid.
● ——●, Counts due to I^{131}, RNA-M-HCY.
△——△, Counts x 10^{-1} due to I^{131}, when a trace of I^{131}-HCY is added to control nucleic acid.
RNA-M-HCY: RNA extracted from macrophages 21/2 hours after injection of I^{131}-HCY in vivo plus Bentonite was layered on a 4-20% sucrose gradient; tube 13 represents the top of the gradient /4% sucrose/ after centrifugation for 31/2 hours

at 34,000 RPM.

Fig.4. Degradation of RNA-M-HCY at 4°C.

Sucrose gradient as in Fig.3. This is another preparation of RNA-M-HCY /as in Fig.3/ left to stand at +4°C overnight.

O ---- O, D_{260} of nucleic acid.

● —— ●, Counts due to I^{131}.

Tube 14 represents the top of the gradient /4% sucrose/.

For discussion in the topic IV.

Possible Relation between Some Results of the Study of Delayed Type Hypersensitivity in Tissue Culture and the Mechanism of Antibody Formation

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Antibody formation and delayed type hypersensitivity are usually considered as two principally different immunological mechanisms, although potential relations between them are often discussed. Most frequently the possibility is considered that delayed type hypersensitivity represents a certain stage in the development of the mechanism of antibody formation or that the state of delayed hypersensitivity is evoked by the binding of special, unconvential antibodies onto cells. Both phenomena /mechanism of antibody formation and delayed type hypersensitivity/ are mostly studied separately, without paying sufficient attention to the fact that both these mechanisms usually exist in the sensitized organism simultaneously and that they both can participate in its immunological reactions.

In the study of delayed type hypersensitivity various methods were intensively developed in the past few years for the demonstration of this type hypersensitivity in vitro, mostly by means of migration inhibition by specific antigen of cells from a sensitized animal. The importance of this reaction, its specificity and reproducibility are beyond any doubt nowadays.

Nevertheless, one basic fact should be kept in mind. In the experiments of this type the reaction of the cellular population of the sensitized animal is followed as a whole, although it

is obvious that only a small percentage of the cells present can be really sensitive to the given antigen. It is, after all, unthinkable that in an organism hypersensitive to several different antigens each of its mesenchymal cells could be specifically hypersensitive to each of these antigens. This conclusion logically ensues from all we know at present on the basis and the manner of the development of immunological reactions.

Recently direct evidence has been brought forward for the correctness of this view. David et al. /1/ have demonstrated that mere 2,5 per cent from a sensitized animal mixed with 97,5 per cent cells from a normal animal are capable of eliciting a state in which the whole cellular population reacts on the addition of specific antigen as though it were taken from a sensitized animal. It is evident that the migration of normal, nonsensitized cells was influenced as a result of the reaction between a small percentage of hypersensitive cells and the anigen; the most probable explanation is that in the course of the specific reaction of hypersensitive cells with the antigen substances possessing pharmacological activity are released which influence the behaviour of the remaining normal cells. We too arrived at similar conclusions on using another experimental approach, i.e. when cultivating simultaneously two spleen fragments, one from a sensitized and one from a normal guinea pig, in a single cultivation chamber in liquid medium /13/. Our results indicated that on adding specific antigen identical migration changes occur in a part of the experiments in the normal fragment taken from a hypersensitive donor. The only possible explanation of this phenomenon is again the presumption that some pharmacologically active substances penetrate through the liquid medium from the hypersensitive to the normal fragment. Analogous con-

clusions ensue also from the recently published study by Pincus et al. /9/.

The mentioned results are in conformity with the general hypothesis presuming that the reaction of hypersensitive cells with antigen induces the formation of pharmacologically active substances /mediator/ which elicit, through an irritation of cells, the actual manifestations of delayed type hypersensitivity /5/.

The second important point is the question, whether the reaction of hypersensitive cells with antigen in vitro always leads only to the inhibition of their function /as it is generally believed since the experiments by Rich and Lewis/, or whether it can manifest itself sometimes, on the contrary, as a stimulation. Evidence can be found here and there in the literature on the stimulatory action of antigen on cell suspension from hypersensitive animals /2, 6, 14, 15/. Also recent studies on the induction of mitotic division in the tissue culture of leukocytes from tuberculin-sensitive subjects following the addition of this antigen /7, 8/ may be a certain reflection of this fact. Nevertheless, most authors still consider cell injury as the only form of the response of hypersensitive cells to the antigen.

At our laboratory this problem has been thoroughly analyzed. We used two experimental approaches, viz. the determination of the migration of hypersensitive guinea pig cells in the presence of graded doses of specific antigen on the one hand /11/, and the study of the dynamics of migration at various time intervals on the other hand /12/. The experiments were performed using our standard technique of spleen fragment cultivation in liquid medium with objective photographic recording of the increasing size of several parallelly cultivated fragments /10/. The results are

expressed as cytotoxic indices /CI/, i.e. as the ratio between the growth in experimental conditions and that in control cultivations.

In the first series of experiments with normal and hypersensitive spleen fragments graded doses of antigen /PPD tuberculin ranging between 50 gamma to 0,02 gamma and of Old Tuberculin from 1 : 100 to 1: 250 000/ were used. Higher antigen doses usually used by most authors studying similar problems regularly result in an inhibition of the migration of hypersensitive cells. The relationship between the quantity of antigen used and the degree of migration inhibition is statistically significant. On using very small antigen doses, i.e. PPD 0,5 to 0,1 gamma, OT 1 : 2 000 to 1 : 50 000 we observe in some cases /in 23 out of 56 experiments with sensitive cells/ a marked stimulation of the migration; this result is statistically significant. Similar stimulation was never observed in control cultivations using nonsensitized cells or on using nonspecifically toxic antigen /purified Salmonella paratyphi B endotoxin/, both in sensitized as in normal cells.

In the second series of experiments antigen doses used were 10 gamma PPD and 1 : 100 OT and migration dynamic was followed at regular time intervals during the incubation of spleen fragments. Cells of normal fragments with or without antigen and cells of fragments from sensitized animals without antigen migrate relatively evenly thoughout the experiment. On the other hand in hypersensitive cells incubated with antigen a stimulation of the migration activity is regularly observed in the first hours, as compared with the control groups, while later cells migration is slowed down or ceases altogether, thus leading to the final result observed after the end of the experiment as the inhibition of

the migration of hypersensitive cells incubated with antigen. Migration stimulation in the first hours of the experiment as well as the final inhibition are statistically significant. In control experiments the effect of purified endotoxin was studied which in low doses causes a moderate stimulation of the migration activity for the duration of the whole experiment, while in higher doses it leads to a permanent inhibition from the beginning up to the end of the experiment, both in normal and in sensitized cells.

The above mentioned results as well as the quoted experiments of other authors permit to conclude that the response of cells from a hypersensitive organism to specific antigen can appear not only in the form of an inhibition, but also of a stimulation of their activity. The result obviously depends on the conditions of the experiment, antigen dose, degree of hypersensitivity, etc. On the basis of single literary data as well as of our own preliminary experiments we believe that the basis of the observed reactions of hypersensitive cells incubated with antigen should be seen in the changes of their metabolic activity.

The above results and conclusions must be brough into connection with the previously discussed hypothesis that a small percentage of hypersensitive cells react specifically with antigen and influence /probably as a result of the release of some mediator or mediators/ the behaviour of the other principally non-sensitized cells. In other words, we have to admit that the reaction of the cellular population to the proceeding reaction of delayed type hypersensitivity can manifest itself by a stimulation of the function of these cells, probably as a consequence of their increased metabolic activity.

This hypothetical conclusion is the very reason for discussing the results of the study of delayed type hypersensitivity

at the Symposium on the Mechanism of Antibody Formation. The exact relationship between delayed type hypersensitivity reactions and the mechanism of antibody formation is not known yet; there is, at least, one fact we must keep in mind, i.e. that in immunized animals usually both mechanisms exist simultaneously. It is known at the same time that the process of antibody formation in vitro /especially the secondary response/ is accompanied by an increased activity of the cellular suspension present, which manifests itself e.g. as a stimulation of DNA synthesis /3, 4/. We might perhaps consider the possibility that in the metabolic changes observed at the beginning of antibody formation in vitro participates the simultaneously proceeding reaction of delayed hypersensitivity. We could even presume that delayed type hypersensitivity reaction connected with the general metabolic stimulation of the cell population constitute the physiological basis for antibody formation; all this is naturally merely a hypothesis.

The main interest of our studies lies exclusively in the field of delayed type hypersensitivity. We think though that some of the reported results can be interesting also for those studying the mechanism of antibody formation.

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The Change in the Molecular Weight of Antibodies Synthesized in Tissue Culture after Second Immunization

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The change in properties of antibodies in the course of immunization is of great theoretical and practical interest. Such a phenomenon has been reported by many authors. It was found in particular that antibodies in sera of hyperimmune rabbits subjected to second immunization with serum albumin exhibit immunochemical properties that are on the fourth day from those displayed by antibodies on the seventh to tenth day after immunization. The former /"earlier"/ antibodies require considerably more antigen for their precipitation than do the latter ones. /7/.

Recently, a number of laboratories have succeeded in showing that after the injection of antigen, sera contain antibodies of a high molecular weight /19 S antibodies/, then their number decreases with increasing number of antibodies of a molecular weight about 160,000 /7 S antibodies/. This has been particularly clearly demonstrated in the case of primary response /1, 3, 13/.

This work deals with possibility of 19 S antibody formation in hyperimmune rabbits by investigating antibodies formed by cells of spleen and lymph nodes on separate cultivation in vitro rather than serum antibodies that are mixtures of antibodies synthesized in different organs. Cells were isolated from rabbit organs on the third day after second immunization, i.e. on the day when one would expect sythesis of antibodies with abnormal

precipitating properties. A comparative study was made of antibodies synthesized on the fifth day after the second immunization. "Heavy" 19 S antibodies were distinguished from "light" 7 S antibodies by filtering through gel Sephadex G-200.

Methods

Rabbits were immunized with a human serum albumin /HSA/, according to the following scheme /6/. They were given increasing amounts of albumin intravenously for three weeks, i.e. one, two and three mg /9 injections/. After two-three months rabbits were injected with 50 mg of HSA each /second immunization/. On the third or fifth day after reimmunization rabbits were exsanguinated and the spleen and inguinal and popliteal lymph nodes removed. These were minced with scissors and passed through sterile capron mesh. The cells were then washed and suspended in Eagle's medium. The final volume of the spleen cell suspension was 6 to 8 ml, that of the lymph node cells 3 ml. Up to 20 % of the serum pf a normal or experimental rabbit was added to the suspension.

Cells were cultured in special small vessels comprising two parts /Fig.1/. A small cup /Fig.1/ was filled with a mixture of Eagles's medium and rabbit serum /about 6 ml. in all/ with a small glass covered magnetic bar /Fig.4/ placed on the bottom. A ring /Fig.2/ with a cellophane membrane /Fig.3/ stretched on it was placed on the top of the cup, both being tightly fastened with rubber rings from outside. 1.5 ml. pf cell suspension with 3 /uc of algae C^{14}-protein hydrolysate, was poured onto the membrane and the asembled vessels were placed in crystalliser /Fig.6/. All vessels were placed under a hood. The chamber was continuously fed with a mixture of O_2 and CO_2 /95% and 5%/.

The medium was mixed in the cups by special magnetic mixers that were switched in at intervals by relay /for further particulars see 10/.

After incubation for 18 or 40 hours at 37°C the cells were collected together with the nutritional medium and homogenized. The homogenate was centrifuged, first at 4,000, then at 40,000 rpm and passed through a column with Sephadex G-25. Cellulose-fixed bovine globulin /as a non-specific immunosorbent/ was added liquid. After its removal by centrifugation cellulose-fixed HSA was added as a specific immunosorbent. Both immunosorbents were washed eight times and then bound antibodies eluated at pH 2.5. The eluate from fixed globulin usually failed to exhibit any radioactivity or the number of impulses did not amount to more than 3 to 5% as compared to that in the eluate from fixed HSA.

To estimate the size of molecules of antibodies synthesized in the cell culture the eluates were filtered through Sephadex G-22 together with the serum of the experimental rabbit /11/ in accordance with the procedure described by Flodin and Killander /5/ using a column 46 x 2.3 cm and a Tris-HCl buffer /0.04 M- with 0.28 M NaCl/. The fractions leaving the column were analysed, their protein concentrations was determined by Lowry method and the radioactivity was counted in a gas flow counter for 500 seconds.

Results and discussion

The cells of the spleen and lymph nodes of hyperimmune rabbits under the conditions of cultivation applied in our experiments had synthesized fairly large amounts of antibodies. By means of immunosorbents in 13 experiments it was found that

that 40 hours' cultivation gave rise to 60 to 360 µg antibody protein per one gram wet weight of the spleen.

The physico-chemical study of antibodies synthesized in the cell culture meets with considerable difficulties because there are soluble substances and fragments of cells disintegrated during cultivation in nutritional medium together with antibodies. This was, in particular, pointed out by Pospíšil and Franěk /12/. The task is, however, facilitated if the synthesized antibodies are isolated by means of specific immunosorbents. This enables one to obtain preparations of high purity. In accordance with this procedure the radioactive antibodies synthesized in vitro were isolated in the experiments below by means of fixed HSA.

The size of the molecules of antibodies formed in the cell culture was determined by filtration through a column filled with Sephadex G-200. According to Flodin and Killander /5/ such a filtration leads to the division of all serum proteins into three main fractions with sedimentation constants 19 S, 7 S and 4 S. Therefore, by labelling a protein, say, with radioactive aminoacids and passing it with serum through Sephadex G-200, it would be possible to estimate approximately the molecular weight of the protein through localization of the tracer in a particular fraction.

In our experiments 1.0 ml. of the solution of radioactive purified antibodies synthesized in vitro was mixed with 0.5 ml. rabbit serum, passed through Sephadex G-200 column and the protein concentration and radioactivity of the fractions measured. The results of a representative experiment with antibodies synthesized by spleen cells on the third day following second immunization are given in Fig.2. It will be seen that the serum proteins display

three clear peaks. The greater part of radioactivity was accounted for by the first peak. This shows that radioactive antibodies had a higher sedimentation constant than that of the proteins of the second peak that are still held by the gel, or higher than 7 S. The rabbit is known to have antibodies with sedimentation constants of 7 S and 19 S only. Hence under conditions of this experiment most synthesized antibodies had a high molecular weight and their sedimentation constant was 19 S.

It was not alwys, however, that the first protein peak accounted for most radioactivity. In a number of experiments the ratio of radioactivity between the first and second peaks was different from above experiment /Fig.3 and 4/. This appears to be due to the fact that the amount of "heavy" antibodies synthesized depends on the individual reaction of the rabbit to the injection of antigen.

The second experimental series dealt with antibodies synthetized by spleen cells taken on the fifth day after the second immunization. In this case practically all radioactivity was acccunted for by the second protein peak /Fig.5/. This means that only antibodies of the 7 S type were synthetized, as was to be expected.

The results obtained in the first experimental series were not due to artefact. This is substantiated by the following consideration. The radioactivity accounted for by the first protein peak did not result from any particles that would have been mechanically sorbed on cellulose during the isolation of antibodies by immunosorbents. As we had already mentioned, non-specific adsorbtion was either absent or very weak. Neither was the tracer in the first protein peak connected with aggregates

of 7 S antibodies that could appear in the process of their isolation. This is shown by sedimentation investigations of rabbit antibodies isolated by immunosorbents from sera on the seventh to tenth days after second immunization. These sedimentation experiments gave only a single peak with a constant 6,1 S /7/. Furthermore, if aggregates of antibodies had appeared on isolation, they would have also been observed in experiments with antibodies synthesized on the fifth day after the second immunization. We have, however, seen from Fig.5 that in these experiments practically all radioactivity was accounted for by the second protein peak, the sedimentation constant being essentially 7 S.

It can therefore be considered as established that on the third day after the second immunization of hyperimmune rabbits the cells of spleen and lymph nodes are capable of synthesizing antibodies of a high molecular weight /about one million/. On the fifth day, however, they cannot yet be found, only antibodies of 7 S type then being synthesized. It cannot be excluded that it is these "heavy" antibodies that exhibit the abnormal precipitation properties mentioned above.

It was suggested that 19 S antibodies were synthesized after second immunization by these cells had not yet come into contact with antigen and for which the secondary introduction of antigen was to be regarded as the primary one /2/.

This point of view however, is contradicted by the quick start followed by just as quick a depression of the synthesis of "heavy" antibodies in our experiments. According to Dixon et al. /4/ antibodies in rabbit serum after the first introduction of serum albumin appear not earlier than on the seventh day, whereas after the second injection they are to be seen on the

second day.

It is, therefore, more likely that "heavy" antibodies after second immunization are synthesized by cells that had already been in contact with antigen. From the third day after second immunization the amount of antibodies formed in vitro is fairly large and it was shown in our experiments that the percentage of "heavy" antibodies may, thus, be rather high. It would be reasonable to suggest that cells that synthesize "heavy" antibodies should have a specific structure similar to that of cells from the early stages of plasmatic series. It has been shown that many large pyronophylic cells resembling plasmablasts in structure can be found among cells of the spleen and lymph nodes of hyperimmune rabbits on the third day after the second immunization with HSA /8/.

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Legends to figures

Fig.1. The chamber and vessels for cell incubation.

Fig.2. Filtration through gel Sephadex G-200 rabbit serum and radioactive antibodies synthesized by spleen cells on third day after second immunization.

1-protein concentration, 2-radioactivity.

Fraction volume 0.9 ml.

Fig.3. Idem

Fig.4. Filtration through Sephadex G-200 rabbit serum and radioactive antibodies synthesized by cells of lymph nodes on the third day after second immunization.

Fraction volume 0.9 ml.

Fig.5. Filtration through Sephadex G-200 rabbit serum and radioactive antibodies synthesized by spleen cells on fifth day after second immunization.

Fraction volume 2.2 ml.

The Hypothesis of "Carrier-Globulins"

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Numerous theories, some quite ingenious, have been formulated in attempts to explain the mechanism of antibody formation. No single theory has ever been universally accepted by all immunologists. It has always been possible to find some fact or facts incompatible with some aspect of each theory thus far brought forward.

It is clear that until we know with some precision the mechanism of protein biosynthesis we shall be obliged to continue to formulate hypotheses as to the manner in which an antigen provokes the synthesis of immunoglobulins. Our ignorance as to the true reason why a given substance should be antigenic forces us also to think in more or less hypothetical terms. In this communication I shall not discuss the final stages of the mechanism of antibody formation, that is the synthesis of molecules of immunoglobulin. It is evident that genetic mechanisms must have an important role in this. Yet I believe that one ought to be too impressed by the great variety of immunoglobulins which can be synthetized if one bears in mind the vast potential of the initial genome. Evolving from the same genome are the gastric epithelial cell which synthesizes pepsinogen, and the thyroid cell which synthesizes thyreoglobulin etc.

When one considers this differentiation one is perforce obliged to envisage selective mechanisms of activation or inhibition of the host of pre-existent synthetic systems in the genome.

While it is tempting to imagine that antigenic substances /or their debris/ intervene in these control mechanisms thus favoring the synthesis of immunoglobulins of particular configurations, I do not believe that, at the moment, we have enough facts at our disposal to accept or to seriously propose such a view.

It seems to me rather more useful to discuss the initial mechanism in the formation of antibody. I should like to consider critically one of the postulates which has served as a cornerstone for several theories of antibody formation and which is almost always admitted without discussion. I speak of the postulate which says that antibodies are formed only against exogenous substances. Now, it has long been observed that antibodies can be formed against endogenous substances and we are witnessing at present a host of experimental observations proving the possibility of formation of auto-antibodies. Attempts to take into account these findings, and at the same time conserve the initial postulate, have necessitated the formulation of ancillary or supplementary hypothese, such as: changes in endogenous substances render them in some way foreign; or that endogenous substances which are potentially antigenic are ordinarily sequestered in the organism out of contact with the immune apparatus etc. I wonder, however, if instead of formulating supplementary hypotheses in order to bring the facts into line with the postulate it might not be more logical to abandon the postulate and admit that native endogenous substances can and do induce the formation of antibody. In order to justify this viewpoint it would be necessary 1.-to prove the formation of antibody to endogenous substances and 2. - explain why in normal serum a multiplicity of antibodies towards the diverse endogenous constituents are not ordinarily seen.

It seems to us superfluous to review the numerous studies showing the existence or the experimental formation of auto-antibodies. Nevertheless, one must ask oneself in each case whether an auto-antibody is truly the cause. The demonstration of antibodies which react with endogenous substances in cases of human pathology could be the consequence of extrinsic factors, e.g. infection, and the reactions observed could be cross-reactions. Similarly, when one provokes the experimental formation of auto-antibodies by the injection of an "endogenous" antigen in Freund's adjuvant, one must question and provoke alterations in the antigen sufficient to transform it into a substance considered as foreign by the organism.

But even without dwelling on the experiments where every precaution has been taken to avoid any modification of the antigen, or on the fact, for example, that anti-thyreoglobulin auto-antibodies can be induced without resorting to Freunds adjuvant, the following argument seems to me irrefutable: if the antibody can be shown to react strongly with unmodified endogenous substances, this proves /even if it is merely a cross-reaction/ that the organism can and does make antibodies possessing a configuration corresponding with antigenic determinants found in the structure of an endogenous substance. In other words, even if antibody formation is facilitated by modification of the antigenic substance, the fact that the antibodies react with the native endogenous substance proves that the organism is perfectly capable of synthesizing specific antibodies directed against antigenic groupings found in normal endogenous substances.

2. Admitting then that endogenous antigens can provoke the

formation of auto-antibodies one is left wondering why the serum of a normal organism does not contain antibodies directed against any and all of its endogenous constituents. There is no simple reply to this question. I would cite on the one hand observations showing the presence of antibodies in normal sera which react with substances found normally in the organism /glycogen, gelatin, nucleic acids/ and on the other hand I can think of two reasons which seem to me valid to explain why antibodies to endogenous substances should not ordinarily be detectable. Indeed, it would be astonishing to be able to show free antibody in the circulation if the antibody is specific for an endogenous constituent with which it should readily react. Secondly the existence of an immunologic tolerance of immunologically competent cells towards the substances with which they normally in contact can be invoked.

The precise mechanism of the tolerance phenomenon is apparently not yet elucidated but we already possess many useful data. Among the principal established facts we should note the following: a/ tolerance cannot be induced to all antigens, b/ tolerance lasts longer when the inducing antigen is phylogenetically closer to the induced organism than when the antigen is phylogenetically distant c/ tolerance can be prolonged by continued administration of the antigen, d/ tolerance can be induced in adult animals by using large quantities of antigen; when the quantities required to induce tolerance in newborn and an adult animal are compared on a weight basis they are found to be closely similar. e/ When tolerance is induced with living cells, their multiplication within the recipient animal can explain the long duration of tolerance. f/ The chemical nature of the antigen can play an important role for some substances persist longer in the organism than others.

g/ Tolerance can be partial, that is to say that it can exist towards certain but not all the determinant groups of a molecule.

Among these facts a certain number resemble those known for adaptative enzyme formation, and I have already had the occasion to point this out and to envisage tolerance as being due to the formation of adaptive enzymes able completely to break down the casual substance.

Unfortunately, we do not know in a precise fashion in what way the antigen is active in antibody synthesis. It seems, on the one hand that beyond a certain point the degradation products of an antigen macromolecule are no longer active, while on the other hand that it is not the macromolecule in its entity which is active. In fact, it is known that antigenic proteins can be attacked by intracellular proteases and yet they provoke antibody formation. We know also that powerful antigens, such as the endotoxins, can be broken down even in vitro in the serum. Such reasoning leads us to think that it is probably the products of partial degradation of an antigen which are active.

If it is so and if tolerance is due to the formation of adaptive enzymes capable of breaking the antigen down completely, the greater part of the findings which we have enumerated can be explained.

We think it useful to recall here an additional fact which is in relation to what has preceded. It has been known for a long time that a cell or a tissue undergoes self autolysis under suitable conditions. This permits the interference that local enzymes able to effect this degradation exist. Embryologists tell us that these autolytic enzymes appear very early in embryonic development. The existence of these enzymes could equally explain the absence of antibodies towards endogenous constituents, for in

normal conditions, such substances would be broken down. On the contrary, in certain circumstances, for example during a pathologic process, if these substances were not completely degraded in situ, they could induce antibody formation-auto-amtibodies.

On several occasions, I have mentioned the hypothesis whereby antibody formation is not looked upon as a special defense mechanism, but a general physiologic mechanism of globulin synthesis able to interact more or less specifically with certain substances and to serve thus as carriers. It is well established now that several serum proteins serve to transport diverse substances: lipids, hormones, vitamins, metals, etc. and that these roles are specific. It thus seems logical to extend this conception of carriers to globulins which are designated by the term immunoglobulins. Normally, these proteins could serve as carriers of products of catabolism. Ordinarily, they would have to exist in very small quantity since the analysis of sera of animals raised in sterile conditions shows minimal but perceptible immunoglobulin levels. Under pathologic circumstances, during inflammation for example, they could quickly enter into action as a clearing mechanism.

I suggest that the abandoning of the postulate of the obligatory exogenous origin of antigens will facilitate our understanding of the mechanism of immunoglobulin formation, and propose the hypothesis of carrier globulins which I believe fits well with the known facts.