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The Mechanism of the Biological Action of Radiation*)

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The aim of the present communication is to draw attention to certain features of decisive importance in the primary effect of radiation, which have hitherto been neglected.

The processes which start the long chain of reactions leading to the final effect of ionizing radiation, begin with the following basic stages:

1. An elementary process,
2. A primary process,
3. An intermediary reaction,

which then leads to the visible reaction by which the action of the radiation is estimated. By the elementary process we mean the interaction between the photon or particle on the one hand and the living matter on the other. The elementary process, therefore, corresponds to the excitation and ionization produced by the absorbed photon or particle. This is followed by the primary reaction, which is of a chemical nature, and which either spreads directly, as in the case of the direct action of radiation, or is brought about indirectly by the action of radiation absorbed through an aqueous medium (action of radicals). The intermediary reaction involves the effect of radiation on certain important components of living matter, which play a decisive role in the resultant visible reaction, namely, the specific action of radiation on protein and nucleic acids.

1. *The Elementary Process*

The absorbed photons of hard radiation or swiftly moving particles impart considerable momentum to electrons or, in rarer cases, to whole atoms. The energy of photons and particles is in most cases greater than the binding energy of the electrons and a large number of excitations and ionizations therefore occur. Excitation can occur only where the energy of the photon or the particles is less than the ionizing potential of the atom. These states of excitation and ionization are very varied and can finally lead to chemical change.

The energy required for extracting an electron from the atom is termed the binding energy and is equal to several eV. This energy depends on the electric charge of the particles concerned and on their distance from one another. The potential energy of particles with one elementary charge and lying 1 Å apart equals 15 eV. The minimal energy required for excitation of external electrons is a few eV, whereas the energy required for excitation of internal electrons is considerable and equals the

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binding energy of the electrons. Chemical bonds develop equal to several eV between the atoms. The forces of cohesion between atoms and molecules in large aggregates and in macromolecules are then weakened still further. The energy required for excitation of the bodily motion of the atom is therefore less than 1 eV.

Excitation differs from ionization in that the excited electron possesses excess kinetic energy and does not break loose from the atomic structure; the atom soon changes over to its basic state, during which it either releases electromagnetic radiation in the form of fluorescent radiation, or internal re-grouping occurs within the atom, leading to a photochemical reaction. The excited external electrons change the inter-atomic bonds and thus the arrangement of the atoms within the molecule and thereby this photochemical reaction develops. In this way the action of weak photons of visible and ultra-violet light brings about a change in the chromophore groups in biologically important molecules, resulting in a specific photochemical reaction. Such chromophore groups are formed by certain biologically important colouring matters (chlorophyll) or by the purine and pyrimidine groups of nucleoproteins, which have a characteristic band at about 2,600 Å. These circumstances are important with regard to the action of radiation on protein. The protein molecule is composed of polypeptide chains, linked in a number of places by hydrogen bonds. The binding energy of these bonds is about 0.2 eV. Pauling (v. Franck and Platzmann 1954) is of the opinion that the simultaneous disruption of these bonds in several places leads to denaturation of proteins.

Similar destruction was recently produced by Ephrussi-Taylor and Latarjet (1955), on irradiating the transforming factor of the pneumococcus, which may be considered as nucleic acid. After X-ray irradiation with 33 KV it was seen that the molecule was destroyed by a single "hit". The authors concede that the energy was dissipated, about 50% in ionization and 50% in excitation, one ionization being equal to about 4.5 excitations of 5 eV each. On this basis, specific absorption in the chromophore groups of nucleic acid is inevitable. Owing to the fact that by certain mutations, the quantum yield with a photon of 5 eV is 100—1,000 times smaller than for ionization, these two authors therefore assume that excitation participates in about only 5% of the transmitted energy and that it can be ignored.

In my opinion this view is not correct, since the external action of ultra-violet light on biological units cannot be compared as a matter of course with excitation, which develops within the protein molecule simultaneously with ionization. This view is also supported in a communication by Watson (1950) and by my own experiments.

Watson succeeded in reactivating phage which had been inactivated by X-ray irradiation. This reactivation was carried out by visible light. It is assumed that bacteriophage can be reactivated because one half of the absorbed energy of the X-rays is dissipated in the form of excitations and the second half in the form of ionizations. A phage inactivated by excitation can be reactivated by visible light.

For the photoreactivation of X-ray damage by visible light, the following circumstances must be taken account: On irradiation with a wave-length greater than 0.4 Å, the photon is completely transformed into a photoelectron. During the Compton effect ($\lambda < 0.4$ Å), besides ionizations, weak photons develop and a large number of excitations are also evident. It may therefore be expected that in radiation produced by a tension lower than 30 kV, photoreactivation will be very small or non-existent, whereas with radiation at 60 KV, photoreactivation increases. This assumption has been confirmed in experiments, which are now being conducted in our laboratory.

Using visible light we succeeded in photoreactivating non-lysogenic bacteria of Escherichia coli B, which had previously been irradiated by X-rays (56 KV). The

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degree of photoreactivation is naturally not so marked as with photoreactivation of damage caused by irradiation with ultra-violet rays. This is due to the fact that only a small percentage of bacteria are damaged by excitation, since in most cases ionization also participates. Details will be given in a separate report. Similar results in lysogenic bacteria were obtained by Latarjet (1951).

In my opinion, excitation, particularly in the case of hard radiation, cannot be ignored.

The energy required for excitation of the electrons may not amount to as much as 20 eV (Fano 1954). This energy is always a few eV lower than the binding energy of the electrons. Excitation of the atom can bring about changes in the molecule which, although they may not lead directly to inactivation, may have an effect on late inactivation.

Bacq and Alexander (1955) cite the communication of Burton, Magee and Samuel, who take the view that the free radicals H and OH develop only by dissociation of excited water molecules. It is an interesting hypothesis, which assumes the development of two types of excited molecules, of which only one is rich in energy and sufficiently stable for the radicals, developed by dissociation, to have sufficient time to escape. It is again seen, however, that excitations are significant for the biological action of radiation. In addition to the facts already mentioned, there is still the further possibility of a shift of energy from one part of a macromolecule to another. These phenomena of energy migration are known to exist in crystals (Riehl 1940) and were found by Wallenstein (1952), to exist in hydrocarbon molecules also. In the case of large molecules, such as insulin, trypsin or chymotrypsin, Pollard assumes that energy migration comes into play in the difference between molecular weight as determined by direct action and by other methods. From this it is already evident that the processes which take place in macromolecules after absorption of radiation are very complex and that it is not sufficient to interpret them by a simplifying conception such as inactivation of a whole molecule by a single ionization.

As far as the action of actual ionization is concerned, these processes are sufficiently well known and will be only touched on here. For ionization of an atom there must be a sufficient amount of energy (in the form of absorbed photons or colliding electrons) to overcome the ionizing potential of the atom. Ionizing potentials have been exactly determined for only some elements and range within the limits of 3.9 eV for CS and 24.6 eV for He (Franck and Platzmann 1954). Ionization of a molecule, on the other hand, is relatively easier. In this case it is necessary to increase the basic electronic state of the molecule. In stable molecules the ionizing potential is between 10 and 16 eV. As already mentioned, in ionization electrons are released which can again be caught up by positive ions. In this case, however, the process is not one of simple supplementation, but the molecule becomes electrically excited. Dissociation may occur or its oscillation energy may be increased. In this connection Franck and Platzmann draw attention to one feature which very sharply differentiates gaseous ionization from aqueous ionization; freshly formed ions in aqueous solutions are very quickly hydrated, and a considerable amount of heat is freed. All these facts lead to the conclusion, with which we agree that the experimentally ascertained "sensitive volume" is not related to some specific part of the molecule, but that it is the average of effective cross-sections for various types of excitation and that this is modified still further by the corresponding probability with which the given biological affect occurs.

A similar point of view was recently adopted by Volkova and Pasynsky (1955), who irradiated protein solutions with ultra-violet and X-rays and found that the quantum yield required for the denaturation of seralbumin in an aqueous solution was 7×10^{-4}, whereas a 37 % dose of X-rays is 10^6r. With these doses of irradiation it was seen that amino nitrogen is more or less stable, so that only $2-3$ peptide links are broken down in the protein molecule (molecular weight 70,000), corresponding to an intensity of hydrolysis of 0.6%. Denaturation of protein following irradiation cannot therefore be explained by disruption of the peptide chains, but by re-grouping of the intramolecular bonds within the protein molecule. This involves the hydrogen bonds already mentioned above. Pasynsky is of the opinion that from 20 to 40 hydrogen bonds need to be destroyed in order to produce probable denaturation of the protein molecule. He opposes the view, however, that the small probability of such simultaneous destruction of the hydrogen bonds should be based on "hitting" a special centre in the molecule. He points out that it is not correct to speak of an ionizing effect of X-rays in relation to the target theory, since the passage of these photons through the molecule leads to a quantity of excitations of the molecules that could possibly exceed the number of ionized molecules and be sufficient to bring about denaturation of the protein molecules.

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There is some evidence that on the track of a swiftly-moving particle not only excitations and ionizations are found but also active radicals, which develop as a result of these elementary physical processes. From this it could be concluded that in some cases further intermediary products following irradiation are also distributed in column formation in the living matter for a small fraction of a second.

2. *Primary Reaction*

As already stated in the introduction, the elementary physical process gives rise to a primary reaction, which covers the group of phenomena occurring in the irradiated area. The primary reaction, is caused either by direct action or indirectly by radicals released in an aqueous medium. These theories, which formerly conflicted with each other, are now regarded as being complementary.

As far as direct action is concerned, there are two things which should be very carefully differentiated. Statistical fluctuations undoubtedly take place in the incidence of elementary physical processes. This is due to the physical character of the whole process and there can be no doubt that it is actually so. Nor can there be any doubt that "sensitivity" is not evenly distributed in living matter. That was demonstrated many years ago in our own experiments (Herčík 1939) and in those of Petrová (1942). Using alpha radiation of varying strengths it was demonstrated that the nucleus was considerably more sensitive than cytoplasm. It is therefore very probable that discontinuous action occurs in living matter the sensitivity of which is uneven. These facts should, however, be distinguished very carefully from a generalisation such as the target hypothesis. According to this hypothesis, a sensitive volume exists in the cell, in which the direct action of one or more ionizations causes a change which leads to its destruction and thus to the death of the cell, or to a profound functional or morphological change. The target theory is incorrect mainly in both these assumptions, which are exclusively of a biological character. As already stated, no objections can be raised to physical facts, but the conception of a single sensitive volume for the whole cell is indubitably incorrect. It cannot be assumed that there are parts of the cell with very low sensitivity and, on the other hand, that it contains only one macromolecule, about 10^{-6} cm. in diameter, which decides on the life of the whole cell. There is no doubt that various processes participate in living systems, in which considerable amplification of the original stimulus occurs, but the hypothetical assumption of a sensitive volume in the cell, limited to a certain structure, is too improbable.

At the present time, the target theory is being subjected to criticism. Fano (1954) states, for example, that many facts are accumulating which oppose this hypothesis. If the unevenness of biological effect depends only on the chance localisation of the primary processes, then the correlation between the two chance phenomena ought to have a definite geometrical structure, i. e., the trend of the curves expressing the relation to the dose ought to be independent of the factors of the environment. Since this is the case, an auxiliary hypothesis is required, which, according to Fano, detracts from the weight of the original working hypothesis. Houtermans (1953) makes a critical analysis of the target hypothesis and points out that the chief substantiation of this—the "single hit" curve—is a formal phenomenon, from which all the others are deduced. I once suggested that instead of the single sensitive volume the possibility of sensitive sites in the cell should be taken into account (Herčík 1946), These sites would change their localisation according to the area where the most sensitive reactions to radiation are taking place. As I see it today, however, this modification of the target theory actually changes its own basis, since it denies the existence of a sensitive. volume. If the various objections to the target theory are taken into account, the only fact which can finally be conceded is that the effective cross-section can be calculated from the relatonship between the biological effect and the radiation dose. The values of the effective average of different types of radiation and different objects have only a formal significance, which in a certain way expresses the sensitivityof a given object to radiation. They have a true significance only in homogenous systems.

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This brings us to the question of whether the target theory also remains valid for determining the size of single objects such as macromolecules, viruses, etc. Lea (1946) verified the target theory when irradiating molecules of myosin and ribonuclease and from the decrease in activity determined the inactivation volume and from this the molecular weight. His results were in agreement with the known molecular volume. Since then, many similar experiments have been carried out. Pollard (1955) established a detailed table giving approximately 40 molecular volumes determined by radiation in contrast to other methods. From these tables, it can be seen that considerable differences exist between molecular weights determined by radiation and other methods. It is not possible, therefore, to share Pollard's optimistic view that this technique has promising prospects.

It cannot be said that the target and radical hypotheses complement each other in any way. The hypothesis of radicals is based on the assumption that the action of radiation on water leads to the development of the radicals OH, HO_2 etc., in the course of the primary reaction, and that these, in the presence of oxygen, are converted into H_2O_2. The presence of these radicals changes according to the structure of the environment. It is assumed that the above-mentioned radicals develop on irradiation of pure water. Their existence with the exception of the radical OH (Dainton 1948), has not yet been determined by a direct method.

In general, it may be said that a primary reaction may be seen in the disintegration of water or of other simple molecules by radiation, which automatically follows the process of elementary absorption in matter.

Densitity of ionizing radiation, such as alpha particals, produces such a concentration of hydroxyl radicals on their track that a molar level is reached (Gray 1955). In such a case the formation of H_2O_2 is more than usually accelerated. On the other hand, around swiftly moving electrons radicals are formed in concentrations of about 1,000 times smaller and there is therefore a preponderance of the reaction $H + OH = H_2O$, by which the formation of radicals is "stabilized" and peroxide can again be formed. Radicals have hitherto not been reliably demonstrated, since they are very short-lived, with the exception of the radical OH, found by Dainton in 1948, so that in pulsed radiation sources they could be demonstrated by microwave spectroscopy, as has already been done in the case of hydrogen atoms in rradiated ice (Gray 1955).

It may be seen from the foregoing that oxygen is of great importance for irradiation. It would be a mistake, however, to evaluate the presence of oxygen only in the light of the hypothesis of radicals. The role of oxygen in respiration and metabolism should not be forgotten. In this connection Gray draws attention to the work of Laser, who found that the radiosensitivity of bacteria ceases to be dependent on the concentration of oxygen from the moment their enzyme system is damaged by poison. The part played by oxygen in chromosome breaks is also very complex. The concentration of oxygen not only has an influence on increased radiosensitivity, but also on the restoration processes which develop in the later phase following irradiation (Giles 1954).

It is not possible to deal here in detail with one question which is still a subject of discussion, namely whether the final product of the primary biological reaction in the presence of oxygen is hydrogen peroxide, which has been demonstrated in pure water, or whether it is the radicals OH and HO_2. It would appear necessary to differentiate the final biological effect. Sufficient material has been collected in favour of hydrogen peroxide, most of which concerns indirect evidence. It must, however, be taken into account that hydrogen peroxide is very rapidly broken down by catalase. Finally, I should, like to stress that all these reactions are of very short duration,

which leads us to the opinion that what we call the primary reaction must of necessity take place within a fraction of a second.

3. *The Intermediary Reaction*

The primary action is followed by the intermediary reaction, which in a certain sense is the most obscure part of the whole process. We should not fail to take into account the great complexity of the reactions which take place in living matter and to label one of them as the intermediary reaction following irradiation by penetrating rays would not be correct. It would seem more likely that qualitatively *different types of reactions* take place, depending on the dose of radiation. In other words, a small dose of radiation produces a qualitatively different reaction from a large dose. Until recently, the majority of radiobiological reactions were tested on very rough indicators, such as death of the cell, chromosome breaks, a decrease in metabolism, etc. In some cases very large doses are required to produce the desired effect. It is known that in order to kill certain Protozoa, doses of hundreds of thousands of r are needed. On the other hand, however, a dose of only 30 r reduces growth of a root after only a few days. A few hundred r cause destruction of the meristem and the root dies in a few days. If a site only 1 cm. from the root tip is irradiated, a dose of 200,000 r is required to reduce mitosis. This means that during 24 hours the sensitivity of the root is changed by 1,000 times (Gray 1955). This demonstrates how important it is to find the correct relationship between the dose and the sensitivity of the object. I should like to draw attention to some circumstances which have a significance for understanding the intermediary reaction in living systems. Latarjet (1951) found that X-rays can evoke the formation of bacteriophage in lysogenic strains. Recently the development of a lytic factor in a non-lysogenic strain of Escherichia coli was evoked by supersonics (Hradečná) and later also by X-ray irradiation of 60 kV (Herčík). Further details are given in separate reports. It has already been known for some time that ultra-violet light has a similar action (Jacob 1950, Hradečná 1952). In Escherichia coli, ultra-violet radiation gives rise to the formation of colicine (Lwoff 1953), an antibiotic produced by a number of strains of intestinal bacteria. It is also interesting that hydrogen peroxide can evoke the formation of phage in lysogenic bacteria and of colicine in colicinogenic bacteria. The intensity of this phenomenon is influenced by the concentration of oxygen. In summing up it may be said that the action of X-ray radiation or the direct influence of hydrogen peroxide brings about changes in bacteria which lead to the formation of bacteriophage (Lwoff). In this connection it should be pointed out that, according to the latest research, the formation of bacteriophage is due to a deviation in nucleoprotein metabolism. It follows from this that radiation or the action of hydrogen peroxide can also bring about a similar deviation in nucleoprotein metabolism. Yamafugi and Osawa (cit. by Lwoff 1953) achieved the formation of insect viruses by the action of hydrogen peroxide and by the influence of other peroxides, such as tertiolutyl. These peroxides, however, are known to be cancerogenic. We therefore arrive at the conclusion that there is a whole series of physical or chemical factors capable of producing a change in nucleoprotein metabolism, which leads to the development of a substance termed a deviant, which in some cases has the character of a virus and in others of a foreign protein.

It must be concluded from the above that quality of the damage caused by ionizing radiation does actually depend on the radiation dose. With a small dose of radiation, there can only be a slight deviation in nucleoprotein metabolism, which may be manifested by a permanent, hereditary change. With larger doses, disintegration

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of nucleoprotein takes place as a result of de-amination and dephosphorylation. This does not mean, of course, that the reaction between radiation and the organism takes place only on the nucleoprotein substrate. Other substances are also involved. We believe that the nucleoproteins are the central axis, as it were, of all these changes.

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Механизм биологического действия облучения

Ф. ГЕРЧИК

Резюме

Процессы, протекающие в живом веществе, подвергавшемся действию ионизирующего облучения, можно разделить на: элементарный процесс, первичную реакцию и интермедиарную реакцию, после которой только следует собственно видимый эффект облучения. У элементарного процесса важна не только ионизация, но и эксцитация, значение которой до сих пор несправедливо недооценивалось. О наличии биологического действия эксцитации говорит и тот факт, что в некоторых случаях удавалось произвести фотореактивизацию действия рентгеновского облучения. По мнению некоторых исследователей, свободные радикалы Н и ОН возникают только в результате диссоциации эксцитированных молекул воды. Особо важное значение эксцитация имеет при воздействии облучением на белки, когда пролетающие фотоны могут вызвать эксцитацию в настолько высокой степени, что молекула ими денатурируется.

В первичном процессе освобождающиеся радикалы имеют большее значение, чем прямой удар. Представление о клетке, как о едином, по всему объёму одинаково чувствительном теле, повидимому, неправильно. Применение гипотезы мишени для определения размеров молекул тоже страдает различными недостатками. Нельзя сказать, что гипотеза мишени и гипотеза радикалов как-нибудь дополняют друг друга.

Интермедиарная реакция наименее изучена. Весьма вероятно, что в зависимости от дозы облучения возникают качественно различные типы реакций. Для течения интермедиарной реакции большое значение имеет девиация метаболизма нуклеопротеидов, возникающая у бактерий при небольших дозах рентгеновского облучения и приводящая к образованию литических факторов. Приводятся примеры, взятые из собственных исследований. Указывается на то, что лизогенный бактериофаг возникает под действием рентгеновского облучения.

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Фаголизис клеток стафилококка

М. РОЗЕНБЕРГ

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В последние годы проблеме бактериофага посвящается повышенное внимание, — несомненно, потому, что бактериофагия тесно связана с проблемами вирусологии, а кроме того имеет и общебиологическое значение. Особенно интенсивно с помощью электронного микроскопа исследовалась морфология бактериофага и механизмы его образования.

Очень подробно эту проблему изучал Герчик (1953), книга которого «Проблема бактериофага» всесторонне знакомит читателя с современным состоянием изучения бактериофагии и с собственными электронномикроскопическими исследованиями автора, результатом которых было создание оригинальной гипотезы о возникновении бактериофага. Сущность гипотезы в следующем: бактериофаг, который адсорбируется бактериальной клеткой, вызывает глубокие изменения ее структуры, характеризующиеся распадом клеточной плазмы на большое количество монодисперсных шариков. Дезинтеграция плазмы в зернистое состояние — это необходимая стадия образования бактериофага. Зернистость обладает способностью группироваться в линейные цепочки и расти в толщину. Сначала в зернистом веществе возникают поля, дворики с ямкой в центре и утолщенными краями. Постепенно утолщаясь и разрастаясь с краев по направлению к центру образования, это вещество формирует тело бактериофага. Строительным материалом для создания бактериофага является монодисперсная зернистость, сущность и качества которой не исследованы. Герчик (1953) считает ее скоплениями живых молекул, которые, как он отмечает, обладают собственным обменом веществ, способностью расти и образовать цепочки.

Изучая лизогенные штаммы стафилококков и изолированные из этих штаммов бактериофаги, мы регулярно сталкивались с этим явлением глобулярного распада. Исходя из предположения Герчика, что глобулярные макромолекулы являются исходным материалом для образования бактериофага, мы попытались установить, обладают ли они уже литической способностью и способностью к репродукции, т. е. могут ли они вызывать у гомологичного штамма бактерий фаголизис, сопровождающийся образованием бактериофага. Для решения этой задачи мы пользовались электронным микроскопом и измерительной ультрафильтрацией.

Материал и методы

При всех опытах мы пользовались бактериофагом стафилококка, изолированным нами из лизогенного штамма LS 2 (Розенберг с сотрудниками 1955). Культивация штаммов и фильтрация производились в бульоне из плацент. Для титрации к нему прибавлялись 2% агара. В нескольких случаях бульон обогащался 1% Протеос-пептона № 3 Difco и 1% аспарагина. В этой среде явления глобулярного распада бывали особенно многочисленны и хорошо заметны.

Применялась обычная техника работ с электронным микроскопом. Молодая культура бактерий, зараженная бактериофагом, центрифугировалась в момент прояснения, дважды промывалась физиологическим раствором и трижды — дестиллированной водой. Потом взвесь бактерий в соответственном разведении наносилась на коллодиевую пленку на подпорной металлической сетке. После высыхания контрастность объекта повышалась путем нанесения искоса в вакууме слоя хрома или сплава золота и палладия по Wyckoff-у и Williams-y (1946). Наблюдения производились с помощью прототипа чехословацкого электронного микроскопа.

Для измерений путем ультрафильтрации мы приготовили набор фильтров с порами различных размеров. Мы пользовались спиртово-эфирным раствором коллодия «Спофа» с примесью ацетона и прибавляли к нему в различных количествах воду и уксусную кислоту. Приготовление и измерения фильтров средней пористости производились по Bauer-у и Hughes-y (1935). Качество фильтров, т. е. вариационный размах диаметров их пор, проверялось по Erbe (1933) и Pisa (1933). Фильтры были удовлетворительного качества, так как полученный нами вариационный размах диаметров пор мало отличался от данных литературы (Grabar 1943). Зараженная бактериофагом взвесь бактерий в бульоне фильтровалась в момент прояснения через коллодиевый фильтр средней пористости (около 800 mμ). Главные измерения производились путем фильтрации через целый ряд фильтров с различной пористостью при постоянном давлении в 200 мм ртутного столба. Отфильтровывалось всегда по 10 мл жидкости. Тесты производились с помощью соответственного чувствительного штамма бактерий. Появление полей негативных колоний бактериофага (plaque) служило доказательством присутствия в фильтрате заразных литических частиц.

Результаты

Электронно-микроскопические наблюдения. Морфологическая картина культуры стафилококка, в которой наш бактериофаг вызвал лизис, весьма разнообразна. В поле зрения находятся наряду с клетками, значительно увеличенными в объёме, — и частью или же полностью распадающиеся клетки. Несомненно, это стадии бактериолиза, характерные для деятельности бактериофага. Они были подробно описаны (Герчик 1953), и мы не будем на них останавливаться.

Форма бактериофага — обычная. К его телу прилегает очень длинный жгутик (200—250 mμ), весьма гибкий и часто различным образом скрученный. Его толщина отвечает диаметру глобулярных макромолекул. Головка бактериофага бывает приблизительно круглая, диаметром в 50—60 mμ. Типичные частицы бактериофага, регулярно встречающиеся в нормальных культурах в бульоне, показаны на рис. 1. В среде, богатой аминокислотами, при дегидратации, головки частью распадаются и сплющиваются (рис. 2, 4). Тем не менее объём головок бывает в обоих случаях приблизительно одинаковый, из чего мы заключаем, что и в гидратированном состоянии их диаметр бывает одинаковый. Типично большое количество однородной зернистости, — совершенно правильно встречающееся и, несомненно, бактериального происхождения. Зернистость располагается иногда на большом расстоянии от клеток, но ее связь с распадающимися клетками несомненна (рис. 2, 4). Диаметр шариков бывает в 20—25 mμ, что отвечает данным Герчика, изучавшего их у E. coli, зараженных бактериофагом. Мы наблюдали также волокнистые образования, диаметр волокон которых говорил о том, что они состоят из таких же шариков (рис. 2). В отдельных случаях удавалось отметить целые поля волокнистых, почти войлокообразно переплетенных образований, которые невозможно считать остатками жгутиков бактериофага (рис. 3).

Результаты ультрафильтрации. С помощью измерительной ультрафильтрации мы попытались установить, к чему приближаются по своим размерам наиболее мелкие заразные частицы, — к бактериофагу, или к глобулярным молекулам. Измерения производились обычным способом: мы измеряли исследуемые взвеси с помощью набора фильтров с известной пористостью и опреде-

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ляли т. н. конечный пункт фильтрации, т. е. диаметр пор такого фильтра, который уже задерживал литические частицы. Конечный пункт фильтрации это величина, на основании которой можно вычислить диаметр измеряемых частиц с помощью коэффициента коррекции Elford-a, которым исправляется несоответствие между диаметром пор задерживающего частицы фильтра и диаметром этих частиц. Для пор в границах 10—100 mμ этот коэффициент составляет 0,33—0,5, для 100—500 mμ — 0,5—0,75, а для 500—1000 mμ — 0,75—1,0. На табл. 1 приводятся результаты измерений с помощью фильтрации с обозначением конечного пункта фильтрации и диаметра литических частиц с поправкой по Elford-у.

Измерения с помощью фильтров показывают, что диаметр самых мелких литических частиц, способных к воспроизведению и вызывающих лизис соответственного чувствительного штамма бактерий, колеблется около 50 mμ.

Дискуссия

Из приведенных результатов четырех ультрафильтрационных измерений очевидно, что диаметр самых мелких литических частиц, встречающихся в куль-

Табл. 1.

Обозначения бактериофага		F 2—3	F 2—13	F 2—14	F 2—24
Обозначения чувствительного штамма бактерий		S 3	S 13	S 14	S 24
Титры литических частиц в 1 мл перед измерением		4,2 . 10⁶	3,0 . 10⁶	3,7 . 10⁴	5,6 . 10⁵
Появление лысых мест (pla-que) в культуре чувствительного штамма, зараженной профильтрированной взвесью	494		+ + +	+ +	+ + +
	407	+ + +			
	310	+ + +	+ + +	+ +	+ + +
	267	+ + +	+ + +	+ +	+ + +
	193	+ + +	+ + +	130	+ + +
	176	+ + +			
Средний диаметр пор применявшихся фильтров в mμ	163	+ + +			
	143	+ +	+ + +	58	+
	118	+	268	2	34
	105	21	0	0	0
	87	0	0	0	0
	74	0	0	0	0
Конечный пункт фильтрации в mμ		87	105	105	105
Коэффициент поправки по Elford-у		0,47	0,5	0,5	0,5
Диаметр измеряемых частиц в mμ		30—50	40—60	40—60	40—60

Пояснения: + + + полный распад
+ + сливающиеся поля распада (plaque)
+ частично сливающиеся, не допускающие точного подсчета поля распада (plaque).

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туре бактерий, зараженной бактериофагом, отвечает величине наименьшего
диаметра головки бактериофага, полученной с помощью электронномикроскопи-
ческих измерений. Это означает, что однородные шаровидные макромолекулы,
из которых, по наблюдениям Герчика, образуется бактериофаг, не обладают
еще способностью вызывать у гомологичных штаммов бактерий образование
заразного вирулентного бактериофага. Мы полагаем, что они не обладают,
и литическим действием. При ориентировочных опытах, при которых ультра-
фильтрат, освобожденный от частиц бактериофага, прибавлялся к гомологичным
чувствительным бактериям, мы не наблюдали с помощью электронного микро-
скопа никаких сколько-нибудь заметных литических клеточных изменений.
Конечно, это метод совершенно недостаточный, — без возможности коли-
чественного контроля.

Тем не менее из наших наблюдений можно сделать вывод, что шаровидные
макромолекулы еще качественно отличаются от бактериофага. Они не обладают
еще способностью к самовоспроизведению в благоприятной среде, т. е. им не-
достает именно того признака, по которому бактериофага относят к живым
организмам. Эта способность возникает, вероятно, как совершенно новое
качество только во время дальнейшего развития бактериофага из этих макро-
молекул. Несмотря на то, что макромолекулы по времени возникновения стоят
между клеткой бактерии и бактериофагом, нам представляется, что они по ка-
честву скорее приближаются к плазме бактерии. Мы полагаем, что большую
пользу могло бы принести серологическое изучение этих соотношений.

Резюме

1. Исследовался фаголизис клеток стафилококка. С помощью электронного
микроскопа изучались жгутиковые формы бактериофага. Диаметр головки
составляет 50—60 mμ, длина жгутиков 200—250 mμ при толщине в 20—25 mμ.
Обычно в культурах наблюдался глобулярный распад клеток. Шаровидные
макромолекулы обладали диаметром в 20—25 mμ, т. е. по своим размерам
отвечали толщине жгутиков.

2. С помощью метода ультрафильтрации был измерен диаметр самых мелких
литических частиц, встречающихся в зараженной бактериофагом гомологичной
культуре стафилококка. Их диаметр составляет 40—60 mμ и отвечает так
диаметру головки бактериофага.

3. Шаровидные макромолекулы, из которых, повидимому, образуется в ре-
зультате постепенного развития бактериофаг, не обладают способностью вызы-
вать образование бактериофага у гомологичного штамма бактерий и каче-
ственно отличаются от бактериофага.

(Табл. XVII, XVIII)

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Lysis of Staphylococcal Cells due to Bacteriophage

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Summary

A study was made of lysis of staphylococcal cells produced by bacteriophage. Flagellate forms of bacteriophage were observed in the electron microscope. The head measured 50—60 mμ in diameter, the tail was 200—250 mμ in length, with a thickness of 20—25 mμ. Globular disintegration of the cells always appeared in the cultures. The globular macromolecules measured 20—25 mμ in diameter and their dimensions corresponded to the thickness of the tail. The ultrafiltration method was used to determine the diameter of the smallest lytic particles which appeared in the same staphylococcal cultures infected with bacteriophage. They measured 40—60 mμ in diameter, the same as the head of bacteriophage. The globular macromolecules from which bacteriophage is probably formed by progressive development, are not capable of inducing proliferation of bacteriophage on a homologous bacterial strain and differ form it qualitatively.

(Plates XVII, XVIII)

FOLIA BIOLOGICA

Динамика распада лизогенных клеток под действием ультрафиолетовых лучей

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Образование бактериофага в лизогенных клетках возможно стимулировать с помощью самых разнообразных неспецифических влияний. Очень эффективно ультрафиолетовое облучение (Jacob 1950, Lwoff и др. 1950, Градечная 1952 и др.), в особенности при длине волны в 2537 Å, которая специфически адсорбируется нуклеопротеиновыми составными частями клетки. Подобным образом действуют и лучи X (Latarjet 1951), аскорбиновая кислота, глютатион (Lwoff, Siminovitsch 1952) и др. вещества.

Под их воздействием бактериофаг в лизогенных клетках образуется не немедленно, а по истечении определенного периода латенции (Lwoff 1952).

При этих процессах чрезвычайно важным фактором является физиологическое состояние лизогенных клеток: в бедной среде эффект стимуляции у лизогенных клеток не наблюдается (Lwoff 1952).

Стимулирующее действие неспецифических импульсов на образование из лизогенных клеток бактериофага имеет большое теоретическое значение, так как показывает, что размножение бактериофага не должно вызываться обязательно только специфическим бактериофагом и что для этого размножения исключительно большое значение имеет прежде всего сама бактериальная клетка.

Целью нашей работы было изучение распада подвергавшихся ультрафиолетовому облучению лизогенных клеток с помощью фазового контраста (in vivo) и сравнение этих процессов с фаголизисом чувствительного штамма бактерий, зараженного бактериофагом. Для инфекции чувствительных клеток применялся бактериофаг, образуемый исследуемым лизогенным штаммом.

Материал и методы

Для опытов использовался лизогенный штамм Staphylococcus aureus (LS 2), изолированный в 1951 г. и в течение нескольких сотен пассажей постоянно остававшийся лизогенным (Розенберг с сотрудниками 1955a, б). Его чувствительность к стимулирующему действию ультрафиолетового облучения показана на графике 1. 5-часовая культура в бульоне облучалась на расстоянии в 50 см с помощью 300 W облучителя с низким давлением. Титр бактериофага определялся через 60 мин. после облучения.

Наблюдения над облучавшимся лизогенным штаммом производились на пластинке агара, закрытой покровным стеклышком и заключенной в парафин. Приток и отток питательной среды делала возможным полоска фильтрационной бумаги. Схема этого устройства приводится на рис. 2. Наблюдения производились с помощью фазового микроскопа Цайсса. Микроскоп с препаратом помещался в особый шкап с автоматической регуляцией температуры (37 ± 0,4 °C).

Перед заключением под покровное стеклышко 5-часовая культура наносилась на пластинку агара, слегка просушивалась и облучалась в течение 30 сек. с расстояния в 75 см 300 W с помощью облучителя низкого давления. При такой экспозиции наблюдалось наиболее сильное образование бактериофага. Как видно, в этом случае оптимальная доза была гораздо меньше, чем при облучении культуры в бульоне, так как здесь не сказывалась сильное поглощение лучей жидкостью. В контрольном опыте к чувствительному штамму S 3 прибавлялся бактериофаг, изолированный из лизогенного штамма LS 2.
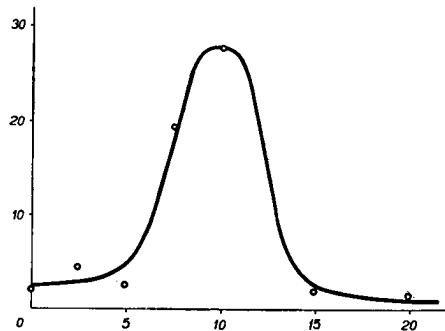
Рис. 1. Зависимость количества выделяемого бактериофага от дозы ультрафиолетового облучения. Ось X: экспозиция в мин., ось Y: титр в 1 см³ . 1 . 10⁴.
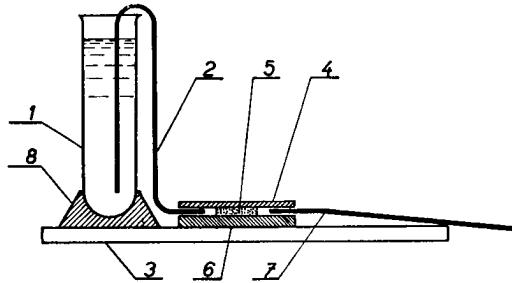
Рис. 2. Схема препарата для наблюдения с помощью фазового контраста. 1 - пробирка с бульоном, 2 - приводящая среду фильтровальная бумага, 3 - предметное стеклышко, 4 - покровное стеклышко, 5 - культура бактерий, 6 - слой агара, 7 - отводящая фильтровальная бумага, 8 - пластелин.

Результаты

Процесс распада чувствительного штамма S 3 под действием бактериофага, изолированного из лизогенного штамма LS 2 (контрольный опыт).

Весь ход этого лизиса показан на рис. 3. Первый снимок был сделан через 10 мин. после прибавления бактериофага к чувствительной культуре. Интервалы между следующими снимками приводятся в таблице.

Течение лизиса отвечает данным, приводимым обычно в литературе (Topley-Wilson 1947). В первый период, когда происходит адсорбция бактериофага чувствительными клетками, наблюдается еще быстрое клеточное деление без сколько-нибудь заметных литических процессов. Около 50-ой минуты после прибавления бактериофага клеточное деление прекращается и начинается постепенное увеличение объёма клеток. В этот период, называемый константным, бактериофаг еще не выделяется из клеток. Массовый распад клеток, сопровождающийся отделением частиц бактериофага, начинается около 80-ой минуты и протекает очень быстро. В течение 10 мин. распадается большинство клеток микроколонии. Клетки, которые за этот период не распадаются, представляются значительно разбухшими. Они, вероятно, тоже пришли в соприкосновение с бактериофагом, но внутриклеточные процессы в них, повидимому, протекают иначе, так как у них не осуществляется ни лизис, ни деление в этой стадии. Возможно, что это — устойчивые клетки, в некоторых случаях становящиеся лизогенными, т. е. способными, как правило, в последующих генерациях выделять бактериофага, с которым они вошли в соприкосновение (Bail 1925, Bordet 1925 и др.).

Распад лизогенного штамма LS 2 в результате ультрафиолетового [облучения

Распад лизогенного штамма стафилококка LS 2 после ультрафиолетового облучения показан на рис. 4. Первый снимок был сделан через 20 мин. после

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облучения, а следующие с 5-минутными промежутками. Литические процессы в одной и той же микроколонии наблюдались в течение 2 час. Путем анализа полученных нами микрофотографий можно установить несколько фактов. Количество клеток, распадающихся под действием ультрафиолетовых лучей, у нашего штамма не слишком велико, — в особенности в сравнении с количеством клеток, распадающихся под действием бактериофага. Для первых 40 минут после облучения характерно земедление или даже прекращение клеточного деления. Клетки увеличиваются в объ ме очень скоро после облучения. Это хорошо
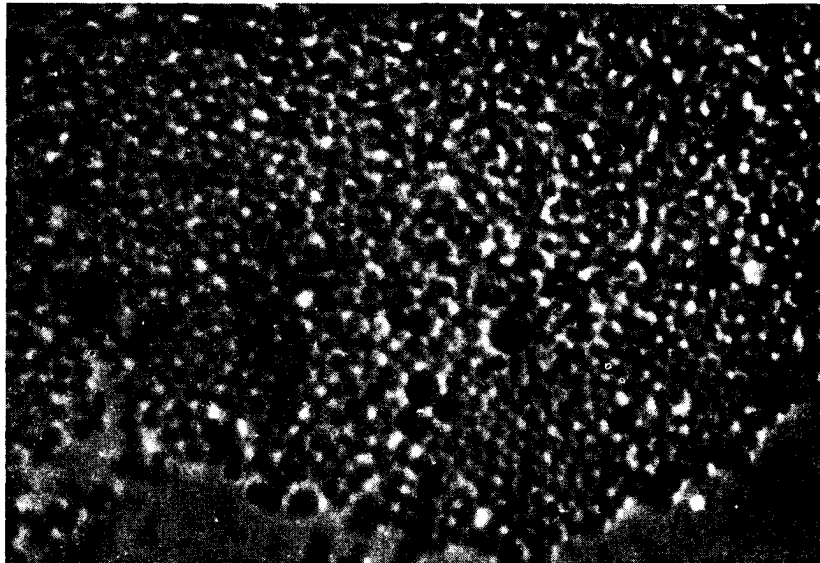

Рис. 5.

заметно при их сравнении с быстро делящимися клетками, снятыми через 120 мин. после облучения. Объ м клеток вплоть до момента распада постоянно увеличивается. У некоторых клеток лизис не осуществляется, и они дорастают иногда до гигантских размеров. Были отмечены клетки, объ м которых был во много раз больше, чем у нормальной клетки (рис. 5). Аналогичное явление описывает Герчик (1949): после ультрафиолетового облучения E. coli росли в виде необыкновенно длинных волокон. Распад клеток бактерий начинается около 40-ой минуты и продолжается приблизительно до 80 минуты. Это наблюдение отвечает данным Lwoff-а (1952). Но мы не можем подтвердить правильность другого его наблюдения, а именно, что еще перед распадом клетки делятся. При самом подробном анализе роста микроколонии нашего штамма ни в одном случае не удавалось отметить отчетливого деления перед клеточным распадом. Конечно, в нашем случае опыты производились с другим видом бактерий и в несколько иных условиях. В период бактериолиза у некоторых бактерий, которые не подвергаются распаду, наблюдается уже клеточное деление, которое через 120 мин. протекает уже с нормальной скоростью. Клетки имеют такие же размеры, как и необлучавшиеся.

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Дискуссия

При сравнении бактериолиза под действием ультрафиолетового облучения с фаголизисом можно заметить некоторое подобие: в обоих случаях клеточному распаду предшествует стадия, характеризующаяся замедлением или даже прекращением клеточного деления, но в первом случае она продолжается около 40 мин., а во втором — около 30 мин. Для этой стадии характерно прежде всего заметное увеличение объёма клетки, приводящее в конце концов к клеточному распаду. Очевидно, это период метаболических изменений в клетке, заканчивающийся образованием бактериофага и его выделением во внешнюю среду. В стадии распада имеются известные различия: при поражении бактериофагом наблюдается лизис большого количества клеток в течение очень которкого времени, — практически минут за 10, — тогда как под действием ультрафиолетовых лучей клетки распадаются в меньшей степени и дольше: отдельные случаи лизиса можно бывает отметить еще через 100 минут после облучения, т. е. более чем через 60 мин. после начала литических процессов. Бактериофаг, как кажется, гораздо более энергично вызывает лизис, чем ультрафиолетовые лучи. Тем не менее аналогия между действием бактериофага на чувствительные клетки и действием ультрафиолетовых лучей на лизогенные клетки велика. Весьма вероятно, что процессы, ведущие в обоих случаях к образованию бактериофага, очень близки друг к другу. Несомненно, главную роль в этих процессах играет бактериальная клетка, так как импульс, вызывающий воспроизведение бактериофага, может быть совсем неспецифическим.

Резюме

1. С помощью фазового микроскопа исследовался лизис лизогенного штамма стафилококка LS 2 после ультрафиолетового облучения. Для сравнения наблюдался распад чувствительного штамма стафилококка S 3 под влиянием бактериофага, изолированного из лизогенного штамма LS 2.

2. Из серии микрофотографий очевидно, что течение бактериолиза в обоих случаях весьма схоже.

3. Облучавшиеся клетки бактерий перед распадом не делятся, но всегда значительно увеличиваются в объёме.

4. Литические процессы, отмеченные в лизогенной культуре после ультрафиолетового облучения, протекают медленнее, чем процессы распада чувствительных клеток, зараженных бактериофагом.

(Табл. XIX, XX)

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The Dynamics of the Breaking-down of Lysogenic Cells
Irradiated by Ultra-Violet Light

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Summary

Lysis of a lysogenic staphylococcal strain was observed in the phase microscope
after irradiation with ultra-violet light. For comparison, the breaking-down of the
sensitive staphylococcal strain S 3 was observed, following the action of bacterio-
phage released by the lysogenic strain LS 2. It is evident from the series of micro-
photographs that the course of bacterial lysis is similar in both cases. The bacterial
cells irradiated by ultra-violet light do not divide before being broken down, but
always considerably increase in size. The lytic processes which were observed in the
lysogenic culture following irradiation with ultra-violet light have a slower course
than following the infection of sensitive cells with bacteriophage.

(Plates XIX, XX)

FOLIA BIOLOGICA

The Character of Leucocyte Reaction Following X-Irradiation

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. After initial leucocytosis in the reaction of the blood to ionizing irradiation has receded, leucopenia develops as a result of the cytostatic action of the X-rays on the tissues of the organism. As a result of regeneration processes, the leucocyte level, after a certain period, returns to normal, the time required for this showing a relationship to the dose used and the manner of administration, to the functional condition and individual resistance of the blood-forming organs, alongside other factors which form a concrete potential for the general capacity to control the consequences of irradiation sickness.

As far as the initial increase in leucocytes is concerned, most radiohaematologists are agreed that its occurrence is a characteristic feature after the administration of high doses of ionizing radiation. On the further course of the post-irradiation leucocyte curve there are altogether three opinions, which are based on experimental results.

One group of authors describes the regeneration phase following a sudden or gradual maximal decrease, as being more or less irregular, with frequent fluctuations and a gradual increase in the white blood components in the surviving animals (Yegorov 1955).

The second group described another phase, within this one—an abortive rise in the leucocytes— which appears between the second and eleventh day after irradiation (Jacobson et al. 1949—in experimental animals) or even later (Hempelmann et al. 1952—in human subjects affected by ionizing rays).

Only a few communications mention that the post-irradiation course of the reaction of the blood is characterised by a periodic, wave-like form. The first to describe this wave-like characteristic were Langendorff and Paperitz (1939), on the basis of their observations on the bone marrow and peripheral blood of irradiated mice. In their series, the minimum and maximum values in the curves are reached after about 40 days. The rhythm they describe is thus of relatively long duration. Regeneration waves in haemopoietic tissue were also observed by Bloom and Murray and by Jacobson and Block. Dérer (1953) observed periodic decreases in leucocytes following X-irradiation of leukaemic patients, with an interval of approximately six days. A periodic leucocyte reaction was also described by Uher (1952) in his communication on the time factor in regulation of the blood and by Thalhammer and Janicek (1951) in their experiments with vitamin D.

The aim of our experiments was to verify the periodic response of the white blood component following irradiation with X-rays and to ascertain the relationship between this response and the dose used and fractionation of the dose and, finally, to study the development of the reaction after irradiation of experimental animals during narcosis (these latter experiments were carried out by Dr Praslička).

Methods

The experimental animals were rabbits, Vienna breed, blue-eyed, weighing from 2,000—3,000 g. Blood was collected from the ear vein daily, at the same time. The blood picture was studied for two months after irradiation. All the animals were subjected to total irradiation with a Super-Sanax lamp

at 180 kV, 15 mA, F = 50 cm., filter 1 mm. Al, 0.5 mm. Cu with a feed of 33 r/min. The single doses were as follows: 200 r (6 animals), 350 r (8) and 800 r (11); on fractionation a total dose of 1100 r was divided into 11 daily doses of 100 r (10 animals), while the narcotized experimental animals were irradiated with a single dose of 350 r (4 animals). The narcotic used was ether, in a concentration which guaranteed deep narcosis during irradiation; narcosis was also maintained for a further 60 minutes after the lamp had been switched off. In every group of experimental animals the blood picture of three control animals was also studied; the initial values before irradiation were determined from eight specimens.

Results

In our case, the curve showing the leucocyte response to the action of ionizing radiation results mainly from two antagonistic processes. On the one hand there is the protracted destructive action of radiation on blood formation, and on the other manifestations of regeneration. The third possible factor, i. e. changes in the distribution of blood, does not participate to any appreciable extent in the prolonged study of changes in the blood, particularly with the method of total irradiation used by us. In studying blood changes we ascertained when the fluctuations in the number of leucocytes took place in the time curve, both from the positive and the negative aspect.

The following findings were made: The incidence of the initial rise in the number of leucocytes, due to an increase in the number of heterophils, shows a relationship to the dose used. It occurs mainly on using higher doses. In our series this first positive deflection was not recorded after 24 hours on using a dose of 200 r. It was, however, recorded in two cases (out of eight) with a dose of 350 r. and in eight cases (out of eleven) with a dose of 800 r. On fractionation of the dose the deflection was not found and in irradiation of narcotized animals with a dose of 350 r, it occurred once (out of four cases). The peak of the first negative phase is related to the individuality of the reaction of the various animals and occurs between the second and fifth day. The maximal decrease occurs most frequently on the third day (50%) or on the fourth day (20%). The incidence of the subsequent positive phase varies still more in the individual animals, its peak being reached between the second and the twelfth day. It occurs most frequently between the fourth and the sixth day (70%). The peak of the following descending phase occurs between the fifth and the 15th day, most frequently between the seventh and the eight day (50%) and then from the sixth to the 12 day (15%). A later incidence of this descending phase is not related to the time incidence of the minimum in the previous wave, but follows retarded commencement of the preceding ascending phase. A further increase again occurs in the number of leucocytes, which reaches its peak, according to the individual cases, between the eighth and the 16th day. With a dose of 200 r, no further deflections occur. Processes of regeneration evidently predominate over destructive processes and the white blood picture tends to regain its normal level. With higher doses (in our case 350 and 800 r) and with fractionation, further deflections often occur.

It is not possible to speak of any regularity, of a chronologically identical, periodic incidence of limit values in the number of leucocytes, either maximal or minimal. The incidence of alternating maximal and minimal values and their time curve tend rather to give a picture of an irregular sinusoid, in which the length of the individual waves varies more or less from case to case with the individual animals. On the basis of our experiments it is impossible to say whether, and how far, the length of these waves depends on the size of the dose of ionizing radiation. With a dose of 200 r it appears to be shorter, since the average length of the interval between the negative deflections is three days and between the positive deflections 4.3 days. With the other methods of irradiation the average length of the intervals was as

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follows: with 350 r, six days between the minima and 7.1 between the maxima; with 800 r, six days between the minima (figures were not obtained for the maxima, since the intervals varied markedly). With fractionated irradiation the average length of the intervals between the minima was 5.4 days and between the maxima 5.3 days.

As far as the absolute values of the decreases are concerned, it was observed that the maximal decrease occurred with doses of 200 r and 350 r in the first negative phase, i. e. between the second and fifth day. Following irradiation with a dose of 800 r, the lowest negative amplitude was mostly found in one of the following negative phases and in fractionation of radiation this was always the case.

The results of irradiation in narcosis did not differ basically from the results with a single administration of the corresponding radiation dose without narcosis.

Discussion

The incidence of the initial rise in the number of heterophils is in agreement with the data of Jacobson et al., who state that a significant increase takes place in the heterophils in the peripheral blood of rabbits after doses of 400 r and more. This does not mean that this increase, which often results in a rise in the absolute number of leucocytes, could not also be manifested in the case of somewhat lower doses (in our case 350 r), as a result of the different individual reactivity of the experimental animals.

With a dose of 800 r, an increase in the number of heterophils was found in all cases. As already stated in the results, in eight cases this led to an increase in the absolute number of leucocytes. In the remaining three cases, the initial decrease in the number of lymphocytes was so sharp and occurred so soon that the increased number of heterophils was unable to equalize the loss in absolute numbers. This is a frequent result in those types of organisms in which the percentual representation of the main white blood components (heterophils and lymphocytes) is mainly in favour of the latter and contrasts with the results in affected human subjects (Hempelmann), in whom, especially after high doses, the phase of initial leucycytosis may be prolonged to as much as seven days. According to Yegorov, the duration of this phase depends both on the dose and also on the type of nervous system of the organism, which is responsible for the individual reaction of the blood-forming tissue.

In the initial changes in the number of leucocytes, an important part is doubtless played by the pituitary-suprarenal system. This factor does not, however, serve to explain the further course of irradiation sickness.

The question of the further trend of the leucocyte curve is a complicated one. The increase in the number of leucocytes described between the second and the twelfth day, which follows a negative phase, is identical with what Jacobson et al. and Hempelmann et al. call the "abortive rise". Bloom and Jacobson associate the etiology of this rise with the incidence of degenerate, temporary forms, which develop in the bone marrow, chiefly in the elements of the myeloid series, and are flooded out into the periphery. Hempelmann tends to take the view that the initial and abortive increases are a secondary, non-specific reaction to damage to the tissue. This reaction appears, for example, after burns, in which the "abortive increase" can be attributed to associated infection. It is certain that the factors of both these hypotheses can be manifested, but it is problematic whether degenerate blood elements appear in such large numbers in the corresponding period; on the other hand, an associated infection is not present in all cases in irradiated animals.

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Langendorff and Paperitz, who described long duration of the periodic character of post-irradiation leucocyte reaction, analyse the morphology of changes in the bone marrow and peripheral blood and come to the conclusion that every type of blood element is characterised by a special wave-like curve of its own. According to them, the effective factor is the length of the life of the individual elements. They evaluate their results at intervals of four days and that is why their rhythm has a prolonged character. Dérer holds the view that the persistent action of cytostatic substances is a result of irritation of the functional centres for blood regulation by tissue changes, which in addition reflexly produce synchronisation of the rhythm by means of which the central nervous system governs the formation and destruction of the blood elements. The exact periodic reaction described after cytostatic action in an organism in which the state of the blood formation is not normal, cannot be verified in normal organisms. Even if one takes the view that the nervous system is largely responsible for the wave-like character of the post-irradiation leucocyte reaction, it cannot easily be conceived that impulses proceeding in a regular rhythm from the central nervous system and possibly caused by a functional modification of the process of internal inhibition due to radiation, should produce absolute regularity of the decreases in the leucocytes in the peripheral blood in circumstances as complex as those which develop following irradiation of the whole organism. This regularity is no doubt disturbed particularly by the positive deflections in the leucocyte curve, which are caused by adaptation phenomena in the organism, associated infection and other factors, and also by the short life of several generations of leucocytes following irradiation.

Summary

Following irradiation of rabbits with a single dose of 200, 350 and 800 r, and also with fractionated irradiation with 1100 r in 11 daily doses of 100 r, alternating positive and negative deflections occur in the leucocyte curve, which on a graph take the form of an irregular sinusoid, in which the individual wave lengths range within considerable limits, according to the individual reactivity of the various animals. The length of the intervals between the maximal and minimum values in the trend of the leucocyte curve varies within an average period of four days with a dose of 200 r and within an average period of about six days with a dose of 350 and 800 r and fractionated radiation of 11×100 r.

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Характер лейкоцитарной реакции после рентгеновского облучения

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Резюме

После однократного облучения кроликов дозами в 200, 350 и 800 г, а также при фракционированном способе облучения в течение 11 дней по 100 г в день, в лейкоцитарной кривой наблюдается чередование положительных и отрицательных волн, которое можно изобразить графически в виде неправильной синусоиды. Длина отдельных волн кривой значительно колеб ется в зависимости от индивидуальной реактивности животных. Продолжительность интервалов между отдельными максимумами и отдельными минимумами лейкоцитарной кривой колеблется при дозе в 200 г около среднего времени в 4 дня, а при дозе в 350 и 800 г и при фракционированном облучении 11 раз по 100 г — около 6 дней.

Автор отстаивает взгляд, что волнообразный характер реакции на облучение обусловливается в значительной мере ролью нервной системы. Известная правильность ритма импульсов, посредством которых центральная нервная система управляет образованием и распадом элементов крови, обусловливается, возможно, функциональными изменениями процесса внутреннего торможения под действием облучения. Правильность колебаний количества лейкоцитов в периферической крови нарушается, главным образом, колебаниями в положительную сторону, — которые связаны с явлениями адаптации организма, — попутными инфекциями и другими факторами, а также сокращением продолжительности жизни нескольких генераций лейкоцитов после облучения.

FOLIA BIOLOGICA

Sporulation of Bacilli

Consumption of Calcium by the Cells and Decrease in the Proteolytic Activity of the Medium during Sporulation of Bacillus megatherium

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The transition of the vegetative cells of bacilli into the resting form, spores, is accompanied by a series of biochemical changes, an analysis of which throws light on the deeper physiological laws of this process. One of these changes is a sharp decrease in the proteolytic activity of medium, which takes place under certain conditions on sporulation of Bac. megatherium.

In the present work a study is made of the influence of calcium and other ions on this process and on the transition of the calcium from the medium into the sporulating cells. An investigation was made also of the effect of adding cysteine to the culture, before sporulation, on the decrease in the proteolytic activity of the medium.

Materials and Methods

Culture: A strain of Bacillus megatherium from the collections of the Institute of Biology of the Czechoslovak Academy of Science was used.

Nutrient medium: Composition — 0.3% casein hydrolysate (Amigen), 0.1% glucose and 0.34% KH_2PO_4. One mililitre of a concentrated mixture of certain salts and important ions was added to the medium, composed as follows: 17.4 g. K_2SO_4, 12.3 g. $MgSO_4 . 7 H_2O$, 0.22 g. $MnSO_4 . 7 H_2O$, 2.0 g. $FeSO_4 . 7 H_2O$, 1.44 g. $ZnSO_4 . 7 H_2O$, 18.3 g. $CaCl_2 . 6 H_2O$ to one litre. The proportion of these salts is similar to that originally suggested by Grelet (1951). Two media were used for culturing—medium A, in which the precipitate of calcium sulphate and phosphate was filtered off, and medium B, which was the same as medium A, but enriched by the addition of calcium (10 ml. $5 . 10^{-3}$ M $CaCl_2$ per litre medium). The pH of the medium was adjusted to 7.2.

Method of inoculation and culturing: The nutrient medium was inoculated with 3 ml. of a suspension of spores freed after autolysis of the sporangia. The suspension was stored at 5° C. From 90 to 100 ml. medium was cultured in 500 ml. flasks, on a shaker (96 deflections/min., length of swing 9.8 cm.) at 27° C. The percentage of spores was determined by direct microscopic observation and counting (Vinter 1955).

Determination of degree of turbidity: The degree of turbidity of the culture was determined in the undiluted solution on a Lumetron colorimeter with a filter of 370 mμ. Clear supernatant fluid was used as the control.

Determination of the proteolytic activity of the medium: The supernatant fluid was incubated with a 1% solution of casein with a pH of 7.0 in a water bath at 37° C. Unless otherwise stated, the length of incubation was 60 minutes. Two methods were used for determining the intensity of proteolysis—the Anson method (1938), in the modification of Chaloupka (1955) and a modified biuret test (Slavík and Smetana 1953). In the case of the first method, the intensity of proteolysis was expressed in amounts of milliequivalents of freed tyrosine, in the case of the second method in amounts of milligrams of digested casein.

Staining of the spores: The spores were stained with malachite green and the cells counterstained with basic fuchsin (Schmidt 1950).

Determination of the calcium content of the cells: Calcium was determined in the cellular sediment (300—400 mg. dry weight), washed with distilled water and 0.01 N HCl, dried and mineralized in a sand bath with a mixture of HNO_3 and $HClO_4$. The actual determination was carried out using a flame photometer (VEB Carl Zeiss, Jena, model III).

Results

Decrease in the proteolytic activity of the medium during sporulation of B. megatherium

Both on media A and B there is a rapid decrease in the proteolytic activity of the medium on sporulation to very low values (fig 1). The curve of the decrease is steepest at the period of mass formation of the spores (fig. 2).

The course of the decrease in the proteolytic activity of the medium differs in the culture and in the supernatant fluid, whether shaking is continued or whether the medium is left at rest. The following procedure was used: two thirds of the contents were removed from the culturing flasks under sterile conditions; they were centrifuged and the remaining third was left in the flask on the shaker. Half the supernatant fluid was placed in a sterile flask of the same size and put on the shaker, while the other half was left at rest at a temperature of 27° C. Proteolytic activity was determined in all three parallel specimens, immediately and after three hours. Sporulation was confirmed by calculating the percentage of spores in the culture on collection (tab. 1). In a culture in which sporulation had not yet commenced (12 to 12 ½ hours culturing), proteolytic activity decreased in the culture after three hours to very low values, as usual; in the supernatant fluid on the shaker it decreased only slightly or even showed an increase, and in the supernatant fluid at rest it showed an evident increase. In the specimen taken at the commencement of sporulation (up to 10% spores), there was a normal decrease in the culture and usually a slight decrease in the supernatant fluid, whether on the shaker or at rest. In the flasks in which mass
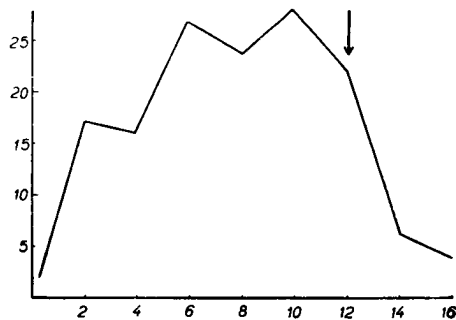

Fig. 1. Relative proteolytic activity in the course of culturing B. megatherium. x - axis: age of culture in hours, y - axis: mg. digested casein per unit turbidity. Commencement of sporulation denoted by arrow.
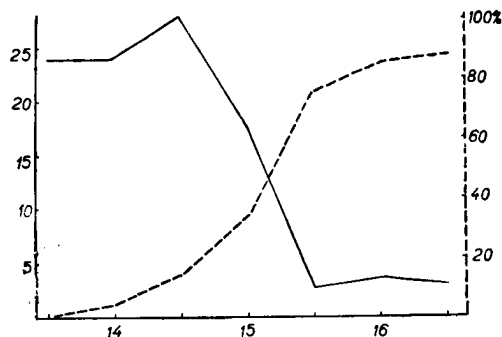
Fig. 2. Relative proteolytic activity of medium during sporulation of culture. x - axis: age of culture in hours, y - axis: left — mg digested casein per unit turbidity ————— , right — % of spores in culture — — — —.

sporulation had begun, the difference between inactivation of the supernatant fluid left at rest and the supernatant fluid on the shaker gradually increased. Although there were considerable differences between the values in the individual flasks, this general tendency is quite evident.

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Table 1. Properties of supernatant fluid taken before and during sporulation. All specimens were incubated for 30 minutes. Proteolytic activity is expressed in a.10^{-4} milliequivalents of tyrosine; the values in the col. headed "After 3 hours" gives the increase or decrease in proteolytic activity as a percentage; proteolytic activity in the supernatant fluid immediately after collection = 100%

$\%$ of spores in culture at moment of collection	Proteolytic activity immediately after collection	After 3 hours		
		Supernatant at rest	Supernatant on shaker at 27°C	Culture
Before sporulation (age of culture 12 hours)	92.00	+ 14.68	— 17.00	— 98.37
Before sporulation (age of culture $12^{1}/_{2}$ hrs.)	94.50	+ 28.20	+ 10.10	— 98.42
3.5	97.00	— 26.30	— 28.90	— 99.13
4.2	128.00	— 11.70	— 21.50	— 99.34
4.5	130.00	— 23.10	— 27.00	— 99.39
5.0	115.00	— 8.70	— 20.90	— 99.44
7.8	76.00	0	— 5.29	— 98.95
9.4	71.50	— 4.20	— 13.30	— 99.17
12.2	91.00	— 4.94	— 30.77	— 98.69
14.5	89.00	— 16.86	— 47.75	— 98.32
29.2	22.65	+ 2.70	— 20.51	— 98.02
29.5	82.50	— 12.74	— 16.40	— 99.04
48.7	5.75	0	— 47.83	— 98.61
48.9	31.35	+ 5.40	— 86.43	— 97.29

Table 2. Determination of the presence of an inhibitor in the supernatant fluid from a sporulating culture. This supernatant fluid is denoted in the table as "Inhibitor". A supernatant fluid from a culture of different age is used as "Enzyme". Proteolytic activity is expressed in miliequivalents of freed tyrosine. 10^{-4}. Incubation: 30 mins. Values: above — activity immediately after mixing: below — after standing for three hours at 27 °C.

% of spores in culture at moment of collecting "inhibitor" supernatant ffluid	Age of culture from which supernatant fluid with "enzyme" activity collected	Proteolytic activity		
		"Enzyme" diluted with same vol. of water	"Inhibitor" diluted with same vol. water	Mixture of same vols. "enzyme" and "inhibitor".
62.8%	9 hrs.	44.00 40.00	1.35 2.05	47.00 42.25
65.9%	$7^{1}/_{2}$ hrs.	29.75 27.10	2.20 2.05	34.60 29.00
70.6%	9 hrs.	40.75 36.25	1.65 1.70	43.60 39.40

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In a further experiment, the possibility of the production of inhibitors of protease during sporulation was tested. The supernatant fluid from a culture 6.5 to 9 hours old, in which it had been demonstrated that proteolytic activity would not change for three hours at rest at a temperature of 27⁰ C, was diluted with supernatant fluid taken in the course of sporulation. Both supernatant fluids were diluted with the same volume of water and the sum of the proteolytic activity of these specimens was compared with the activity of a mixture of the same volumes of both supernatant fluids. The culture fluid was collected in the course of sporulation at the moment when proteolytic activity was very low. If an inhibitor were present in this supernatant fluid in an effective form, then the reaction between this inhibitor and the active enzyme would result in a decrease in the activity of the mixture. In actual fact, no decrease in activity occurred; on the contrary, there was usually a slight increase, which was equalized after three hours' interaction at 27⁰ C (tab. 2).

Effect of the addition of certain ions on the decrease in the proteolytic activity of the medium.

All the ions named below were added after 12 hours' culturing, i. e. about 1—2 hours before sporulation commenced. Culturing was carried out on an A medium.

The addition of calcium ions in a concentration of $5 . 10^{-3}M$, which proved the optimal concentration for stability of proteases of actinomyces (Chaloupka 1955) prevents a decrease in the proteolytic activity of the medium, while the course of sporulation remains normal. The calcium was added in the form of a solution of $CaCl_2$. A ten times smaller concentration of Ca·· ions, $5.10^{-4}M$, also prevents a decrease (fig. 3). A lower concentration of calcium in the medium $(5 . 10^{-5}M)$ only delays the commencement of the decrease in proteolytic activity, while a concentration of $5 . 10^{-6}M$ has no influence on it (tab. 3).

Table 3. Influence of the addition of calcium in lower concentrations on the decrease in proteolytic activity of the medium. Proteolytic activity expressed in mg. casein digested in 30 mins.

Sample		Age of culture		
		12 hrs	14 hrs.	16 hrs.
Control (with added H_2O)	Prot. act. Sporulation	28.3 0%	1.2 66.8%	0.3 81.3%
Ca·· ions added in concentration $5 . 10^{-5}$ M	Prot. act. Sporulation	26.2 0%	26.8 37.7%	0.6 85.2%
$5 . 10^{-6}$ M	Prot. act. Sporulation	24.0 0%	0.5 67.9%	0.6 85.9%

Mg·· ions, which are known to have a mildly stabilizing effect on bacterial proteases (Gorini 1950) when added to the culture in the form of $MgSO_4$ (concentration $5.10^{-4}M$) had only a weak stabilizing influence. The addition of Mn·· ions, which, according to the data in the literature, are necessary in the presporulation period in Bac. subtilis (Weinberg 1955) and, according to other data, activate proteases of Bac. megatherium (Levinson and Sevag 1954), had no influence, with media of our composition, either on proteolytic activity or on sporulation.

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Influence of the addition of calcium ions on the stability of proteases in the
supernatant fluid.

The supernatant fluid was collected in the period of advanced sporulation and was
divided between two flasks. Calcium was added to one of these in a concentration
of 5.10^{-4}M and the same amount of distilled water to the other; both flasks were
then shaken at 29^0 C. In the flask to which calcium had been added, the decrease
which took place in the proteolytic activity of the supernatant fluid during shaking
was considerably less than in the flask containing no calcium (fig 4). The calcium
therefore clearly increased the stability of the proteases in the supernatant fluid
against shaking.

The addition of $Ca^{..}$ ions to the specimens
of supernatant fluid collected in the course
of sporulation does not lead to an increase in
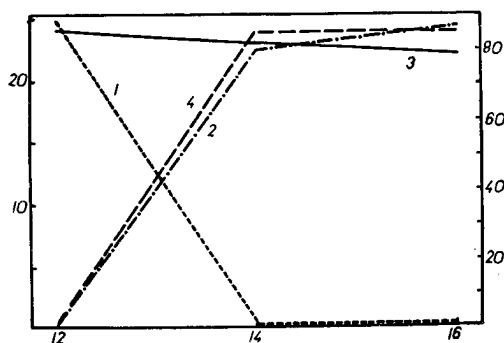proteolytic activity as compared with the
controls with H_2O.

Fig. 3. Influence of addition of $Ca^{..}$ ions to culture
before sporulation on further proteolytic activity. x -
axis: age of culture in hours, y - axis: left—proteolytic
activity in mg. casein digested in 30 mins., right—%
of spores in culture. 1 - - - - - - Proteolytic activity in
culture with added H_2O. 2 —.—.—.— % of spores in
culture with added H_2O. 3 ——— Proteolytic activity
in culture with added $Ca^{..}$ ions. 4 — — — — % of spores
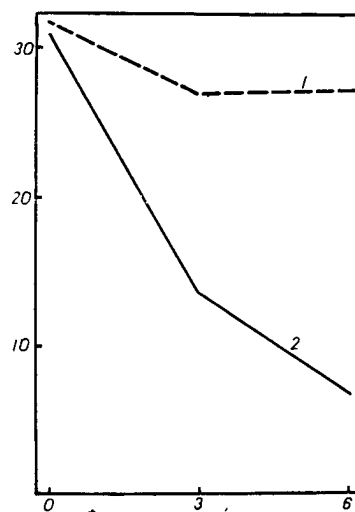in culture with added $Ca^{..}$ ions.

Fig. 4. Influence of $Ca^{..}$ ions on stability
of enzymes in supernatant fluid taken in
period of advanced sporulation (29.6%
spores). x - axis: duration of shaking of
supernatant fluid on shaker at 29° C, in
hours; y - axis: proteolytic activity in mg.
casein digested in 30 mins. 1 — — — —
Supernatant fluid with added $Ca^{..}$ ions
2 ——— Supernatant fluid with added H_2O.

Uptake of calcium from the medium into the sporulating cells

The calcium content of the cells before and after sporulation was determined by
means of spectrochemical analysis. On the A medium, the calcium content of the
cells increased during sporulation from 0.011% to 0.041%. The addition of 2 mg.
calcium per 1 litre medium (B medium) resulted in a considerable increase in the
differences in the calcium content before and after sporulation, but was not sufficient
to maintain stability of the proteolytic enzymes in the medium during sporulation.
The association between the decrease in proteolytic activity and exhaustion of the
calcium from the medium was thus maintained.

Spectrochemical analysis showed that between the 12th and 15th hour of cultur-
ing, i. e. in the period when the spores are being formed, a considerable increase
took place in the calcium content of the cells (fig. 5). The spores formed in the cells at

this period have all the appearance of spores, but they are still immature and do not yet possess the property of retaining a stain on decoloration. This property is acquired only after further hours of culturing.

When calcium was added before sporulation in considerably higher concentrations, no increase occurred in the calcium content of the cells which completed sporulation, with concentrations higher than $5 . 10^{-4}M$ (fig. 6).
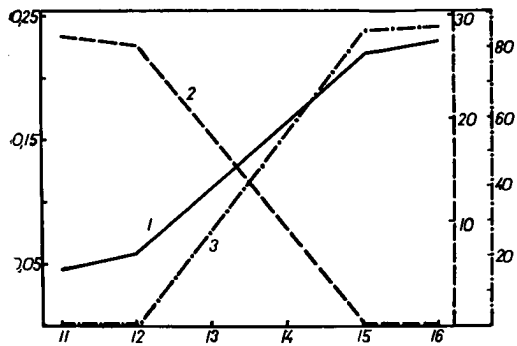

Fig. 5. Consumption of calcium by sporulating cells and influence of this process on proteolytic activity of medium. x - axis: age of culture in hours, y - axis: calcium content of cells in % of dry weight. 1 ————— Calcium content of cells in % of dry weight. 2 — — — — — Prot. activity in mg. casein digested in 30 mins. 3 —.—.—.—.— % of spores.
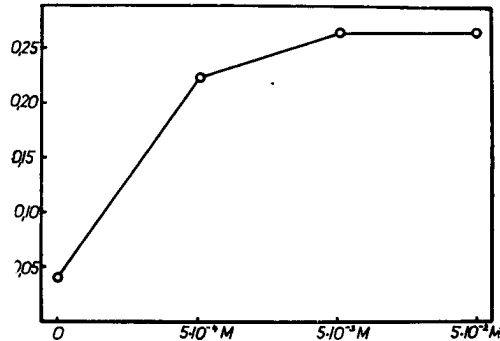
Fig. 6. Calcium content of cells completing sporulation in presence of varying concentrations of calcium. x - axis: concentration of Ca added after 12 hours culturing (10 ml. to 90 ml. culture). y - axis: calcium content of fully sporulated cells (17 hours culturing) in% of dry weight.

Influence of the addition of cysteine to the culture before sporulation on the proteolytic activity of the medium and on sporulation

After 12 hours' culturing, 10 ml. 0.01 M l-cysteine solution (pH 7.0) were added to flasks containing 90 ml. A medium, so that the final concentration of the cysteine in the culture was 0.001 M. Proteolytic activity was determined immediately after mixing and further after two and four hours' shaking. In the control flasks, 10 ml. distilled water was added and the determination carried out in the same way as for the actual specimens. In the intervals, the state of the cells in the culture was also controlled microscopically. Whereas in the control flasks, to which water had been

Table 4. Influence of the addition of cysteine to the culture before sporulation on the proteolytic activity of the medium and on sporulation of B. megatherium. Concentration of cysteine in the culture $1.10^{-3}M$. Proteolytic activity expressed in mg. digested casein.

	12 hrs. (immed. after mixing)		14 hrs.		16 hrs.	
	Prot. act.	Sporulation	Prot. act.	Sporulation	Prot. act.	Sporulation
Control (with 10 ml. water added)	20.40	0	2.60	53.1%	2.50	89.3 %
Specimen (with cysteine solution added)	19.30	0	17.00	0	16.50	0

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added, the usual decrease in proteolytic activity and normal sporulation took place, in the flasks containing cysteine, no decrease occurred in proteolytic activity and no spores were formed during the four hours (tab. 4). Similar results were obtained on adding cysteine in a 10 times weak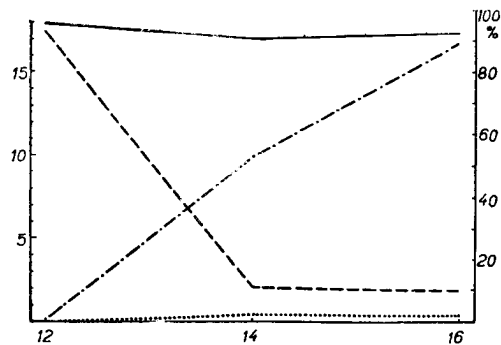er solution, when the final concentration in the medium after mixing was 0.0001 M (fig. 7). The only difference was that during the first two hours about 2% roundish, less shiny spores were formed. By the end of the fourth hour they had not increased in number.

Fig. 7. Influnece of the addition of cysteine to culture before sporulation on proteolytic activity of the medium and on sporulation of B. megathcrium (concentration of cysteine 1.10^{-4}M). x - axis: age of culture in hours: y - axis: left—mg. digested casein (flasks with water added — — — — — ,flasks with cysteine ————), right—% of spores in culture(flasks with water—.—.—.—, flasks with cysteine ....).

We also influenced the supernatant fluid from cultures of varying ages by 1-cysteine solution. Tab. 5 shows the values of proteolytic activity of supernatant fluids from 7, 13 and 14.5-hour-old cultures, immediately after adding water or a solution of cysteine in varying concentrations. The table shows that in a supernatant fluid taken during vegetative growth of the culture, cysteine brought about only a slight decrease in activity; in a supernatant fluid taken after a decrease in proteolytic activity, the values remained too low for accurate estimation.

Cysteine was also added to supernatant fluid taken in the course of sporulation and its influence determined after further shaking. The whole supernatant fluid was equally divided between two 500 ml. flasks, to one of which cysteine was added and to the other distilled water. Proteolytic activity was determined immediately and after a certain period of shaking. The supernatant fluids were diluted so that the final concentration of the cysteine was 1.10^{-4}M (tab. 6). The results show that the decrease in the proteolytic activity of the medium during shaking is almost the same in the mixture containing the cysteine and in the control containing water and that in this case the cysteine exerted no "protective influence".

Table 5. Influence of cysteine on proteolytic activity of supernatant fluid from 7,13 and 14.5 hours of culturing. Proteolytic activity expressed in mg. casein digested in one hour. Values in brackets represent proteolytic activity of supernatant fluid after being diluted with water.

Age of culture from which super-natant fluid was taken	Proteolytic activity of supernatant fluid with varying concentrations of cysteine		
	5×10^{-3}	3×10^{-3}	1×10^{-3}
7 hrs.	13.70 (14.90)	14.70 (15.00)	15.00 (15.20)
13 hrs.	8.90 (10.50)	10.00 (11.10)	10.36 (10.50)
14 1/2 hrs. (65% spores)	1.20 (0.90)	—	1.20 (0.80)

Table 6. Influence of cysteine (1.10^{-4} M) on activity of supernatant fluid containing proteases labilized on sporulation, shaken at 27^0 C.

$^0/_0$ of sporu-lation	Period of shaking in hrs.	Proteolytic activity in mg. casein digested in 1 hr.			
		Immed. after mixing		After shaking	
		Water	Cysteine	Water	Cysteine
11.5	6	37.00	36.80	28.00	29.50
13.8	6	52.00	51.00	34.80	34.00
42.0	3	16.70	17.70	3.00	5.60

Discussion

From the data in the literature we know that certain ions and salts are necessary for the sporulation process in bacilli and clostridia (Curran and Evans 1954, Fabian and Bryan 1933, Foster and Heiligman 1949, Charney, Fisher and Hegarty 1951, Grelet 1952a, 1952b, Knaysi 1945, Leifson 1931, Weinberg 1955) and also for the activity and stability of bacterial proteases (Gorini, Fromageot 1949, Gorini 1950, Gorini and Crevier 1951, Levinson and Sevag 1954). The present work aimed at ascertaining whether, in Bac. megatherium, certain ions necessary for the stability or activity of proteolytic enzymes were not exhausted from the medium before or during sporulation.

The results of the experiments show that in the presence of a small amount of calcium in the medium an associatión is displayed between sporulation and the state of the proteolytic enzymes in the medium. If the concentration of calcium in the nutrient medium is higher than that required by the sporulating cells, this association is not seen. The question arises as to whether the small calcium content in the A and B medium used by us was not the cause of the lability of the proteolytic enzymes throughout the whole period of culturing and not only during sporulation and whether the decrease in proteolytic activity during sporulation was not only the result of the termination of the production of proteases. In that case the high proteolytic activity in the culture before sporulation would have been due only to the continuous renewal of the level of the enzyme in the medium, in replacement of the denatured molecules of the enzyme. This possibility is suppressed by the following findings:

1) Sensitivity of the proteases to shaking is not noticeably increased until during the actual sporulation process.

2) The decrease in proteolytic activity is far more rapid in the presence of sporulating cells than in the shaken supernatant fluid.

3) The considerable increase in calcium in the cells during sporulation on the B medium is evidence that up to the time of sporulation, this amount of calcium was "available" for the stability of the proteolytic enzymes in the medium.

4) The interruption of the sporulation or presporulation mechanism by cysteine results in high proteolytic activity being maintained in the medium, even the A medium.

It is also known from the literature that the spores of bacilli contain more calcium than vegetative cells and the ability to accumulate calcium is mentioned in association with the thermoresistance of spores which have developed (Curran, Brun-

stetter and Myers 1943, Sugiyama 1951, Grelet 1950, 1952, Powell 1953, Tinelli 1955a, 1955b). Our work shows that the uptake of calcium occurs during formation of the spores and that this exhaustion of calcium may lead to significant changes in the medium.

From the data in the literature it is also known that a number of proteases require the SH group for functioning. The results of our experiments show that in this case the cysteine does not act on the proteases but on the actual sporulation process.

Summary

1) In the course of the sporulation of Bacillus megatherium a sharp decrease can take place in the proteolytic activity of the medium to negligible values.

2) The decrease in the proteolytic activity of the medium on sporulation is not due to the production of an inhibitor by the cells into the culture medium.

3) In a medium deficient in calcium, an increase occurs during sporulation in the sensitivity of the proteolytic enzymes in the medium to shaking.

4) The proteolytic enzymes produced during vegetative growth and after it has ended, require calcium to maintain their stability. A decrease in proteolytic activity in a calcium-deficient medium can be prevented by the addition of an excess of $Ca^{..}$ ions to the culture before sporulation.

5) Spectrochemical analysis showed that after the formation of young spores, the cells of Bac. megatherium contained several times more calcium than the vegetative cells before the commencement of sporulation.

6) A decrease in proteolytic activity takes place during sporulation of Bac. megatherium in a nutrient medium which does not contain more calcium than is required by the cells for the formation of the spores.

7. The addition of cysteine to the culture before sporulation (concentration $1.10^{-4}M$) destroys the ability of the cells to form spores and prevents a decrease in the proteolytic activity of the medium.

8. A concentration of cysteine of $1.10^{-4}M$ does not prevent a decrease in the proteolytic activity of supernatant fluid taken in the period of advanced sporulation and shaken on a shaker at $27^{)}$ C. Nor does cysteine increase the proteolytic activity of the supernatant fluid at rest.

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Спорообразование бацилл

Переход кальция в клетки и падение протеолитической активности среды при спорообразовании Bacillus megatherium

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Резюме

Мы культивировали штамм Bacillus megatherium на качалке в питательной среде с гидролизатом казеина и с небольшим содержанием кальция. Этот штамм выделял в среду значительные количества протеаз. В процессе спорообразования наблюдается резкое снижение протеолитической активности среды — вплоть до самых незначительных величин. Это снижение не обусловлено выделением клетками в культивационную среду ингибитора.

Протеолитические энзимы, выделяемые в среду в течение вегетативного роста и после его окончания, для своей устойчивости нуждаются в кальции. С помощью спектрального анализа мы установили, что после образования спор клетки Bac. megatherium содержат в несколько раз больше кальция, чем вегетативные клетки перед началом спорообразования. Этот переход кальция в клетки в течение процесса образования спор в бедной кальцием среде вызывает лабильность протеолитических энзимов среды, проявляющуюся в течение спорообразо-

вания постепенным повышением чувствительности протеаз по отношению к встряхиванию на качалке при 27 °C. Предупредить снижение протеолитической активности в бедной кальцием среде можно путем прибавления в культуру перед началом спорообразования избытка ионов $Ca^{..}$. Подобным образом, прибавляя дополнительно избыток кальция, можно стабилизировать протеазы супернатантной жидкости, взятой в период развернутой споруляции и далеко зашедшей лабильности протеаз. Таким образом, падение протеолитической активности при спорообразовании наблюдается в такой питательной среде, которая содержит не больше кальция, чем сколько требуется для образования спор.

Переход кальция в спорообразующие клетки протекает в период, когда возникают молодые споры, обладающие уже оптическими свойствами спор, но отличающиеся от зрелых спор по своей окрашиваемости.

Далее в работе описывается влияние прибавления в культуру перед спорообразованием цистеина на снижение протеолитической активности среды и на спорообразование Bac. megatherium. В концентрации $1 . 10^{-3}$ и $1 . 10^{-4}$ M цистеин нарушает способность клеток к образованию спор и полностью подавляет снижение протеолитической активности среды. Факт, что цистеин действует на способность клеток к спорообразованию, подтверждается и тем, что цистеин не препятствует снижению протеолитической активности супернатантной жидкости, взятой в период развернутой споруляции и встряхиваемой на качалке при 27 °C, и не влияет на протеолитическую активность супернатантной жидкости и при ее хранении в покое.

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On the Transglucosidatory Activity of Enzymatic Preparations of the Fungus, Aspergillus niger

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If a fluid culture or extract of a culture of Aspergillus niger is incubated with maltose, certain oligosaccharides are formed in addition to the hydrolysis of maltose (Pan, Andreasen and Kolachov 1950, 1951). The containing of a 1.6-glucopyranose bond is characteristic for these substances (panose, isomaltose). In addition, there are usually also traces of maltotriose (Burger and Beran 1956, a ,b).

There are still too few facts available to be able to say by what mechanism these products develop. The solution of this problem is important for several basic questions connected with the interaction of the substrate with the enzyme in the hydrolysis of poly- and oligosaccharides.

It is now known that the formation of these oligosaccharides takes place through transglucosidation, i. e. by transferring the glucose residue from maltose or starch to the corresponding acceptor (maltose, glucose etc.) (Pan, Nicholson and Kolachov 1952). It is interesting that in the experiments of Pan et al., the series of oligosaccharides, apart from maltose, did not act as substrate for the formation of isomaltose or the higher oligosaccharides with a 1.6-glucopyranose bond (Pan, Nicholson, Kolachov 1952).

The aim of the present work was to ascertain whether reducing oligosaccharides with a 1.6-glucopyranose link are formed from certain substances after incubation of short and long duration with a preparation of Aspergillus niger.

Methods

Enzymatic preparations used. An enzymatic preparation from a culture of the fungus Aspergillus niger was used for the experiments. The preparation of the cultures on bran and the extraction of the enzymes from these cultures has already been described in a previous communication (Burger and Beran 1956a). The characteristic property of this preparation is that it has a high maltose activity.

Working method. Incubation of the individual substrates by the enzymatic preparation was carried out for 75 minutes and 18 hours at 30° C. The total volume of the incubated mixture was 1 ml. and contained 3% enzyme solution, an acetate buffer in a final concentration of $6.5 . 10^{-2}$ M and with a pH of 4.5 and in some cases glucose in a final concentration of 0.3%. The glucose was added to the substrate as a possible acceptor. In order to prevent growth taking place during incubation, a few drops of toluene were added to the mixture. Enzymatic activity was arrested by placing the test-tube in a bath of boiling water for five minutes. Of the specimens, 20 μl. was dripped on to Whatman 1 chromatographic paper.

Analytical Methods. The incubation products were ascertained by the chromatographic method of Greene and Stone (Green and Stone 1952) with our own modification (Burger and Beran). A sample of maltose from incubation with an enzymatic preparation of A. niger was taken as standard. The composition of the sugars of the sample is known (Burger and Beran 1956 b).

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Results

Action of the Enzymatic Preparation on Various Oligosaccharides

Fig. 1. gives a chromatographic analysis of the products produced after 75 minutes' incubation of the enzymatic preparation with maltose, saccharose, lactose, raffinose and cellobiose. During this period hydrolysis of these substrates took place to varying degrees. In the case of raffinose, it could be seen that the breaking-down of fructose takes place with greater intensity than hydrolysis of melibiose. A spot of raffinose and saccharose appeared on the paper obviously after hydrolysis of these sugars, during development of the chromatogram. In the case of maltose, the formation of panose and isomaltose is evident, in the case of cellobiose the formation of isomaltose could be seen and also of another product, which was situated on the paper between maltotriose and panose. In the case of the other oligosaccharides no reducing sugar was produced. Since, with the method used for developing the chromatogram, lactose is situated in the same place as isomaltose, it can be stated that this oligosaccharide does not form higher reducing oligosaccharides.

Fig. 2 shows the situation after 18 hours' incubation. Maltose and cellobiose were by now virtually completely hydrolysed. Panose and an unidentified oligosaccharide which developed from cellobiose had also been broken down and only isomaltose remained. Not even after this period did raffinose (or melibiose), lactose and saccharose produce reducing oligosaccharides with a 1.6-glucopyranose link similar to those which developed from maltose.

The chromatogram further shows that from maltose and cellobiose traces of oligosaccharides developed, which were situated on the paper between maltose (or cellobiose) and isomaltose. These are probably oligosaccharides with a bond other than 1.4- or 1.6-glucopyranose and they still remain to be identified.

As far as raffinose, lactose and saccharose are concerned, our findings confirm those of Pan et al. (Pan, Nicholson and Kolachov 1952), who also found no reducing oligosaccharides after incubation of a fluid culture of A. niger with the sugars named above. As regards cellobiose, our findings are at variance with those of the authors mentioned, who found no oligosaccharides after the incubation of cellobiose with a fluid culture of A. niger (Pan, Nicholson and Kolachov 1952).

Fig. 3 gives results following the incubation of trehalose (α-D-glucopyranosyl-, α-D-glucopyranose) with an enzymatic preparation of A. niger.

With this oligosaccharide also there was no formation of a new product apart from glucose. As is seen from the illustration, trehalose gives a spot between maltose and isomaltose. As a non-reducing oligosaccharide it completely reduced silver following preliminary hydrolysis on paper during development of the chromatogram. Fig. 3 shows that even after 18 hours trehalose was hydrolysed only to a small degree. The traces of fructose and pentoses on the chromatogram are from the enzymatic preparation. .

Action of the Enzymatic Preparation on Some Heterosaccharides

Further experiments dealt with the action of an enzymatic preparation of A. niger on methyl-α-D-glucose, methyl-α-D-mannose and glucose-l-phosphate. The results are given in figs. 4 and 5.

As shown in fig. 4, the results are similar to those in the previous experiments. Hydrolysis of methyl-glucose occurred within 18 hours, but even after this period oligosaccharides were not formed. Fig. 4 also shows that only traces of methyl-mannose were hydrolysed.
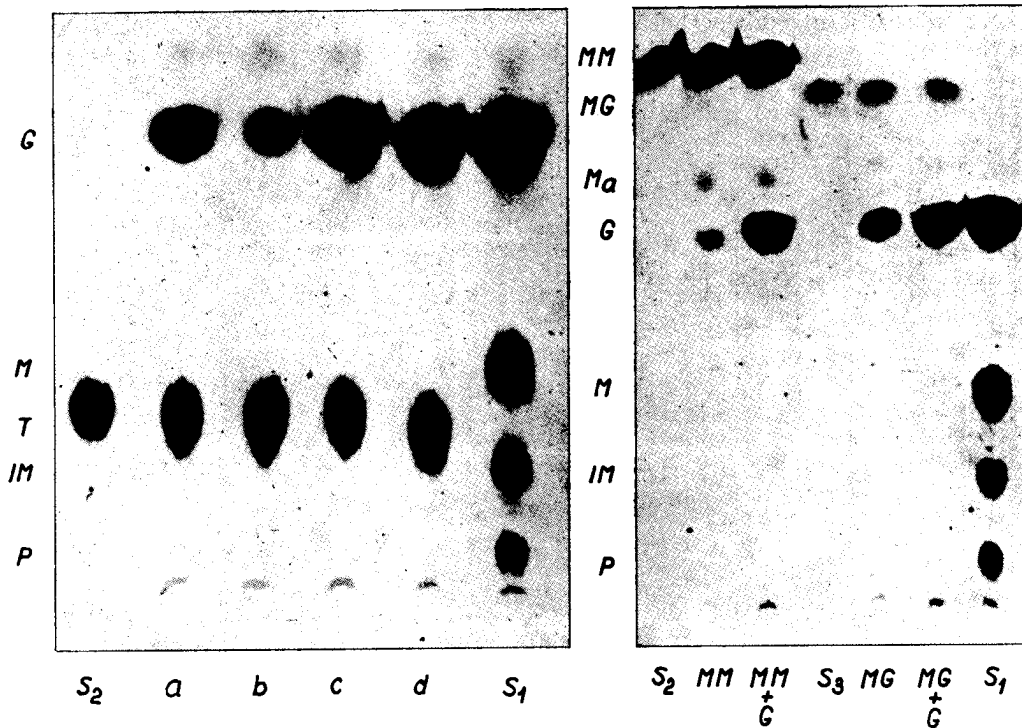
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| S_2 | a | b | c | d | S_1 |

Fig. 3.

| S_2 | MM | MM + G | S_3 | MG | MG + G | S_1 |

Fig. 4.

Fig. 3. The Action of an Enzymatic Preparation of A. niger on Trehalose. S_2 = specimen of trehalose, a = trehalose with glucose after 75 minutes' incubation, b = trehalose after 75 minutes' incubation, c = trehalose with glusose after 18 hours' incubation, d = trehalose after 18 hours' incubation, G = glucose, M = maltose, T = trehalose, IM = isomaltose, P = panose.

Fig. 4. The action of an Enzymatic Preparation of A. niger on Methylmannose and Methylglucose. S_2 = specimen of methylmannose, MM = methylmannose, MM + G = methylmannose with glucose, S_3 = specimen of methylglucose, MG = methylglucose, MG + G = methylglucose with glucose, Ma = mannose, G = glucose, M = maltose, IM = isomaltose, P = panose.

Similar results were also obtained on the incubation of glucose-1-phosphate with an enzymatic preparation (Fig. 5). Not even after 18 hours was any new product formed, apart from glucose. Even hydrolysis of glucose-1-phosphate was not complete under our experimental conditions.

Action of the Enzymatic Preparation on Glucose

In the previous experiments a study was made of the transfer of the glucose radical from holo- or heterosides to a carbohydrate acceptor.

Further experiments aimed at ascertaining whether direct synthesis of the oligosaccharides mentioned above takes place. Since it was assumed that possible synthesis would take place as a reversal of the hydrolytic process, the experimental conditions were adapted in such a way that the synthetic reaction could take place as far as possible. For this purpose the active concentration of water was reduced by adding an excess amount of glucose or by adding glycerin to the mixture. The final concentration of glucose was 5, 10, 30, 40, 50 and 60%. The glucose was therefore both

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substrate and acceptor in the mixture and, in excess concentrations, it also absorbed water from the mixture. Otherwise the conditions of incubation were the same as in the previous experiments. The results after 18 hours are given in fig. 6 and 7.
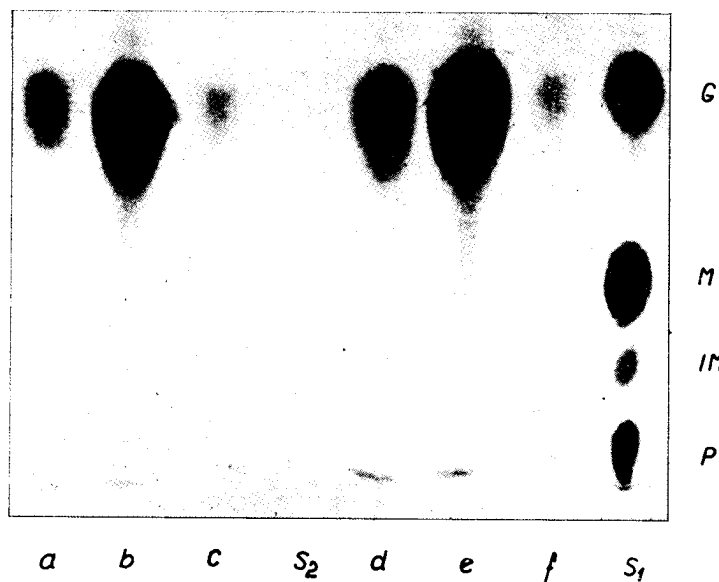

Fig. 5. The Action of an Enzymatic Preparation of A. niger on Glucose-1-Phosphate a = glucose-1-phosphate after 75 minutes' incubation, b = glucose-1-phosphate with glucose after 75 minutes' incubation, c = enzymatic preparation after 75 minutes' incubation, S_2 = specimen of glucose-1-phosphate, d = glucose-1-phosphate after 18 hours' incubation, e = glucose-1-phosphate with glucose after 18 hours' incubation, f = enzymatic preparation after 18 hours' incubation.

Fig. 6 shows that under our experimental conditions, synthesis of two oligo-saccharides from glucose occurred, viz. maltose and isomaltose. It should be noted that whereas maltose was formed only in the presence of higher concentrations of glucose (from 30%), isomaltose was already formed when only 5 and 10% glucose was present in the mixture.

After 150 minutes' incubation, the chromatogram presented a similar picture to that after 18 hours, the only difference being that the amount of products formed was smaller.

Fig. 7 shows, in addition to the incubated mixtures, control glucose solutions in the same concentrations as in the mixture. Fig. 7 therefore shows that the oligo-saccharides which developed were formed by enzymatic synthesis and were not present in the specimens of glucose in the form of impurities.

Discussion

In the previous work a number of facts were given, demonstrating that the formation of isomaltose and panose on the incubation of maltose with an enzymatic preparation of A. niger, is not the result of the activity of a special transglucosidase, but that the development of the oligosaccharides named from maltose is due to the catalytic action of maltase (Burger and Beran 1954, 1956b). It is known, however, that maltase also hydrolyses a number of substrates, in which the formation of oligo-

saccharides was not found (Summer and Myrbäck 1950) (e. g. methylglucose and trehalose). The question arises as to why the formation of the oligosaccharides mentioned did not take place with these substrates under our experimental conditions. It is known, and was also clearly demonstrated on our chromatograms, that these substrates are hydrolysed by maltase much more slowly than maltose.

It follows therefore that the affinity of maltase for trehalose and methyl glucose is much less than its affinity for maltose. It is probable that the same will apply if a comparison is made of the affinity of maltase for the substrates concerned and of its affinity for water. The transglucosidation products catalysed by maltase can develop, however, only if an acceptor with a sufficiently greater affinity for maltase than water is present in the system. It is clear that trehalose and methylglucose do not possess this affinity. Nor could glucose be the acceptor in these experiments, since there was a low concentration of glucose in the solution.

The formation of isomaltose and cellobiose is interesting, since from these facts it can be assumed that in this case isomaltose is formed by a similar, or identical mechanism, as from maltose. No further experiments are, however, available for confirming this assumption.
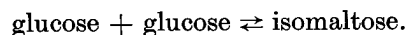
As far as saccharose is concerned, it is evident that hydrolysis took place by means of invertase (β-fructosidase) and that development of the reducing oligosaccharides which develop on the hydrolysis of maltose was therefore not possible (Bealing and Bacon 1953).

It must be emphasized that the method employed by us (4-fold development of the chromatogram) does not reveal oligosaccharides with more than four or five glucose fractions in the molecule. The formation of higher saccharides in our experiments is therefore not excluded.

The experiments in which varying amounts of glucose were incubated demonstrated that the enzymatic preparations studied by us are capable of resynthesizing isomaltose from glucose, and that if the concentration of water is reduced to a sufficient limit, resynthesis of maltose also occurs. The formation of isomaltose from maltose did not take place as a secondary process, as is demonstrated by the fact that with a lower concentration of glucose (from 5%), only isomaltose was formed and no maltose. It follows, therefore, that an enzymatic preparation of A. niger catalyses the synthesis of isomaltose not only by the route of transglucosidation:

$$\text{maltose} + \text{glucose} \rightleftarrows \text{isomaltose} + \text{glucose,}$$
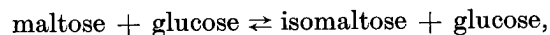
but also on the basis of the following equation:

$$\text{glucose} + \text{glucose} \rightleftarrows \text{isomaltose.}$$

The calculations show that these reactions are thermodanymically possible, if it is borne in mind that the isomaltose or maltose were formed in amounts over 1,000 times less than the concentration of glucose.

(Plates XXIV, XXV)

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К вопросу трансглюкозидирующей деятельности энзиматических препаратов Aspergillus niger

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Резюме

Исследовалось образование восстанавливающих олигосахаридов, обладающих 1,6-глюкопиранозной связью (изомальтоза и паноза) при инкубации энзиматического экстракта плесени А. niger (культивировавшейся на отрубях) с растворами различных олигосахаридов и глюкозы в различной концентрации. Хроматографический анализ исследуемых олигосахаридов производился после краткосрочной и длительной инкубации.

В соответствии с произведенными ранее исследованиями Pan-а с сотрудниками мы установили, что из мальтозы образуется как изомальтоза, так и значительные количества панозы. При длительной инкубации накопляется только изомальтоза. Из рафинозы, лактозы, сахарозы и глюкозы-1-фосфата вышеназванные олигосахариды не образовались, хотя их гидролиз осуществлялся.

В отличие от данных Pan-а и др. мы убедились, что из целлобиозы образуется изомальтоза и точнее не определенный нами олигосахарид, который на хроматограмме помещается между мальтозой и панозой.

Из других изучавшихся нами веществ (трегалоза, метил-глюкоза, метил-манноза) восстанавливающие олигосахариды с 1,6-глюкопиранозной связью также не образовались, хотя у них осуществлялся по крайней мере частичный гидролиз.

При инкубации более или менее концентрированных растворов глюкозы ресинтетизируется изомальтоза (начиная с 5% глюкозы и больше) и мальтоза (начиная с 30 и более процентов глюкозы). Образование изомальтозы из глюкозы осуществлялось не через мальтозу, а прямо из глюкозы. Это доказывает, что образование изомальтозы может — кроме реакции трансглюкозидации из мальтозы — осуществляться также путем ресинтеза из глюкозы.

(Табл. XXIV, XXV)

FOLIA BIOLOGICA

Das Auftreten von Ölmohnbakteriosen in der Tschechoslowakei

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Der Ölmohn *(Papaver somniferum* L.) gehört unter unseren Züchtungsbedingungen zu den landwirtschaftlichen Produkten mit sehr schwankenden Ernteerträgen, deren Höhe unter anderem vom Gesundheitszustand der Pflanzen, also von der Summe verschiedener Krankheits- und Schädlingseinflüsse abhängt.

Bei Untersuchungen von Zuchtkulturen des Mohns (1953) erweckte unser Interesse das Auftreten und die Verbreitung zweier unterschiedlicher Bakteriosen, deren Merkmale uns zwar bereits aus früheren Jahren bekannt waren, bisher aber nicht gebührend betrachtet wurden. In der Praxis wurden diese Bakteriosen meistens übersehen und die Erkrankung der Pflanzen anderen Ursachen zugeschrieben. Wir sahen uns daher zu ihrem eingehenden Studium veranlasst.

Nach ihren typischen Erscheinungsformen bezeichnen wir diese Erkrankungen im weiteren als "Blattfleckenbakteriose des Mohns" und als „Stengelbakteriose des Mohns".

Die Blattfleckenbakteriose des Mohns

Die in der Literatur angeführten Blattbakteriosen betreffen vorwiegend wild-wachsende Mohnarten und ihre Varietäten, die in den USA und in England als Zierpflanzen gezüchtet werden (Clinton 1909, Bryan und Mc Whorter 1930, Dowson 1949, Nance 1951). Der Krankheitserreger wurde von Bryan und Mc Whorter als *Bacterium papavericola* n. sp. beschrieben und nach der späteren Taxonomie als *Xanthomonas papavericola* eingeordnet. Die Blattfleckenbakteriose kommt nach Berichten auch beim Ölmohn in der UdSSR vor (Beloselskaja und Silvestrov, 1953, Gorlenko 1953).

Die Pflanzen werden hauptsächlich in den Sommermonaten befallen, also zu einer Zeit, in der unter dem Einfluss abwechselnder Regenfälle und stärkerer Besonnung im Boden und in den Kulturen ein dunstiges Mikroklima herrscht. Der Mikro-organismus gelangt zunächst aus dem Boden durch abprallende Regenspritzer oder vom Wind angewehte Teilchen der Ackerkrume auf die untersten Blätter. Die weitere Verbreitung erfolgt durch gegenseitige Berührung der Pflanzen, durch Regenspritzer oder auch durch Insekten. Die feuchten Blattflächen ermöglichen das Vordringen des Mikroorganismus durch die Spaltöffnungen in das Gewebe. An der Infektionsstelle entstehen zunächst helle, wässerige, sich vergrössernde Flecke, die, von der Nervatur begrenzt, eine unregelmässig eckige Form einhalten. Später nehmen sie eine gelbliche Färbung an, trocknen ein und bleiben, zum Unterschied von

den sehr ähnlichen, durch Helminthosporiose hervorgerufenen Flecken, transparent. Die schütter verstreuten Flecke sind gewöhnlich grösser (5—10 mm), rundlicher und lassen manchmal eine Zonenbildung erkennen. Bei dichtem Besatz gehen sie ineinander über und das Blatt vertrocknet schnell. Eine Verbreitung durch die Gefässbündel auf grössere Entfernung wurde nicht beobachtet. Unter günstigen Bedingungen, hauptsächlich bei genügender Feuchtigkeit und Wärme, kommt es zu einer epidemischen Ausbreitung der Krankheit auf die ganze Kultur. Der Verlust der Assimilationsfläche übt bei stärkerem Befall einen bedeutenden Einfluss auf die Entwicklung der Mohnkapseln und Qualität des Samens aus. Beim Klatschmohn werden vom Mikroorganismus nicht nur die Blätter, sondern auch Stengel, Knospen und Kapseln befallen, was bei Ölmohnkulturen nicht beobachtet wurde.

Eine Verbreitung der Blattfleckenbakteriose wurde bisher nur gelegentlich in einigen Gebieten der Republik festgestellt.

Der Mikroorganismus konnte im allgemeinen leicht aus frischen, noch nicht eingetrockneten Flecken isoliert werden, wo er in einer fast einheitlichen Assoziation vorzufinden ist. Das Ausgangsmaterial für die reinen Schalenkulturen bildeten auf dem Nähragar die an der Randzone der ausgestanzten Blattfleckenstücke oder in den Ausstrichen des im Wassertropfen zerriebenen Blattfleckenmaterials entstehenden Kolonien. Mit den Subkulturen wurden die Blätter junger, im Beet gezüchteter Pflanzen durch Bestreichen mit verdünnter Bakterienemulsion infiziert. Die Pflanzen wurden durch Glaszylinder mit Kaliko-Verschluss durch zwei Tage hindurch verdeckt. Nach 4—5 Tagen machte sich die Infektion in dichten kleinen Flecken bemerkbar, die stellenweise in zusammenhängende Flächen übergingen. Die Identität des Mikroorganismus wurde durch Rückisolierung nachgewiesen.

Die Infektion des Stengels wurde mit einem sehr dünnen Kapillarröhrchen durchgeführt, das 1—2 mm³ wässerige Bakteriensuspension enthielt und bis zum Bereich der Gefässbündel eingestochen wurde. Nach 7—9 Tagen trat nur eine lokale Oberflächennekrose unmittelbar um den Einstich herum und eine Nekrose des inneren Gewebes bis zu 2 cm ober- und unterhalb des Einstichs in Erscheinung. Nach Ablauf von 2—3 Wochen breitete sich die Nekrose in schmalem Streifen nach oben und unten auf die Entfernung von einigen cm aus. Diese Pflanzen können zwar in ihrer Entwicklung geschwächt sein, doch tritt keine Fäule ein wie bei der Stengelbakteriose des Mohns.

Beschreibung des Mikroorganismus

Die Bakterien haben die Form eines kurzen, ovoiden, 0,9 × 0,6 μ grossen Stäbchens; sie sind beweglich, gramnegativ, besitzen eine einzige, polständige Geissel und bilden bei älteren Kulturen Hüllen, aber keine Sporen.

Auf Fleischextrakt-Peptonagar wachsen im Verlauf von 24 Stunden 1 bis 1,5 mm grosse, runde und gewölbte Kolonien von senfgelber glänzender Färbung und mit geradem Rand; im durchscheinenden Licht sind sie braungelb und gegen das Zentrum zu dichter granuliert. Auf Schrägagar bilden sie einen dünnen, sattgelben Belag von schleimiger Konsistenz. Auf Kartoffelagar verläuft ihr Wachstum üppiger und die Färbung ist sattgelb; Stärke wird hydrolysiert. Bei Milch erfolgt am zweiten Tag Koagulation, am sechsten Tag Peptonisation. Der Mikroorganismus bewirkt Gärung ohne Gasbildung bei Dextrose, Galaktose, Saccharose, Fruktose, Laktose, Maltose, Arabinose, Mannit, Mannose und Glyzerin, reduziert Nitrate, verflüssigt Gelatine langsam (in 6 Wochen bis zur Hälfte der Normalsäule) und produziert Ammoniak sowie Schwefelwasserstoff, nicht aber Indol. Der Mikroorganismus ist oxybiotisch; im Eintrocknungszustand beträgt seine Lebensbeständigkeit 7 — 9 Monate. Temperaturoptimum 25 — 30° C; pH Wert 6,5 — 7,5.

Nach diesen Eigenschaften wird als Erreger der bei uns verbreiteten Blattfleckenbakteriose des Ölmohns bestimmt:

Xanthomonas papavericola (Bryan et Mc Whorter) Dowson.

Synonymum: *Bacterium papavericola* Bryan et Whorter.

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Die Stengelbakteriose des Mohns

Diese durch Schwarzwerden und Nassfäule des Stengels in Erscheinung tretende Krankheit wurde ursprünglich beim Opium- und Klatschmohn in Indien festgestellt (Hutchinson 1916, Ram Ayyar 1917); ihr Erreger wurde erst später unter der Bezeichnung *Bacillus papaveris* n. sp. beschrieben (Ram Ayyar 1927).

In unseren Kulturen beobachteten wir Pflanzen mit Merkmalen, die jenen glichen, die von diesen Autoren angeführt wurden. Die ersten Krankheitssymptome machten sich bei den Pflanzen im Stadium ihrer stärksten Entwicklung zu Beginn der Knospenbildung bemerkbar. Die Erkrankung beginnt mit dem Welken der Gipfel und breitet sich binnen 2—3 Tagen über die ganze Pflanze aus. Der Infektionsbeginn verrät sich durch eine dunkle, weiche Stelle, am häufigsten in der oberen Stengelhälfte, an der gewöhnlich der Stengel brüchig wird. Im Querschnitt des Stengels erscheint das Mark gebräunt bis schwärzlich und ist zum grössten Teil zerstört; die Innenwand ist von weisslichem bakteriellem Schleim bedeckt, der aus der Bruchstelle fliesst. Es folgt dann der Zerfall aller übrigen, bisher nicht betroffenen Pflanzenteile. Jüngere Pflanzen unterliegen dem Zerfall bedeutend schneller, sodass oft nur auf der Erde liegende vertrocknete Blattreste übrigbleiben. Die in ihrer fortgeschrittenen Entwicklung befallenen Pflanzen werden zwar nicht brüchig, sterben aber ebenfalls schnell ab. Beim Klatschmohn sind die Krankheitssymptome ähnlich. In Kulturen werden zunächst einzelne Pflanzen befallen, von denen aus dann die Krankheit nestartig um sich greift, wobei besonders feuchte Böden ein Reservoir dieses Mikroorganismus bilden. Bei der Infektion scheinen eine grosse Rolle beissende und saugende Insekten zu spielen, die in der weiten Umgebung des Standortes einen Bestandteil der Biozönosen der Mohnkulturen und der Phytozönosen bilden. Die beträchtliche Menge des bakteriellen Schleims, mit dem die Pflanzen im vorgeschrittenen Fäulezustand bedeckt sind, ermöglicht eine Übertragung der Infektion, die, wie experimental nachgewiesen wurde, auch durch die Weichwanze *Calocoris norvegicus* Gmel. erfolgen kann.

Die Stengelbakteriose des Mohns wurde bisher bei einer grösseren Zahl von Lokalitäten in Mähren und in der Slowakei beobachtet.

Zur Isolierung des Mikroorganismus wurde das Material der Schleimsubstanz verwendet, die das Innere der faulenden Stengel bedeckt. Die künstliche Infektion zahlreicher Pflanzen verlief durchwegs positiv und rief alle Begleitsymptome natürlicher Infektionen hervor. Nach 48 Stunden begann das Welken der Pflanzen, am dritten Tag erschienen am Stengel die ersten Bruchstellen und im Verlaufe einer Woche waren 90 % der Stengel von Versuchspflanzen brüchig, was auf eine grosse Pathogenität des Mikroorganismus hinweist.

Nach Infektion durch Auflegen eines befallenen Gewebeteils in die Blattachsel kam es unter typischen Erkrankungserscheinungen zum Absterben der Pflanze bereits nach 12 Tagen. Ebenso wurden ganze Pflanzen nach Infektion des Hauptnerven eines Blattes befallen, die ungefähr 5 cm von der Insertion entfernt gesetzt wurde; dabei konnte eine Verbreitung der Bakterien durch die Gefässbündel beobachtet werden. Das Aufstreichen der Suspension auf Blätter rief keine Fleckenbildung hervor, die bei der vorherbeschriebenen Blattfleckenbakteriose erzielt werden konnte. Die infizierten Knospen trieben entweder noch aus und entwickelten verschiedentlich deformierte Mohnkapseln, oder wurden schwarz und vertrockneten, wobei die Infektion oft durch die Gefässbündel auch auf den Stengel übergriff und manchmal bis in die Wurzel vordrang. Ähnlich verlief die Infektion in den verschiedenen Organen des Klatschmohns und unter den verwandten Pflanzen beim gemeinen Erdrauch (*Fumaria officinalis* L.), während sie beim Schölkraut (*Chelidonium maius* L.) und beim Jungfernherz (*Dicentra spectabilis* L.) negativ blieb.

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Beschreibung des Mikroorganismus

Die Bakterien haben die Form kurzer, 0,4 × 0,5 bis 2,7 µ grosser Stäbchen mit abgerundeten Enden. Sie sind beweglich, gramnegativ, seitenständig begeisselt und bilden weder Hüllen noch Sporen. Die Kolonien erreichen auf Fleischextrakt-Peptonagar eine Grösse von 0,5 bis 1,5 mm und sind schwach kuppelförmig, rundlich mit geradem hellem Rand, gräulich weiss, glänzend, schwach blau opalisierend, im durchscheinenden Licht gelbbraun und feinkörnig. Ältere Kolonien haben ein annähernd stürzenartiges Aussehen mit erhöhtem gelblichem Zentrum und unregelmässig gewelltem, radial gefaltetem Rand. Auf Schrägagar bilden die Kolonien einen üppigen, grauweiss glänzenden und im durchscheinenden Licht gelbbraunen Belag mit fein gewölbtem Rand, auf Kartoffelagar einen schwach erhöhten, grau-weissen, stark glänzenden Belag mit gelapptem Rand. Bouillon wird nach 24 Stunden in der ganzen Säule getrübt, Milch am dritten Tag ebenfalls in der ganzen Säule gefällt. Der Mikroorganismus vergärt Dextrose, Fruktose, Saccharose, Laktose, Mannose, Glyzerin, Xylose, Mannit und Arabinose unter schwacher Gasbildung, die am dritten Tag ausgeprägter ist. Maltose gärt nicht oder nur sehr schwach und ohne Gasbildung. Positive Reduktion von Nitraten. Gelatine wird anfänglich schüsselartig, nach zwei Tagen sackartig und am sechsten Tag in der ganzen Säule verflüssigt. Ammoniak und Schwefel-wasserstoff wird nur sehr schwach, Indol überhaupt nicht produziert. Sehr schwache Hydrolyse der Stärke. Milch mit Lakmus und Methylenblau färbt ab. Der Mikroorganismus ist fakultativ anaerob, seine Lebensfähigkeit beträgt im Eintrocknungszustand ungefähr 4—5 Monate mit einem Wachstums-optimum bei 26—30° C und pH 7—8.

Im Hinblick auf diese Eigenschaften gehört der Erreger der bei uns beobachteten Stengelbakteriose des Ölmohns zur Gruppe der pathogenen Stämme, die taxonomisch repräsentiert wird durch die Art:

Bacterium carotovorum (L. R. Jones) Lehmann, Neumann.

Synonyma: *Bacillus papaveris* Ayyar,
    Erwinia papaveris (Ayyar) Magrou,
    Erwinia aroideae (Towsend) Bergey.

Die erkannte Tatsache, dass beide Krankheiten auch wildwachsende Mohnarten befallen und durch Insekten übertragen werden können, erhöht die Bedeutung dieser Bakteriosen unter unseren Züchtungsbedingungen. Vom Standpunkt des Pflanzen-schutzes sind vor allem Präventivmassnahmen notwendig, also die Auswahl nicht allzu feuchter Böden, Einhaltung einer richtigen Fruchtfolge und Verwendung von gut geerntetem und gereinigtem Saatgut aus nur gesunden Kulturen. Durch rechtzeitige Bekämpfung der Schädlinge mit Insektiziden kann auch die Ausbrei-tung dieser Bakteriosen eingeschränkt werden.

(Bildtafeln XXVI, XXVII, XXVIII, XXIX)

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Бактериозы сеянного мака, наблюдавшиеся в Чехословакии

Я. РОЗСЫПАЛ и Ф. МРАЗ

Резюме

Приводятся сведения о нахождении и распространенности в ЧСР двух различных бактериальных заболеваний сеянного мака, которые необходимо присоединить к известным до сих пор хозяйственно важным заболеваниям этого широко распространенного у нас масличного.

По признакам мы обозначаем их как «бактериальную пятнистость листьев мака» и «бактериоз стебля мака».

Первое заболевание появляется в летние месяцы, когда при чередовании дождей и интенсивного солнечного облучения почва и посевы запревают. Микроорганизмы попадают на растения из почвы вместе с отскакивающими дождевыми каплями и навеянными ветром частицами земли. Являясь подвижными, они легко проникают по влажной поверхности листа в устьица и в межклеточное пространство. Здесь возникают желтоватые, позднее буреющие и засыхающие, ограниченные по контурам жилками пятна неправильной формы и различных размеров. При соответственных условиях болезнь эпидемически распространяется по всей культуре. Утрата большей части ассимилирующей поверхности резко влияет на развитие маковок и качество зерна. Бактериоз наблюдался пока в нескольких областях ЧСР. Бактерии не трудно изолировать из молодых, еще не засохших пятен, где они образуют почти однородное сообщество. Растирая эмульсию чистой культуры на листья, не трудно вызвать искусственное заражение.

Бактерии имеют форму короткой яйцевидной палочки размерами в 0,9 : 0,6 μ. На мясопептонном агаре они чере 48 час. образуют выпуклые круглые колонии с гладкими краями, размерами в 1—1,5 мм, горчично-желтые, блестящие, на свет коричнево-желтые, к центру более густозернистые. На косом агаре они образуют яркожелтый слизистый налет. Бактерии медленно разжижают желатин; вызывают свертывание, а позднее пептонизацию молока; сбраживают декстрозу, галактозу, фруктозу, сахарозу, лактозу, мальтозу, арабинозу, манит, манозу и глицерин без образования газа; восстанавливают нитраты; выделяют аммиак и сероводород, но не индол. Бактерии кислородолюбивы. В засушенном состоянии они сохраняют жизнеспособность в течение 7—9 месяцев. Оптимум роста при 25—30 °C, рН 6,5—7,5.

По этим признакам возбудителем наблюдавшейся нами бактериальной пятнистости листьев мака является Xanthomonas papavericola (Bryan & Whorter) Dowson.

Синоним: Bacterium papavericola Bryan & Whorter.

Второй бактериоз мака проявляется почернением и мокрой гнилостью стеблей, — чаще всего в период наиболее бурного роста, в начале периода

образования бутонов. Болезнь проявляется завяданием зеленых растений, начиная с верхушек. О начале заболевания говорит более темное и мягкое место в верхней части стебля, где он обычно надламывается. На разрезе видно, что мякоть стебля почернела, большей частью разрушена, а стенка стебля с внутренней стороны покрыта беловатой слизью бактерий. В посевах болезнь появляется сначала на отдельных растениях, вокруг которых распространяется гнездообразно. Резервуаром микроорганизма является, главным образом, влажная почва. Значительную роль при инфекции играют, как кажется, колющие и сосущие насекомые. На опыте была подтверждена возможность передачи инфекции клопом Calocoris norvegicus Gmel. Искусственное заражение многочисленных растений взвесью чистой культуры (в стебель, бутоны и маковки) всегда давало положительные результаты. Вкладывая кусочки зараженной ткани в пазуху листа тоже можно вызвать заболевание. Растирание взвеси по листьям не вызывало пятнистости. Это свидетельствует о том, что в устьица листьев бактерии не проникают.

Возбудитель — это весьма подвижные палочки, размерами в 0,4 : 0,5—2,7 μ, с закругленными концами и несколькими перитрихальными жгутиками, грамотрицательные и не образующие ни спор, ни капсул. На мясопептонном агаре через 24 часа вырастают круглые, слегка куполообразные колонии с более светлыми гладкими краями, размерами в 0,5—1,5 мм в диаметре, серовато-белые, блестящие, отливающие синеватым, на свет желто-коричневые, мелкозернистые. Старые колонии имеют несколько выпуклую форму, с неправильно волнообразными, лучеобразно сморщенными краями. Разжижение желатина проходит быстро, — на 6-ой день по всему столбику. Бактерии сбраживают декстрозу, фруктозу, сахарозу, лактозу, манозу, глицерин, ксилозу, манит, арабинозу при незначительном образовании газа, но не сбраживают мальтозы; восстанавливают нитраты. Выделение аммиака и сероводорода весьма незначительно, индол не выделяется. Бактерии могут жить анаэробно, в засушенном состоянии сохраняют жизнеспособность в течение 4—5 месяцев. Оптимум роста при 26—30 °C и pH 7—8.

По этим признакам возбудитель наблюдавшегося нами бактериоза маковых стеблей принадлежит к группе патогенных штаммов, таксономически представленных видом Bacterium carotovorum (L. R. Jones) Lehmann, Neumann.

Синонимы: Bacillus papaveris Ayyar.
Erwinia papaveris (Ayyar) Magrou
Erwinia aroideae (Towsend) Bergey.

Наблюдение, что оба заболевания поражают и дикорастущий сорный мак и могут передаваться и насекомыми, повышает их значение для наших посевов. С точки зрения защиты необходимо заботиться прежде всего о профилактических мероприятиях, подборе не слишком влажного участка, соблюдении правильных сроков и способов посева и об использовании правильно собранных и очищенных семян только из здоровых культур. Своевременное применение инсектицидных средств может ограничить распространение этих бактериозов вредителями.

(Табл. XXVI, XXVII, XXVIII, XXIX)

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К вопросу участия неоплодотворяющих живчиков в половом процессе

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Открытие, что живчики можно найти в парафиновых срезах тканей полового тракта самок (Herwerden 1905, Kohlbrugge 1910, 1911, Генин 1950, 1951, 1953 и 1955, Хурый и др. 1954, Войтишкова 1955) вело к заключению о наличии причинной зависимости между проникновением живчиков в соматические ткани полового тракта самки и явлениями телегонии и ксении, а отчасти эффективностью гетероспермии (Саркисян 1952, Кушнер 1954).

Мы сконтролировали подобные случаи позитивного диагноза с помощью видоизмененного метода и ряда моделей опытов, результаты которых свидетельствуют о том, что случаи нахождения живчиков в тканях — и нами, и другими цитируемыми авторами — являются артефактами и обусловлены [способами гистологической обработки.

Экспериментальная часть

I. Половой тракт курицы

А. *Парафиновые и целлоидиновые срезы.* Куры, изолированные за 45 дней до опыта от петуха, искусственно осеменялись (Seiden 1947, Орел 1949) дозами в 0,4—0,8 мл эякулята за 26 час., 6 ч. 30 м., 4 ч. 30 м., 2 часа и, наконец, за 10 мин. до умерщвления. После быстрой препаровки весь яйцевод курицы погружался в 10% формол, через 2 часа вынимался, разделялся на блоки и после окончательной фиксации подвергался дальнейшей обработке. Часть каждого отрезка яйцевода мы заливали в парафин, а часть — в целлоидин. Толщина парафиновых срезов бывала 6—8 μ, целлоидиновых —10 μ. Окраска производилась большей частью гематоксилин-эозином по модификации Harris-а.
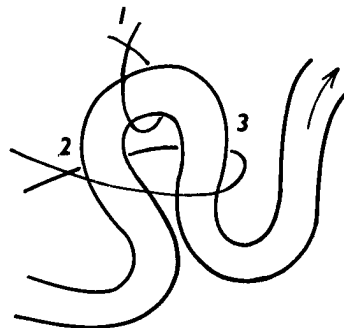
Рис. 2. Схема подвязывания петель яйцевода. 1 - место наложения первой лигатуры; 2, 3 - места наложения второй, общей лигатуры. Стрелкой обозначен ход яйцевода по направлению к яичнику.

Б. *Исследования перитонеальной поверхности и внешних слоев яйцевода курицы после осеменения.* Три курицы были на 40—75 дней изолированы от петуха. Чтобы исключить возможность проникновения живчиков на перитонеальную поверхность яйцевода из его просвета через полость брюшины, проиводилась следующая операция: все куры оперировались под уретан-барбиталовым наркозом (10 г уретана, 0,75 г барбитуровой кислоты, 100 мл H_2O в полость брюшины за 2—3 часа до операции). После лапаротомии производилось двойное подвязывание петли яйцевода в его секреторной части (двойной шелковой нитью поверх

толстого слоя марли — рис. 2). В пункте 1 была наложена первая лигатура, в пунктах 2 и 3 — вторая, общая. На второй день после операции у всех 3 кур было произведено искусственное осеменение. Потом у 1-ой курицы яйцевод был отпрепарирован и вынут из брюшной полости, а у 2 других — только развязан, но оставлен в брюшной полости. С поверхности яйцевода, матки и isthmus tubae тонкими ножницами или тонким крючковым пинцетом отрезались маленькие частицы ткани, которые растирались и наносились в форме мазков на предметное стеклышко. После приготовления каждого препарата инструменты заменялись чистыми. Предметные стеклышки нумеровались в зависимости от места взятия образца и количества образцов, взятых с одного и того же места яйцевода. После этого вскрывался яйцевод и делались мазки-отпечатки слизистой, к которой прикладывались предметные стеклышки. Яйцевод вскрывался в следующем порядке: мы вели продольный разрез сначала между лигатурами в пунктах 1 и 3, потом между лигатурами 1 и 2 и, наконец, перед пунктом 2.

II. Половой тракт мыши и крысы

Мы случали на 10 час. (на ночь) 50 белых мышей (по 1 самцу на 2 самок) и 40 крыс (по 2 самца на 3 самок), после чего у всех самок делались влагалищные мазки, которые окрашивались по Giemsa. Наличие живчиков было установлено только у 1 мыши и 8 крыс. Этих 9 самок было умерщвлено. Интервал между осеменением и умерщвлением был не больше 12 час. Половые тракты этих самок (влагалище с обоими углами матки и яйцеводы) были отпрепарированы и обработаны (путем заливки в целлоидин).

III. Модели опыта

А. *Реакция тканей на впрыскивание спермиев.* Мы исходили из предположения, что такие большие количества живчиков, какие мы находили на парафиновых и целлоидиновых срезах половых органов курицы, должны были бы вызывать бурную реакцию фагоцитоза, если бы живчики активно проникали в ткани живого, способного реагировать организма самки. Так как мы не наблюдали подобной реакции, мы поставили следующий опыт: четырем цесаркам было введено под кожу, в двойную кожную складку между плечом и костьми предплечья по 0,5 мл взвеси промытых живчиков петуха породы Леггорн в физиологическом растворе. Способ промывки: полученный эякулят трижды промывался в 5—7 раз большим количеством физиологического раствора. Осадок разводился в соотношении приблизительно 2 части живчиков на 3 части физиологического раствора. Цесарки убивались через различные промежутки времени после впрыскивания живчиков: через 1, 6, 24 и 48 час. У всех 4 цесарок место укола было заметно макроскопически. У первой и второй цесарок на крыльях прощупывались флюктирующие утолщения величиной с небольшую горошину. Так было и у цесарки, убитой через 24 часа, а у убитой через 48 час. на крыльях прощупывались плотные, нефлюктирующие утолщения величиной с горошину. Покраснения не наблюдалось. Складки кожи вырезались всегда далеко от места укола и на 2—3 часа погружались в 10% формол. Только после того, как бывала обеспечена по крайней мере первичная коагуляция и не грозила уже опасность, что живчики будут занесены в окружающие ткани, складки кожи разрезались до требуемых размеров и после окончательной фиксации заливались в парафин.

Б. *Живчики в скелетной мышце.* Скелетная мышца (бедренная мышца курицы или 1-дневного цыпленка) погружалась до ¹/₃ длины на 2 часа при комнатной температуре в эякулят. Некоторые мышцы погружались в нормальный эякулят с живыми живчиками, которые через 2 часа оставались еще нормально подвижными. Другие мышцы помещались в эякулят, к которому была прибавлена 1 капля 8% формола, немедленно убивавшая живчиков. Через 2 часа мышцы обрабатывались гистологически, путем заливки в парафин и целлоидин.

В. *Показ искусственного привнесения чужеродных клеток в ткани.*

а) Эритроциты кролика в куриной мышце. Куриная мышца погружалась на 2 часа при комнатной температуре в цитратную кровь кролика. Кроличья кровь позволяла отличить собственные (ядерные) красные кровяные тельца цыпленка от могущих проникнуть сюда безъядерных эритроцитов кролика. Через 2 часа мышца вынималась, споласкивалась физиологическим раствором и заливалась в целлоидин.

б) Живчики из фиксационной жидкости на целлоидиновых срезах мышцы. Мышца цыпленка фиксировалась нормальным способом в 10% формоле, к которому мы прибавляли свежий эякулят (0,5 мл эякулята на 100 мл 10% формола) и перемешивали встряхиванием. После 12-часовой фиксации мышца в течение 24 час. промывалась в текущей воде и заливалась в целлоидин.

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Результати и дискуссия

Главная разница между картинами парафиновых и целлоидиновых срезов тканей полового тракта осемененной курицы заключается в отсутствии живчиков в эпителии и слизистой оболочке на целлоидиновых срезах. В отличие от парафиновых, на целлоидиновых срезах не были найдены проникающие живчики, а только живчики, приставшие к ресничкам. Картина спермиев, находимых в мышечной и соединительной ткани, тождественна с картиной парафиновых срезов (рис. 1). Наличие живчиков в эпителии и слизистой оболочке и присутствие ровных, как бы вытянутых живчиков в просвете, близ поверхности эпителия на парафиновых срезах можно объяснить механическим их протаскиванием ножом микротома при разрезе. Однако мы полагаем, что и другие живчики могут попадать на срез в результате намывания из просвета яйцевода во время удаления парафина перед окраской, так как на свежезалитых в бальзам препаратах часто встречаются свободно движущиеся спермии, а массивные их скопления встречаются и сравнительно высоко над плоскостью разреза. Кроме того мы показали, что в парафиновых срезах находятся и мертвые, и живые спермии, — даже в тех случаях, когда блоки разрезаются по направлению от не погружавшейся в эякулят поверхности мышцы, откуда возможность механического перенесения живчиков на срез хотя и не исключена, но по крайней мере ограничена. Этот взгляд подтверждается тем, что на целлоидиновых срезах живчики не встречаются ни в эпителии, ни в слизистой, ни в виде скоплений над уровнем среза.

Из наличия живчиков в мышечной и соединительной ткани на парафиновых и целлоидиновых срезах вытекает еще одна возможность искусственного, пассивного попадания живчиков в ткани: и механического, при разрезах свежей ткани перед фиксацией, и вместе со впитывающейся в ткань фиксационной жидкостью. Поэтому для следующего опыта мы избрали такой прием, который позволял бы исключить это побочное действие гистологической обработки. Мы исходили из следующего предположения: если живчики проникают в эпителий и в глубже расположенные мышечный и серозный слои яйцевода в результате активного движения, мы можем попытаться найти их там, не применяя перед взятием образцов каких бы то ни было вмешательств или реагентов. Результаты этого опыта: т. е. установленное нами отсутствие живчиков в этих слоях яйцевода у всех 3 кур, как и их отсутствие в просвете дистальной трети секреторной части яйцевода курицы, осемененной за 20 или 10 мин. до умерщвления, — доказывают искусственный характер, во-первых, попадания спермиев в мышечную и соединительную ткань на парафиновых и целлоидиновых срезах, а во-вторых, и наличия спермиев в парафиновых срезах проксимальных участков яйцевода и стромы яичника курицы, убитой через 10 мин. после осеменения, — так как за такой короткий срок живчики не способны проникнуть так высоко вдоль полового тракта курицы. Вероятно, здесь сыграли роль те же факторы, как и при модели опыта, когда мы фиксировали мышцу в формоле с прибавлением эякулята и когда в целлоидиновом срезе тоже были найдены живчики (рис. 8). Отрицательные результаты дала, наконец, и обработка in toto яйцеводов мышей и крыс (рис. 3), при которой исключается и вдавливание спермиев в срезы ножом, и их всасывание в межтканевые щели.

Чтобы проверить механизм пассивного внесения живчиков в ткани, мы поставили еще два модельных опыта (кроме упомянутого уже опыта фиксации в формоле с прибавлением эякулята). При первом опыте мы установили, что живчики проникают в ткани также in vitro (рис. 5), при втором же убедились, что практически так же ведут себя и мертвые спермии и даже кроличьи эритроциты

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(рис. 6, 7). Разница здесь только количественная: мертвых спермиев в срезах находится гораздо меньше, чем живых. Эта разница объясняется тем, что живчики, убитые формолом и впитавшиеся в межтканевые пространства при последующей обработке (главным образом, промывке), большей частью снова вымываются, тогда как живые спермии, подвергающиеся массовой коагуляции только под действием фиксирующей жидкости, не вымываются. Подтверждением этого мог бы служить и тот факт, что и эритроцитов из цитратной крови находится в тканях гораздо больше, чем убитых живчиков. Мы полагаем поэтому, что во всех случаях дело идет лишь о пассивном движении живчиков, — безразлично, живых или убитых. Наконец, косвенным доказательством не в пользу активного проникновения живчиков в ткани полового тракта самки in vivo является, быть может, и то, что в этих тканях не бывает целлюлярной реакции. Так как при подкожном введении спермиев такая реакция и фагоцитоз живчиков, действительно, наблюдаются (рис. 4), можно было бы ожидать, что аналогичная реакция наступит и в тканях полового тракта.

Данные Kohlbrugge (1910, 1911), находившего спермии в эпителии матки у различных животных, критиковал уже Sobotta (1911, 1920). Источник ошибок Kohlbrugge он видел в лейкоцитах, пикнотические ядра которых Kohlbrugge принимал за увеличенные головки спермиев. Однако главным недостатком работ Kohlbrugge и других цитируемых нами авторов была, как кажется, применявшаяся ими методика заливки в парафин. Таким образом, у Kohlbrugge, бесспорно, речь идет о живчиках, искусственно привнесенных в ткани.

Наши первые опыты с курами (Войтишкова 1955) с применением заливки в парафин привели нас к таким же выводам, какие сделали и остальные цитируемые авторы. К ряду несоответствий, которых никто из них не объяснил, принадлежит, напр., и то, что невозможно, чтобы спермии так быстро продвигались по яйцеводу и уже через 10 мин. после случки попадали в стенки полового тракта: движение живчиков продолжается обыкновенно несколько часов, прежде чем они доходят до яйца (2—4—8 часов у овец — Dauzier, Wintenberger 1952, Buisson, Dauzier 1955; 4 часа у кролика — Chang 1951). Kohlbrugge (1910, 1911) и Генин (1950, 1951, 1953, 1955) наблюдали, как и мы, в парафиновых срезах спермии, направляющиеся к поверхности эпителия или проникающие в эпителий. По поводу слов Генина, что в эпителии живчики встречаются редко, но зато в соединительной и мышечной ткани — в больших количествах, можно сказать только то, что различия в количестве живчиков, занесенных на срез из просвета ножом, и живчиков, впитавшихся в межтканевые щели, вполне понятны, как мы показали на модели опыта. Что касается утраты жгутиков, якобы наступающей при проникновении живчиков в ткани, следует заметить, что спермии со жгутиками и без них можно найти в просвете полового тракта самок очень скоро после случки.

Причину наблюдавшейся Гениным фрагментации живчиков в тканях (по его мнению, они якобы закономерно теряют ту часть головки, которая в момент проникновения направлена к эпителию) легко открыть в том, что фрагментация головок наступает уже в просвете, так что среди живчиков, занесенных в ткани из просвета, должны встречаться самые разнообразные обломки.

Результаты опытов Генина с проникновением живчиков в различные ткани in vitro это тоже артефакты, как мы уже показали.

«Доказательство» присутствия живчиков в стенках матки путем определения гиалуронидазы тоже основано на артефакте. Авторы (Ципер и Генин 1952) разрезали матку осемененных мышей на несколько частей, промывали их так долго, пока в последних двух фракциях жидкости для промывания уже не было живчиков. Но этим они не только не исключили возможности, что ряд живчиков

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приставал к эпителию, но, главным образом, механически вдавливали живчики в стенки матки при ее делении, а также делали возможным проникновение живчиков из жидкости для промывания в межтканевые щели. Само собой, так получался положительный диагноз гиалуронидазы в стенках матки.

Теперь остается вопрос, что происходит с неоплодотворяющими живчиками в половом тракте самки. Этого вопроса касается Sobotta (1920), который подчеркивает роль фагоцитирующих клеток в половом процессе и отмечает, что через несколько часов после случки у мышей наступает интенсивный лейкоцитоз и массы лейкоцитов продвигаются через эпителий матки в просвет, в котором находится эякулят. По мнению этого автора, лейкоциты у мышей фагоцитируют живчиков, однако этот фагоцитоз играет лишь незначительную роль при устранении живчиков, которые в большей своей части выделяются через влагалище.

Данные Sobotta о состоянии половых путей у мышей после случки подтверждают наблюдения Königstein-a (1908), который однако, в отличие от Sobotta, подчеркивает значение лейкоцитов для удаления живчиков из ̧матки, а также данные Попа и Марза (1929). Косвенное доказательство резорбции спермиев приводит также Moxter (цит. по Kohlbrugge 1910), иммунизировавший животных живчиками других животных. Когда он потом снова впрыскивал этим животным чужеродных живчиков в полость брюшины, живчики немедленно погибали. Waldstein и Ekler (1913) также приводят биологическое доказательство резорбции спермиев организмом самки. Они установили, что в крови кролика нормально отсутствуют ферменты, которые разрушают ткань testes, однако через несколько часов после случки в крови самок появляется фермент, который специфически действует на тестикулярную ткань. Способность крови разрушать testes сохраняется в течение всего периода беременности и вплоть до puerperium. Если же после случки беременность не наступает, положительная реакция крови через 2 недели угасает.

Таким образом, телегония, ксении и гетероспермия, которые на основании нахождения живчиков, проникших в соматические ткани полового тракта самок считались уже объясненными, так что оставалось только выяснить их механизм, — с точки зрения наших данных опять становятся неясными. Если они действительно существуют, то, повидимому, могут иметь только две причины: или влияние развивающегося плода на матку, или влияние резорбируемых живчиков на ее метаболизм. В своей следующей работе мы попытаемся решить этот вопрос.

Резюме

1. Спермии, попадающие при половом процессе в половые пути самки, не проникают в ее ткани, а повидимому, в большей своей части фагоцитируются. Мы не исследовали ни способа и течения фагоцитоза, ни судьбы фагоцитирующих элементов.

2. Живчики, которые встречаются в тканях половых путей самки, заносятся сюда искусственным путем.

3. Артефакты возникают при гистологической обработке тканей полового тракта, а именно:

а) механически, при разделении органов до фиксации на блоки требуемой величины;

б) путем впитывания спермиев вместе с фиксирующей и другими жидкостями, с которыми блок приходит в соприкосновение при гистологической обработке;

в) механически, при разрезании парафиновых блоков ножом микротома;

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г) намыванием живчиков из просвета при удалении парафина перед окраской срезов.

4. Возникающих описанными способами артефактов можно избежать только при обработке и заливке органов в целлоидин in toto.

(Табл. XXI, XXII, XXIII)

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The Question of the Participation of Non-fertilising Sperms in the Sexual Process

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Summary

The finding of sperms in paraffin sections of the genital tract of the female (Her-werden 1905, Kohlbrugge 1910, 1911, Genin 1950, 1951, 1952, 1953, 1955, Churý et al. 1954, Vojtíšková 1955) led to the conclusion that there is a causal relationship between the phenomena of telegony and xenia and also, partially, heterospermia (Sarkisjan 1952, Kušner 1954) and penetration of the sperms into the somatic tissues of the genital tract of the female.

These positive findings were subjected to re-examination by further methods and in a series of model experiments, the results of which indicate that our own positive findings and those of the other authors cited above are artefacts, which develop during the making of the histological preparations.

Experimental Section

IA. The genital tract of a hen, artificially inseminated (Seiden 1947, Orel 1949) five times, from 26 hours to 10 minutes before killing, was treated histologically, partly by embedding in paraffin, partly in celloidin.

B. In three hens, two of which were inseminated four times (four hours to 10 minutes) and one, twice (20 and 10 minutes) before killing, the following procedure was carried out: all the hens were operated on under urethane-barbital narcosis (10 g. urethane, 0.75 g. barbituric acid, 100 ml. H_2O), administered intraperitoneally, from two to three hours before the operation. After laparotomy, the loop of the oviduct was double ligatured in the secretory part. Ligaturing was carried out with double silk thread over a thick strip of gauze (fig.2), the first ligature being applied at site 1 and the second, joint, ligature at sites 2 and 3. On the second day after operation all three hens were artificially inseminated. After the hens had been killed, minute portions of tissue were taken from the surface of the uterus and the isthmus using fine scissors or fine hooked forceps, and crushed on a slide. The instruments were changed the making of each preparation. Finally, the lumen of the oviduct was opened and smears were made by applying a slide to the mucous membrane.

II. The genital tract of eight rats and one mouse, in which sperms were found in vaginal smears after coitus, were treated in toto (vagina, both horns of uterus and ovaries) by embedding in celloidin.

III A. 0.5 ml. of a washed suspension of sperms from a Leghorn cock in physiological saline were injected subcutaneously into four guinea fowl. The guinea fowl were killed after 1, 6, 4 and 48 hours, and the skin folds were examined histologically (paraffin sections).

B. Hen skeletal muscle was dipped for one third of its length into an ejaculate of living sperms and also into an ejaculate of sperms which had been killed with 8% formol. Part of the muscles were embedded in paraffin and part in celloidin.

C. After being steeped for two hours in citrated rabbit blood, hen muscle was treated by embedding in celloidin.

D. Hen muscle was normally fixed on 10% formol to which fresh ejaculate had been added (0.5 ml. ejaculate/100 ml. 10% formol) and treated further by embedding in celloidin.

Results and Discussion

The main difference between the picture in paraffin and celloidin sections of the genital tract of the inseminated hen is the absence of sperms in the epithelium and mucous membrane in the celloidin sections. In contrast to paraffin sections, no penetrating sperms were found in celloidin sections but only sperms adhering to the cilia. The findings of sperms in the muscles and connective tissue, however, are the same as those in paraffin sections (fig. 1). The finding of sperms in the epithelium and mucous membrane and of straightened sperms in the lumen near the surface of the

epithelium in paraffin sections can be explained by mechanical transfer by the microtome on cutting the sections. We also postulate however that further sperms can get on to the section by being washed out from the lumen on dissolving the paraffin before staining, since in preparations freshly mounted in balsam we often find freely moving sperms and also, relatively high above the level of the sections, there are massive aggregates of sperms. We further showed that there were dead and living sperms in paraffin sections, even when the blocks were cut from a muscle which had not been immersed in ejaculate, so that the possibility of a mechanical transfer on to the section was not actually excluded but was at least decreased. This is confirmed by findings in celloidin sections in which there were no sperms in the epithelium, or the mucous membrane or in aggregates above the level of the section.

The finding of sperms in muscle and connective tissue in paraffin and celloidin sections, however, indicates the further possibility of artefacts arising from the passive mechanical transfer of sperms into the tissue on separating the fresh tissue before fixing and also by sperms being drawn into the tissue with the fixing fluid. In a further experiment we therefore selected a method which would exclude all such effects of histological elaboration. Our reasoning was based in the following assumption: If the sperms make their way into the epithelium and the deeper layers of the oviduct, i. e. the musculature and the serosa, by active movement we should attempt to find them there without the use of any histological reagents before actually preparing the material. The result of this experiment, that is to say, absence of sperms in the layers of the oviduct mentioned above, in all three hens, and absence of sperms in the lumen of the distal third of the secretory part of the oviduct of a hen inseminated 20 and 10 minutes before killing, demonstrates both the artificial character of the finding of sperms in the musculature and connective tissue in the paraffin and celloidin sections and also that the finding of sperms in the proximal parts of the oviduct and in the stroma of the ovary of a hen killed ten minutes after insemination, which were treated by embedding in paraffin, is also an artefact since it is impossible for the sperms to travel so far up the genital tract of the hen in so short a time. Perhaps the same factors were active in this case in the model experiment in which the muscle was fixed in formol to which ejaculate had been added and in which sperms were also found in celloidin sections (fig. 8). There were also negative results with the elaboration of the oviducts of the mouse and rats in toto (fig. 3), in which the possibility of transfer of the sperms into sections by the microtome and the indrawing of sperms into the tissue spaces was excluded.

In order to confirm the mechanism of the passive transfer of sperms into the tissues, further model experiments were carried out (in addition to the experiment already desribed with fixing in formol to which ejaculate had been added). In the first experiment it was found that sperms also penetrate into the tissues in vitro (fig. 5). In the second it was found that dead sperms and even rabbit erythrocytes behave in very much the same way (fig. 6, 7). The differences are only quantitative — far fewer dead sperms are found in a section than living sperms. This difference is explained by the postulation that most of the sperms which have been killed by formol and drawn into the tissue spaces are washed out in the subsequent processes (chiefly washing), whereas the living sperms which are coagulated in masses by the fixing fluid are not washed out.This assumption is supported by the fact that far more erythrocytes from the citrated blood are found in the tissue than dead sperms. It is therefore assumed that in all cases the movement of the sperms is passive, and that it makes no difference whether they are alive or dead. Lastly, indirect evidence against the active penetration of sperms into the tissues of the genital tract of the female in vivo is provided by the fact that no cellular reaction takes place in these tissues. Since such a reaction actually does take place on the subcutaneous injection

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of sperms, which undergo slight phagocytosis (fig. 4) a similar reaction might have been expected, by analogy, in the tissues of the genital tract.

Kohlbrugge's results (1910, 1911) on the finding of sperms in the epithelium of the uterus in various animals were early subjected to criticism by Sobotta (1911, 1920), who thought that the source of all Kohlbrugge's errors was to be found in the leucocytes, the pyknotic nuclei of which Kohlbrugge apparently regarded as the thickened heads of sperms. It would appear, that the main weakness of Kohlbrugge's work and of the work of the other authors cited was the use of embedding in paraffin. In Kohlbrugge's case also, therefore, sperms had evidently been transferred into the tissues as artefacts.

The first experiments carried out by us in hens (Vojtíšková 1955) using embedding in paraffin, led us to the same conclusions as those of the other authors cited. One of the inconsistencies which were never adequately explained, is the fact that it is impossible for sperms to travel so rapidly up the oviduct as to penetrate the walls of the genital tract as early as 10 minutes after coitus; as a rule it takes several hours for the sperms to reach the ovum (2—4—8 hours in the sheep—Dauzier, Wintenberger 1952, Buisson, Dauzier 1955; 4 hours in the rabbit—Chang 1951).

Kohlbrugge (1910, 1911) and Genin (1950, 1951, 1952, 1953, 1955) also observed, as we did, in paraffin sections, sperms directed towards the surface of the epithelium or penetrating into it. With reference Genin's conclusion that the sperms are rarely observed in the epithelium but that they are found in large numbers in the connective tissue and muscle, it may be said that there is understandably a disproportion between the number of sperms which are introduced into the section from the lumen by the microtome and the number drawn into the tissue spaces, as shown in the model experiment. With reference to the loss of the flagellae, which is assumed to occur, on penetration of the sperms into the tissue, it must be objected that sperms with and without flagellae can be found in the lumen of the genital tract of the female shortly after coitus.

The cause of Genin's findings of fragmented sperms (according to him the sperms are bound to lose the part of the head which at the moment of penetration is directed to the epithelium), can easily be explained by the fact that fragmentation of the heads already takes place in the lumen and since the sperms in the tissue are sperms which have been forced in from the lumen, they will include all possible types of fragments.

The results of Genin's experiments on penetration of sperms into various tissues in vitro are also artefacts, as has already been demonstrated by us.

The "proof" of the presence of sperms in the wall of the uterus by a demonstration of hyaluronidase is also based on an artefact. The authors (Ciper and Genin 1952) sliced the uteri of inseminated mice into a number of parts and washed them until there were no sperms in the last two fractions of the washing solution. By doing this they not only failed to exclude the possibility of a number of sperms still adhering to the epithelium, but what is more, on slicing the uteri they transferred sperms mechanically into the walls and made it possible for the sperms to be drawn into the tissue spaces from the washing solution. The positive finding of hyaluronidase in the wall of the uterus was therefore a logical result.

There still remains the question as to what happens to non-fertilising sperms in the genital tract of the female. This question was touched on by Sobotta (1920) who emphasized the part played by phagocytic cells in the sexual process and stated that a few hours after coitus in mice intensive leucocytosis occurred and that masses of leucocytes made their way through the uterine epithelium into the lumen which contained the ejaculate. In mice, leucocytes do actually phagocytose sperms but

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according to Sobotta the participation of this phagocytosis in eliminating the sperms is not significant but that the latter are for the most part lost via the vagina.

Sobotta's results on the conditions in the genital tract in mice following coitus were later confirmed by the results of Königstein (1908), who, however, in contrast to Sobotta, laid great stress on the role of leucocytes in removing sperms from the uterus; Sobotta's results were also confirmed by Popa and Marza (1929). Indirect evidence on the absorption of sperms was also given by Moxter (cited by Kohlbrugge 1910) who immunised animals with the ejaculate of other animals. When these animals were injected intraperitoneally with foreign sperms, the latter immediately perished. Waldstein and Ekler (1913) also bring biological evidence of the absorption of ejaculate by the female organism. They found that no ferments capable of breaking down testicular tissue are normally present in female rabbit blood. A few hours after coitus, however, a ferment appears in the blood, which acts specifically on testicular tissue. The blood retains this ability throughout pregnancy and into the puerperium. If pregnancy does not occur after coitus the positive reaction of the blood is lost after 14 days.

Telegony, xenia and heterospermia which were already regarded as having been explained on the basis of findings of the penetration of sperms into the somatic tissues of the genital tract of the female, of which only the mechanism remained to be explained, are therefore, as far as our findings are concerned, once again obscure. If they actually do exist then they can only have two possible causes: the effect of the developing foetus on the mother or the effect of the absorption of sperms on the metabolism of the female. An attempt will be made to resolve these problems in further work.

Conclusions

1. Sperms which enter the genital tract of the female in the sexual process do not penetrate into the tissues but are to a great extent phagocytosed. The manner of action, the time relationship and the fate of the phagocytosing elements was not observed.

2. The sperms found in the tissues of the genital tract of the of the female are artefacts.

3. Artefacts develop during the histological treatment of the genital tract as follows:
   a) Mechanically, on slicing the organs into blocks of suitable size before fixing,
   b) By the indrawing of sperms together with the fixing fluid and others with which the block comes in contact in the course of histological elaboration,
   c) Mechanically, on making sections from paraffin blocks with a microtome,
   d) By the flooding out of sperms from the lumen on dissolving the paraffin before staining the sections.

4. The development of artefacts in the manner described above can be avoided only by treating the organs in toto and by embedding in celloidin, also in toto.

(Plates XXI, XXII, XXIII)

FOLIA BIOLOGICA

The Influence of Intra-embryonal and Repeated Post-embryonal Injections on the Formation of Heteroagglutinins

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A description was given, in an earlier communication, of depression of the immune response in hens, embryonal parabionts, which during embryogenesis exchanged their blood (Hašek 1953). This adaptation to a foreign antigen was termed immunological approximation. The degree of immunological approximation against the blood cells of other species is usually much weaker and sometimes no decrease in the ability to form antibodies is found. Hanan and Oyama (1954), however, succeeded in obtaining immunological areactivity, even to a distant antigen, by the repeated administration of equine albumin to newborn rabbits.

In the present communication the following questions are resolved: 1. whether immunological approximation can be evoked in ducks by an intra-embryonal injection of hen blood and 2. whether the same influencing of immunological areactivity described by Hanan and Oyama can also be evoked against blood cells of a distant species.

The experimental animals were divided into four groups: the first group was given only one intravenous injection during embryogenesis (tab. 1, series I), the second group was given one intravenous injection during embryogenesis and a series of post-embryonal injections after hatching (tab. 1, series II), the third group was given only a series of post-embryonal injections (tab. 1, series III) and the fourth group consisted of control animals which had received no injections prior to immunisation at the age of eight weeks (tab. 1, series IV). A further group contained ducks, which received a series of injections at adult age (tab. 2).

Methods

Embryonal Injections: 15-day-old duck embryos received an intravenous injection of 0.3 ml. of fresh citrated hen blood (1 part 3.8% sodium citrate to 9 parts blood).

Post-embryonal Injections: Ducks were injected from the first, second, third, etc. day after hatching (some on alternate days, others every fifth day), with fresh citrated hen blood from various donors, which was administered into the leg vein in doses of 5×0.3 ml. 5×0.4 ml., 5×0.5 ml. etc., for a period of four to eight weeks. Control birds of the same age were given injections for the first time at the age of eight weeks, when all the ducks were immunised with four doses of 1.5 ml. hen blood administered intravenously on alternate days. Blood was collected on the 5th and 10th day after the last injection. After a further 2—3 weeks the birds were reimmunised with a further four doses of 1.5 ml. hen blood administered intravenously on alternate days. In some birds, turkey blood was administered together with hen blood on reimmunisation (1.5 ml. + 1.5 ml. $4 \times$). Adult ducks received a series of injections of 1 ml. hen blood and four doses of 1.5 ml. administered intravenously on alternate days.

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Agglutination Tests: One drop of 2% suspension of erythrocytes, washed three times in physiological saline, was added to two drops of duck serum. The reaction was read off after 5, 30 and 60 minutes at room temperature. The titre given in the tables is the inverted value of the last dilution of the serum, in which discernible agglutination was found after 60 minutes.

Blood Transfusions: 80 ml. blood were collected from ducks and 100 ml. citrated blood were then injected slowly into the leg vein.

Results

1. Ducks injected intra-embryonally with hen blood formed immune hetero-agglutinins at the age of eight weeks in a titre actually insignificantly higher than the control birds (tab. 1, series I and IV). There was a significant difference between them after the first immunisation (t = 2.56, P < 0.02), but this was no longer found after reimmunisation (t = 1.42, P > 0.10). Immunological approximation was not, therefore, found in ducks as a result of a single intra-embryonal injection.

2. In ducks in which the repeated, prolonged injection of hen blood in the post-natal period was commenced shortly after hatching, a substantial decrease occurred in the formation of immune agglutinins. After the first immunisation series with hen blood, which was carried out at the age of exactly eight weeks, agglutinin titres were found both in ducks injected intra-embryonally and later in the post-natal period (tab. 1, series II) and also in ducks injected only in the post-natal period (tab. 1, series III), at the level of the natural heteroagglutinins found in the control ducks before immunisation (difference not demostrable, t = 0.821, P > 0.3). Although a protein medium (20% equine serum albumin) was used as a replacement for normal saline no differences were found in the titres. A comparison of these two groups of ducks — those injected post-natally and the control group—after the first immunisation (tab. 1, series IV), shows a statistically demonstrable difference (t = 6.29, P < 0.001). After reimmunisation the difference was still maintained. In all ducks in which the injections were commenced up to the fourth day after hatching, the titres remained at the level of natural hetero-agglutinins. In two ducks out of four which were given a series of post-embryonal injections, commencing on the fifth day after hatching, an increase in the titre was found following reimmunisation, which varied within the limits of the titres in the control birds.

3. In adult ducks (tab. 2), which were given a similar series of repeated injections over a long period, as in the newborn birds, no decrease was found in the formation of immune agglutinins as compared with the controls (t = 0.654, P > 0.51 < 0.7).

4. The influencing of reactivity appears to be specific for species, as in the case of immunological approximation between species. If the birds were immunised simultaneously with hen and turkey blood cells, the ducks which had been given injections over a long period normally formed immune heteroagglutinins against the turkey cells, but showed no increase in the titre of heteroagglutinins against the hen cells (tab. 3).

5. The ducks used in our experiments were developed to approximately the same degree. In evaluating the weight of all the two-month-old ducks used, no statistically demonstrable difference was found in the weight of the individual groups (series I—III) and the control group (series IV)—(I. t = 1.07, P = 0.30, II. t = 0.169, P > 0.80, III. t = 0.4, P = 0.7).

6. The transfusion of a large amount of hen blood (100 ml.) to ducks in which reactivity had been influenced (Nos. 373 and 389 from group II) led to a decrease in the titre of heteroagglutinins to zero immediately after the transfusion, and in birds injected over a long period then reached the original values. In the controls the titre

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Table 1.

Group:	No. of birds:	Day on which po-st- embr. injs. com-menced:	No. of injs., inter-vals and total amt. of blood admin. before immunisation	Titres before after imunisation at 8 weeks		Titres after reimm. at 12−13 weeks	Titres before after further reimmunisation at age	
I. Ducks inj. i. v. with 0.3 ml. hen blood in embryo-genesis (15th day)	11	—	—	1, 2, 2,* 2 1, 2, 1, 1, 2, 2, 1	64, 32, 16* 64, 8, 16, 64 32, 8, 8, 64	64, 32, 64,* 64, 64, 64, 64, 64, 32, 8, 64	1* 20 weeks	32*
II. Ducks inj. i. v. with 0.3 ml. hen blood in embryogen. and series of post-embr. inj. after hatching	1 6 8 2	1 2 3 4	22, alt. days, 8.2 ml. 22, alt. days, 8.2 ml. 22, alt. days, 8.2 ml. 11, ev. 5 days, 7.5 ml. 22, alt. days, 8,2 ml.	1 1, 2, 1, 2 2, 1 1, 2, 2, 2, 2 2,* 8, 2 1, 1	1 1, 2, 2, 1, 1 1 1, 2, 2, 2, 2, 1,* 4, 1 1, 2	1 1, 1, 2, 1, 1, 1 1, 2, 2, 2, 2 2,* 2, 2 1, 2	T 2(2) T 2(2) 27 weeks R 2* 33 weeks	2(2) 2(2) 8*
III. Ducks given series of post-embryonal injections	8 2 1 1 4	1 2 · 3 4 5	24, alt. days, 10.2 ml. 11, alt. days, 3.5 ml. 11, alt. days, 3.5 ml. 11, alt. days, 7.6 ml. 11, ev. 5 days, 7.5 ml. 11, alt. days, 3.5 ml.	1, 2, 2, 1, 1 2, 1, 2* 4, 8 4° 2* 2, 4, 0, 1°	1, 2, 1, 2, 2 2, 2, 2* 2, 1 0° 0* 4, 2, 2, 2°	1, 2, 1, 2, 2 1, 1, 1* 2, 1 2° 2* 32, 8, 2, 1°	R 2* 33 weeks 2° 20 weeks 2* 25 weeks 2° 20 weeks	2* 2° 2* 2°
IV. Control ducks	— 4	—	immun. with doses of 1.5 ml. at 33 weeks	2, 1, 1, 2, 1 1, 2, 0, 1,* 0' 2, 2, 1, 4, 2" 0, 1, 2, 2	16, 16, 8, 8 16, 8, 2, 8, 4* 8,' 1, 1, 2, 8 4" 2 64, 32, 256	32, 64, 32, 16 64, 32, 32, 8 32,* 16',2, 4 128, 64, 128" 64, 64, 128 4	2* 2' 2" 20 weeks	32* 32' 32"

The last column gives the titres before and after reimmunisation in the birds denoted by the various signs in the preceding columns. A T denotes that at the age of 22 weeks the ducks were given a transfusion of hen blood and that 40 days later (at 27 weeks) they were given four immunisation doses of hen blood. The figures denote the titres before and after this reimmunisation and those in brackets denote the titres before and after transfusion. An R denotes ducks which, after the second reimmunisation, were given further injections twice weekly, under the conditions of a long-term experiment. A reimmunisation series at the age of 33 weeks and collection of blood were carried out in the same way as in the other birds.

Table 3.

Group	Titre agt. hen blood cells		Titre agt. turkey blood cells	
	Before immun.	After immun.	Before immun.	After immun.
Injected ducks	2,2	2.2	1,1	128,16
Controls	1, 2, 0, 2,	32, 2, 64, 256	1, 1, 0, 1	64, 4, 64, 128

Table 2.

Group	No. of birds	Day on which injection comenced	No of inj., interval and total amt. of blood administered before immun.	Titres	
				natural hetero agglutinins	after imm. with 4 doses 0.5 ml. hen blood on alt. days
Ducks given series of injections in adult life	4	113 114 159 175	15, alt. days, 15 ml. 15, alt. days, 15 ml. 30, alt. days, 30 ml. 30, alt. days, 30 ml.	2 1 0 2	16 16 16 32
Control ducks, adult, selected at random	4	—	—	2 1 2 1	16 32 32 16

fell immediately after the transfusion from the value 1:1 to zero, rose again, however, to 1 : 8, again fell, and after immunisation five weeks after the transfusion again rose to 1 : 8, whereas in birds which had been receiving injections it did not change. Hen blood cells were not demonstrated in the blood of the ducks, by means of immune duck serum, a few hours after the transfusion.

7. In birds injected between the first and fourth day after hatching up to the age of four or eight weeks, the same degree of effect on reactivity was found in some as long as four months after completing the repeated post-embryonal injections (tab. 1). In birds given a transfusion of hen blood at the age of 22 weeks, there was no change in the titre of heteroagglutinins, even after reimmunisation at the age of 27 weeks.

Discussion

A single intra-embryonal injection of hen blood does not lead to a decrease in the formation of immune heteroagglutinins in eight-week-old ducks; in duck embryonal parabionts with hens, however, Hašek, Hraba et al. (1955) found a significant decrease in the formation of immune heteroagglutinins. These results corrrespond to findings made hitherto of a difference in the effectiveness of immunological approximation following embryonal parabiosis and a single embryonal injection.

On making a comparison of the influence of intra-embryonal injections of hen blood in ducks with the influence of the long-term administration of blood cells from other species in post-embryogenesis, a manifest difference is found. Ducks injected with

hen blood from birth do not form immune heteroagglutinins, after a series of immunisation doses at the age of eight weeks, in a titre higher than that of natural heteroagglutinins in ducks. This depression of antibody formation was found in several ducks still under observation at the age of 27 weeks and, in birds in which the injections had been continued, at the age of 33 weeks. Our findings following the injection of blood cells may be compared with the results of Hanan and Oyama (1954) in rabbits injected from birth, over a long period, with equine serum albumin, which later did not form antibodies against this antigen. In rabbits which were not given injections until after the age of 16 weeks, on the other hand, the authors found permanent active immunity after 12 weeks' administration of the antigen. Similarly, Dixon and Maurer (1955) found specific depression of antibody formation after the prolonged administration of large doses of heterologous plasma to rabbits from birth, which they term "immunological unresponsiveness" and which was maintained long after all the detectable proteins had disappeared (10—11 months). They also found protracted depression of antibody formation in adult rabbits following repeated injections of heterologous plasma, on administering the antigen to animals irradiated by X-ray. In six out of seven normal adult rabbits, however, they did not succeed in producing permanent inhibition of antibody formation by means of repeated injections. They take the view that in normal adult rabbits the development and duration of depression of antibody formation depends on the presence in the tissues of the antigen, which directly blocks the formation of antibodies. Johnson (1955) also studied the effect of a large amount of equine albumin on the immunity response in adult rabbits and in the discussion postulates that a high concentration of antigen results in complete non-synthesis of the antibodies. These findings correspond to those of Felton (1949), who inhibited immunity mechanisms in mice by large doses of antigenic pneumococcal polysaccharide. What he terms "immunological paralysis" is explained today by some authors as being due to the prolonged, persistent presence of non-metabolised antigen in the organism. Inhibition of antibody formation in adult animals, following on after a phase of increased antibody formation after the long-term administration of a specified amount of antigen, was also found by Šterzl (1956).

In our opinion, the mechanism by which areactivity develops in our ducks and also in the experiments of Hanan and Oyama and of Dixon and Maurer, following prolonged administration of the antigen immediataly after birth, is different from the inhibition evoked by large doses of antigen, from adaptation to repeated irritation on daily immunisation and from Felton's "immunological paralysis". In our view, it is rather a matter of adaptation of the organism to the antigen in the antenatal and immediate post-natal period (the "adaptive" period), i. e. at a time when the organism does not form antibodies and when immunological approximation can still be effected. Dixon and Maurer (1955) also assume in connection with their results in newborn rabbits, that large doses of antigen entered the body before the rabbit acquired the capacity of producing a defence reaction. In our experiments, prolonged depression of antibody formation was produced against blood cells, as compared with the results hitherto, which were obtained against dissolved proteins. After injection, the hen blood cells were always soon destroyed. Even after a transfusion of 100 ml. of hen blood, no hen blood cells were found in the blood of non-reactive ducks after only a few hours. According to the results obtained hitherto, as our experiment was carried out, the amount of antigen administered (3.5—10 ml.), the number of doses (11 or 24), the period of administration of the injections (four or eight weeks) and the intervals between the injections had no decisive influence (tab. 1). It is, however, conspicuous that, although in 29 ducks which were given repeated injections from the fourth day after hatching the titre after reimmunisation was not

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higher than 1 : 2, in four ducks given injections from the fifth day, the titre after reimmunisation in two of these was the same as in the controls. The first few injections were probably given still in the adaptive period, which in ducks is to some extent carried over into post-foetal life, as regards tissue antigens also, as shown by an analysis using skin homografts (Hraba, Hašek 1956). The relatively strong effect of the repeated administration of blood as compared with a single intra-embryonal injection may therefore be due to the larger amount of blood which is administered in a number of injections. The further injections may intensify immunological approximation.

Summary

Intra-embryonal and post-embryonal injections of hen blood were administered in ducks. Specific suppression of antibody formation against foreign blood cells was found after repeated injections of hen blood in ducks, commencing from the first to fourth day after hatching, which still persisted four months after completing the series of injections. After a single intra-embryonal injection of hen blood, no effect on antibody formation in the ducks was found in post-foetal life. In our opinion, this is an example of adaptation of the organism to the antigen in the adaptive period, when the organism does not form antibodies and immunological approximation can still take effect.

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Влияние интраэмбриональных и многократных постэмбриональных впрыскиваний крови на образование гетероагглютининов

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Резюме

В своей работе мы решали вопрос, может ли однократное внутризародышевое или многократное постэмбриональное внутривенное введение куриной крови повлиять на нормальное образование гетероагглютининов у уток. Мы установили, что: 1. после внутризародышевого введения 0,3 мл крови иммунные гетероагглютинины у утят в возрасте 8 недель образовались даже в несколько более высоком титре, чем в контроле. После реиммунизации мы уже не наблюдали разницы (табл. 1); 2. многократные длительные впрыскивания куриной крови утятам, начиная с 1—4-го дня после выклевывания (11—22 по 0,3—0,6 мл через день или на каждый 5-ый день), подавляли образование антител так, что после иммунизации в возрасте 8 недель титр гетероагглютининов у этих утят был не выше, чем бывает титр естественных гетероагглютининов. У 2 из 4 уток, у которых серия уколов была начата на 5-ый день после выклевывания, после реиммунизации титр агглютининов поднялся до уровня титра иммунных агглютининов в контроле (табл. 1). 3. У взрослых уток после длительных выскиваний таких же количеств не наблюдалось снижения образования иммунных агглютининов в сравнении с контролем (табл. 2). 4. Против эритроцитов индюка подопытные утки образовали гетероагглютинины в нормальном титре (табл. 3). 5. После однократного введения подопытным уткам 100 мл куриной крови мы через несколько часов не могли отметить присутствия куриных эритроцитов. 6. Наблюдения над подопытными особями показали, что специфическое угнетение образования антител у них сохраняется еще через 4 мес. после окончания серии уколов.

В дискуссии мы сравниваем различные типы торможения образования антител с полученными нами результатами. Несмотря на то, что однократное внутризародышевое введение крови другого вида не вызывало иммунологического сближения, мы полагаем, что угнетение образования антител после серии постэмбриональных инъекций связано с адаптацией организма на антиген в адаптивный период, т. е. в период, когда организм не способен еще к образованию антител, но еще возможно иммунологическое сближение. Серия уколов, из которых один или несколько относилось еще к адаптивному периоду, оказывала более значительное действие.

Influencing the Transplantability of the Virus of Rous Sarcoma by Immunological Approximation in Turkeys

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The transfer of tumours from one species to another is usually not successful. As in the case of the transfer of normal tissues, the cause of this is transplantation immunity. Various authors (Greene 1955, Ahlström and Bergmann, 1954, Shrigley et al. 1945, and others) have been successful in transferring tumours into the anterior chamber of the eye and into the brain of phylogenetically distant species, but the success of this heterotransplantation is explained by the special anatomical relationships in the brain and the anterior chamber of the eye.

For overcoming transplantation immunity to tumorous tissue in a heterotransplant transfer, methods of influencing the recipient were used (e. g. the "enhancing effect", elicited by means of lyophilised tissues — Kallis and Borges 1952, Snell 1954). Some findings in tumour transplantation are also closely associated with immunological approximation. Gross (1950a, b) found that a leukaemic tumour can be transferred from the strain A_k to newborn mice of the strain C_3H or C_{57}, although it does not grow in adult mice of these strains. Following the inoculation of one-day-old rats with a homogenate of the liver of leukaemic mice of the strain C_{57}, Říman (1956, not published) observed atypical leukaemoid changes associated with deformities in the skeleton and the finding of a tumour in the abdominal cavity. These findings are a direct demonstration that immunological approximation occurred during the adaptive period. They are at the same time an example of elicitation of immunological tolerance by means of malignant cells.

Many authors have also studied heterotransplants of virus tumours. In 1932, des Ligneris described the positive transfer of a homogenate of Rous sarcoma from a hen to a turkey; he also found that in this case filtered preparations are ineffective. Duran-Reynals (1943) made a more detailed analysis and supplemented the findings of the preceding author by producing tumours in turkeys up to three weeks old by means of injections of a non-cellular extract of Rous sarcoma.

Immunological approximation results in specific depression of the ability of the recipient to make an immunological response. A high degree of immunological tolerance to foreign cells, however, is found only on using intra-embryonal administration of the iso-antigen. The degree of immunological tolerance to hetero-antigens is much lower (Hašek, Hraba 1955).

Up to now, a relatively high degree of suppression of immunological response has been found only after embryonal parabiosis between turkey and hen (Hraba 1956). In parabiosis, however, a large amount of foreign blood is transmitted to the embryo

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over a long period and re-population of the foreign cells can take place, thus increasing still further the amount of the cellular stimulus. The effects of immunological approximation found after the administration of a single injection of heterologous blood are far smaller (Simonsen 1955, Hraba et al., 1956, Hašek 1956).

Immunological tolerance, which was originally studied in relation to normal cells (Billingham, Brent, Medawar 1953, Hašek 1953, Ripley 1953) has also been used in the study of the transplantability of malignant cells (Bollag 1955, Grozdanovič 1956, Koprowski 1955, Simonsen 1955). Bollag found that in rats injected during embryonal life with normal mouse cells, resistance to Crocker mouse sarcoma was lowered in post-embryonic life. Similar results were obtained by Grozdanovič in rats injected with a homogenate of normal tissue on the first day after birth. Koprowski found that on evoking immunological approximation by means of normal cells of the strain C_3H, it is possible to abolish resistance of the strain ICR to ascitic tumour, which normally grows on mice of the strain C_3H. A tumour developing on a tolerant mouse of the ICR strain actually undergoes antigenic transformation and can even be transplanted onto a normal adult animal of the ICR strain. Simonsen showed that turkeys injected with hen blood during embryogenesis are sensitive to the agent of Rous sarcoma. He found that turkeys which had immunological tolerance to hen blood, developed tumours visible even at the age of eight weeks, after being inoculated with a non-cellular extract of Rous sarcoma.

In the present work, immunological approximation was obtained, as by Simonsen, by means of a single injection of hen blood into turkey embryos. Some of the birds were also given repeated injections of hen blood in post-embryonic life. Hašková and Pokorná (1956) found that in ducks which had undergone prolonged injection with hen blood from the commencement of post-embryonic life, there was very marked suppression of the immunite response to hen blood cells. This method was also used in our own experiments.

A hen skin transplant was made in some of the birds, the formation of immune hetero-agglutinins was determined in another group and all the birds were inoculated with the virus of Rous sarcoma.

Material and Methods

The turkeys were injected with the blood of adult hens. Turkey embryos were injected intravenously on the 15th day of incubation, 0.3 ml. of citrated blood being injected with a number 20 needle into the allantochorionic vein through the shell membrane, which had been made transparent with paraffin oil. The details of this method have been given in a previous communication (Hašek, Lengerová, Maternová 1955).

In newly-hatched turkeys, amounts of 0.4 ml. citrated blood were injected into the wing vein. Further injections into the wing vein were administered at intervals of two to three days. The dose of blood was increased progressively and by the end of three weeks was 1 ml. This amount was administered up to three to four days before inoculation with the Rous virus.

Each series of experiments included control birds of the same age as the experimental birds. The experimental birds were given either a single intra-embryonal injection of hen blood, or an intra-embryonal injection followed by a series of injections in post-embryonic development, while a further group received only a series of injections after hatching.

In the first group of turkeys, the hen skin transplant was made at the age of 17 days, the donor being a chick aged 28 days. The skin grafts were fixed to the back of the turkeys with collodion, without suturing. Details on the method of transplantation were given in an earlier communication (Hašek 1954).

In the fifth series of experiments, immunisation was carried out at the age of 90 days with a single dose of 1 ml. citrated hen blood, administered intravenously. Blood was collected on the 6th, 8th, 10th, 13th and 15th day after immunization and the titre of agglutinins against the donor's erythrocytes was determined. For the agglutination reactions, citrated plasma was used (1 part 3.8% sodium citrate to

9 parts blood), which had been frozen before use at −13° C. In the test-tubes, one drop of a 2.5% suspension of erythrocytes in physiological saline was added to two drops of plasma. Agglutination was read off after 15 minutes and two hours at room temperature.

The tumour used in preparing the inoculum for the first three series was taken from a 6-months-old Leghorn hen, which had been inoculated in the breast. It had appeared 10 days after inoculation and was collected after 22 days' growth. After removal, the tumour was stored at −13° C. The birds in the fourth and fifth series were injected with an inoculum prepared from a further passage on a Leghorn cockerel. The tumour was removed after 20 days' growth.

Preparation of non-cellular extract: The tumour was homogenized for 20 minutes in a mortar and diluted with physiological saline in the proportion of 1 : 5. This homogenate was then centrifuged for 20 minutes at 3,000 RPM in silicon test-tubes. After transferring the supernatant fluid by pipette into new silicon test-tubes, centrifugation was repeated under the same conditions. The supernatant fluid was then used for inoculation. In the second series, silicon test-tubes were only in the second centriufgation. In the third series silicon glass was not used at all.

Siliconisation was carried out with a 2.5% solution of methylsiloxane of Czechoslovak production in distilled benzene. Clean test-tubes were dipped into this solution and after they were removed the fluid was allowed to drip off, thus giving an even coating. The film was fixed for two hours at 250° C.

All the birds in series 2, 3, 4 and 5 were given intramuscular injections of 1 cc. of the extract in both legs. In the first series half of this quantity was used.

Evaluation of the tumours: The experimental birds were killed five weeks after inoculation, those in the fourth and fifth series being killed eight weeks after inoculation. All the birds were postmortemed, including those which died during this period. The tumour was found in its typical form as a palpable new growth at the sites of inoculation, with metastases in the liver, spleen and lungs.

Metastases were evaluated as positive when they were discernible as whitish, compact nodes which frequently projected above the surface of the organ and penetrated deep into the parenchyma. In some cases the material was examined histologically and evaluated. In the microscopic preparations, typical tumour cells were observed fusiform and also polymorphous in character.

Results

The results are given in tab. 1 (experimental series 1—5).

Skin transplants: In the first experimental series, these survived for almost the same period as in the controls; only in bird No. 8/401 did the transplant survive longer (15 days).

Table 1. Table of Results

RL: denotes whether tumour developed at site of injection on right leg. LL: denotes same for left leg. ML: presence of metastases in liver. MS: presence of metastases in spleen. MP: presence of metastases in lungs.

a = lung metastases not tested in this series
b = metastasis almost completely destroyed liver paremchyma and prevented the organ from functioning
c = solitary metastasis on one lobe of liver; area about 1 cm., penetrating maximally 0.5 cm. into organ
d = tumours appeared at sites of injection in controls 8/917, 8/915, 8/762, but later regressed. Size of tumour in control 8/460 also decreased before the bird was killed

Series 1

No. of bird	Hatched out	Method	Age at time of skin transpl.	Survival of transp. (days)	Age on inoculation (days)	No of days fr. inoc. to death	RL	LL	ML	MS	MP
8/596	30. 8. 55	embr. inj.	17	8	46	—	—	—	—	—	a
8/401	30. 8. 55	postem. inj.	17	15	46	28	+	+	+	+	
8/462	30. 8. 55	postem. inj.	17	8	46	34	+	—	+	+	
8/403	30. 8. 55	postem. inj.	17	9	46	30	—	+	+	+	
8/405	30. 8. 55	contr.	17	8	46	—	—	—	—	—	
8/406	30. 8. 55	contr.	17	8	46	—	—	—	—	—	
8/407	30. 8. 55	contr.	17	7	46	—	—	—	—	—	

Series 2

No. of bird	Hatched out	Method	Age on inoculation (days)	No. of days fr. inoc. to death	RL	LL	ML	MS	MP
8/410	5. 9. 55	embr. postem. inj.	58	24	+	+	+	+	+
8/412	5. 9. 55	,,	57	25	+	+	+	+	+
8/415	5. 9. 55	,,	57	26	+	+	+	+	+
8/418	5. 9. 55	embr. inj.	58	24	+	+	+	+	+
8/425	5. 9. 55	,,	58	19	+	+	+	+	+
8/423	5. 9. 55	,,	58	34	+	+	+	−	+
8/417	5. 9. 55	,,	57	24	+	+	+	+	+
8/404	30. 8. 55	po tembr. inj.	64	29	+	+	+	+	+
8/409	30. 8. 55	,,	64	24	+	+	+	−	+
8/416	5. 9. 55	control	58	−	−	+	−	−	−
8/422	5. 9. 55	,,	57	−	−	−	−	−	−
8/419	5. 9. 55	,,	58	−	−	−	−	−	−
8/411	5. 9. 55	,,	57	−	−	−	−	−	−

Series 3

No. of bird	Hatched out	Method	Age on inoculation (days)	No. of days fr. inocul. to death	RL	LL	ML	MS	MP
8/475	13. 9. 55	embr. inj.	55	24	+	+	+	+	+
8/477	13. 9. 55	,,	55	30	+	+	+	+	+
8/451	13. 9. 55	,,	55	24	+	+	+	+	+
8/460	13. 9. 55	control	55	−	−	+d	−	−	−
8/462	13. 9. 55	,,	55	−	−	−	−	−	−
8/453	13. 9. 55	,,	55	−	−	−	−	−	−
8/449	13. 9. 55	,,	55	−	−	−	−	−	−

Series 4

No of bird	Hatched out	Method	Age on inoculation (days)	No. of days fr. inocul. to death	RL	LL	ML	MS	MP
8/841	20. 9. 55	po tembr. inj.	126	24	+	+	+	−	+
8/842	20. 9. 55	,,	126	24	+	+	+	+	+
8/840	20. 9. 55	,,	126	16	−	−	+b	−	−
8/839	20. 9. 55	,,	126	−	−	−	+	−	+
8/813	28. 9. 55	embr,+ postem. inj.	119	26	+	+	+	+	+
8/915	20. 9. 55	control	126	−	d	−	−	−	−
8/917	20. 9. 55	,,	126	−	d	−	−	−	−
900	28. 9. 55	,,	119	−	−	−	−	−	−
8/762	28. 9. 55	,,	119	−	d	−	−	−	−

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Series 5

No. of bird	Hatched out	Method	Titre of agglut.		Age on inocul. (days)	No. of days fr. inocul. to death	RL	LL	ML	MS	MP
			Before immuu.	After immun. *							
8/719	28. 9. 55	emb. inj.	—	32	118	22	+	+	+	+	+
8/865	28. 9. 55	,,	2	(4	118	24	+	+	+	+	+
8/898	28. 9. 55	control	1	16	118	—	—	—	—	—	—
772	28. 9. 55	,,	—	16	118	—	—	—	+c	—	—
780	28. 9. 55	,,	—	16	118	—	—	—	—	—	—
782	28. 9. 55	,,	—	32	118	—	—	—	—	—	—

* with 1 ml. hen blood at age of 90 days

In the fifth series, the formation of immune heteroagglutinins was the same in the inoculated birds as in the controls. The titre of agglutinins given in the table was determined after collecting blood on the eighth day after immunisation.

After inoculation with the virus, very definite results were obtained. All the experimental birds, with the exception of the controls and birds Nos. 8/596 and 8/839, died on an average 25.4 days after inoculation with demonstrable tumours and metastases. The birds which received intra-embryonal injections died on an average in 22.8 days, birds which were given repeated post-embryonal injections in 23.2 days and birds which were given injections intra- and post-embryonally in 25.2 days.

Discussion

The results obtained with immunological approximation to normal tissue antigens are in agreement with previous findings. If hen blood cells which are injected in amounts of 0.3 ml. into the blood stream of the turkey embryo take effect on it, by the age of 90 days no decrease is found in the ability to respond to an injection of hen blood by the formation of antibodies. Simonsen (1955) found reduced ability to form agglutinins in turkeys injected intra-embryonally with hen blood, but this was in birds immunised at the age of six weeks.

The survival of skin transplants in the turkeys of the first series was also noticeably prolonged only in turkey No. 8/401. The transplants were already made, however, on the 17th day after hatching. The degree of immunological tolerance in turkeys injected with hen blood is therefore very low.

Very convincing results, however, were obtained in ascertaining susceptibility to the Rous sarcoma virus in turkeys in which immunological approximation had been produced. In three birds inoculated with tumour extract at the age of eight weeks, Simonsen obtained tumours in two, but these did not cause the death of the birds, even after five weeks. The results obtained by us were more definite. We explain the difference in the results mainly by the fact that in our case the initial tumour grew on a Leghorn hen and not on a Plymouth Rock, which is the original host of the virus of Rous sarcoma. Simonsen (1955) does not state which breed he used, but he refers to Duran-Reynals (1943), who used only Plymouth Rocks. A similar example of increased affinity of the Rous virus, which was not cultured on the original host (Plymouths) for birds phylogenetically still more distant, is given by Carr (1953). This author writes that the virus never produces tumours in pigeons

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when cultured in hens, but that the duck variant of the virus produces the tumour in these birds.

In the turkeys given repeated injections from the day of hatching we also found the same effective increase in susceptibility to the virus as after intra-embryonic injections. Hanan and Oyama (1954), Dixon and Maurer (1955) and Cinader and Dubert (1955) were successful in influencing antibody response to proteins by means of repeated injections from the day of birth. Hašková and Pokorná (1956) obtained a similar result in ducks against heterologous erythrocytes. Increased susceptibility to Rous sarcoma was also found in all turkeys which had received only postembryonic injections, in spite of the fact that in turkeys, in contrast to ducks, the duration of the adaptive period is approximately the same as in hens (Hraba, Hašek 1956).

There is, however, another possibility to be considered: In the birds injected in post-embryonic life we cannot exclude the possibility that the hen blood elements, particularly the monocytes, which were injected into the blood stream of the turkey, may have been infected by the injected virus and continued to act as a cellular inoculum, which always gives a positive result on transplantation. A transfer of the virus by the monocytes alone from a bird with the tumour to a healthy bird was already described by Haagen (1928, cited by Mauer 1938).

Interesting results were obtained on comparing the survival of a heterotransplant of normal hen skin tissue and the growth of the Rous sarcoma on turkeys in which immunological approximation had been produced by the injection of hen blood. In the first experimental series, we observed the degree of immunological tolerance in these birds, first by means of a skin heterotransplant and then by inoculation with the virus of Rous sarcoma. Turkey No. 8/401, on which the heterotransplant survived longest (15 days), also produced large tumours on both legs at the site of the injection, after inoculation with the virus, whereas a further two post-embryonic injections produced only small tumours on one of the legs. The birds died, therefore, chiefly as a result of metastatic proliferation. The manner in which the tumour grew on turkey No. 8/401 resembled more a typical hen tumour, since large tumours were produced directly in the muscular tissue of the turkey.

In the fifth experimental series, it was found that the injected birds formed immune agglutinins as well as the controls. In spite of the fact that a high titre of agglutinins was found in all birds, those in which immunological approximation had been produced by an intra-embryonal injection of blood died, in contrast to the controls, 22 to 24 days after being inoculated with the virus.

The acquired susceptibility of turkeys to the virus of Rous sarcoma is most probably the result of immunological tolerance to the normal hen blood cells. The finding of immunological approximation to Rous sarcoma is therefore in agreement with the view that the virus contains a component of "hen species specifity".

The finding of the normal formation of immune agglutinins in the treated turkeys of the fifth series and fully preserved susceptibility to the Rous virus can be explained by the postulation that the "hen" antigenic component of the virus does not correspond to the erythrocytic antigens, but to the antigens of the tissue cells responsible for transplantation immunity in transfers of other tissues. Attention has already been drawn to this antigenic difference or inequality between the erythrocytes and antigens of the cells of the skin transplant (Hašek, Lengerová, Maternová 1955, Billingham, Brent, Medawar 1953, Hašek, Hraba, Esslová 1956). It is therefore possible that tolerance for erythrocytic antigens could no longer be found, but that tolerance for leucocytes was preserved, but this was not tested in our experiment. There may also be a quantitative difference between the susceptibility of normal and tumor cells to a transplantation immunity reaction. The tumour can

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evidently grow and destroy the host as a result of the greater growth aggressiveness of the tumorous cells, even in the presence of slight immunity to the tumour. This is borne out by the large series of results in which transfers of tumor cells were far more successful than the transfer of normal tissues. It may therefore be assumed that the testing of immunological tolerance by means of tumor cells is a more sensitive method than by means of normal cells.

Finally, in an interpretation of acquired susceptibility in turkeys, one should bear in mind the possibility that there may be a decrease in the formation of anti-bodies against the antigen of the virus of Rous sarcoma possibly present in the hen blood used for the injections, in sub-infeetive amounts, or another virus may be present which is immunologically identical with or related to the virus of Rous sarcoma. This last problem was resolved in normal hens by Duran-Reynals (1953) for the virus RPL 12; this author found that it was not the formation of antibodies against this virus, but actually its presence, which had an inhibitory effect on the Rous virus, i. e. that in this case there was antagonism between the two viruses. The possibility of producing immunological approximation to a virus was also previously demonstrated by the results obtained by Traub (1936, 1938, 1939), who found that the embryos of mice which are infected with the virus of lymphocytic choriomeningitis in utero, suffer from an atypical form of this disease in post-embryogenesis, without the formation of antibodies. Similar results have been obtained more recently by Komrower, Williams and Stones (1955).

(Plates XXX, XXXI)

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Воздействие иммунологического сближения на передаваемость вируса саркомы Rous-a у индеек

Я. СВОБОДА и М. ГАШЕК

Резюме

Мы добивались у индеек иммунологического сближения по отношению к куриным антигенам, в результате которого наступает полное подавление или ослабление иммунной реакции против применявшихся для сближения антигенов, — во-первых, с помощью инъекций 0,3 мл цитратной куриной крови в аллантохорион зародышей индейки, а во-вторых, путем внутривенных впрыскиваний крови индюшатам, начиная с момента выклевывания вплоть до прививки, с 3-дневными интервалами. Бесклеточный экстракт гомогенизированной опухолевой ткани саркомы Rous-a для прививки в икроножную мышцу приготовлялся путем ее разведения физиологическим раствором 1 : 5 и повторного центрифугирования в течение 20 мин. при 3000 об/мин. Для опыта брались индюшата в возрасте 6—18 недель. У контрольных в большинстве случаев после прививки вируса не наблюдалось никаких макроскопически заметных изменений, в небольшом количестве случаев нам удавалось наблюдать доброкачественное заболевание регрессирующего характера. Из 23 сближавшихся индюшат у всех, кроме двух, заболевание протекало злокачественно, и все они погибли в среднем через 25,4 дня с выразительными опухолями и метастазами в легких, печени и селезенке. Индюшата, получавшие внутризародышевые уколы, погибали в среднем через 22,8 дня, при повторных постэмбриональных инъекциях — через 23,2 дня, а при эмбриональных и постэмбриональных — через 25,2 дня.

Повышение восприимчивости индеек по отношению к вирусу саркомы Rous-a, по всей вероятности, наступает как реакция иммунологического сближения на антигены куриной крови, применявшейся во всех случаях для сближения. Это подтверждается и наблюдением, что вирус саркомы Rous-a содержит куриный антиген. Наконец, при постэмбриональных впрыскиваниях не следует забывать и о возможности прямой инфекции впрыскнутых в кровяное русло индейки элементов куриной крови, выступающих потом в качестве клеточного inoculum, которое губительно действует и на нормальных животных. Мы принимали во внимание и возможность нахождения в применявшейся для уколов куриной крови субъинфекционных количеств вируса саркомы Rous-a или другого иммунологически родственного вируса. Таким образом, сближение могло бы осуществляться и непосредственно в ответ на вирусный антиген. В последней серии опытов мы перед прививкой вируса иммунизировали индеек куриными эритроцитами и убедились, что у сближавшихся особей иммунные агглютинины образуются в таком же титре, как в контроле. Тем не менее сближавшиеся животные после прививки вируса погибали в отличие от контрольных через 22 и 24 дня.

(Таб. XXX, XXXI)

M. Розенберг: Фаголизис клеток стафилококка.　　　　　　Табл. XVII.
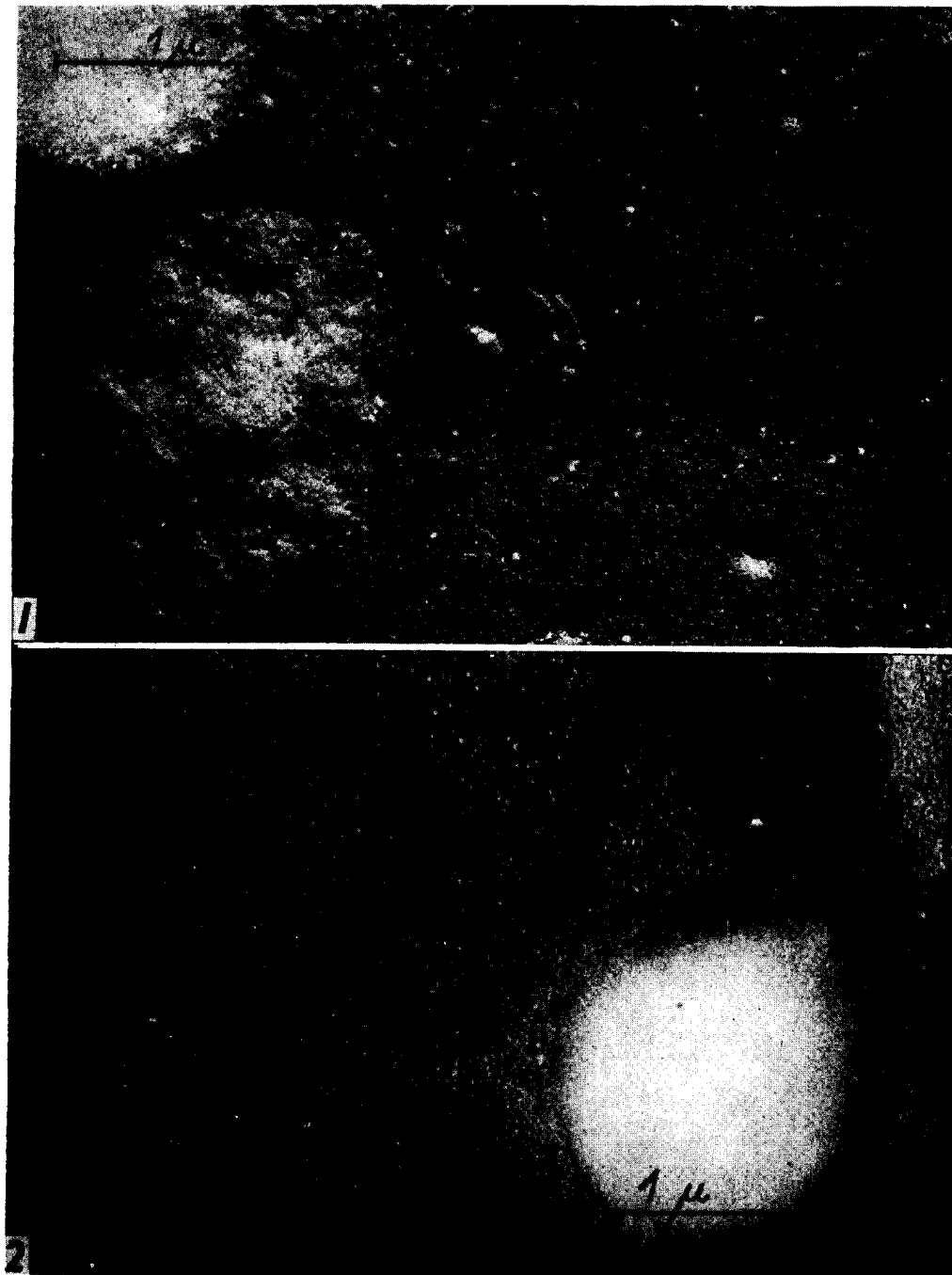
Рис. 1, 2.

M. Розенберг: Фаголизис клеток стафилококка.   Табл. XVIII.
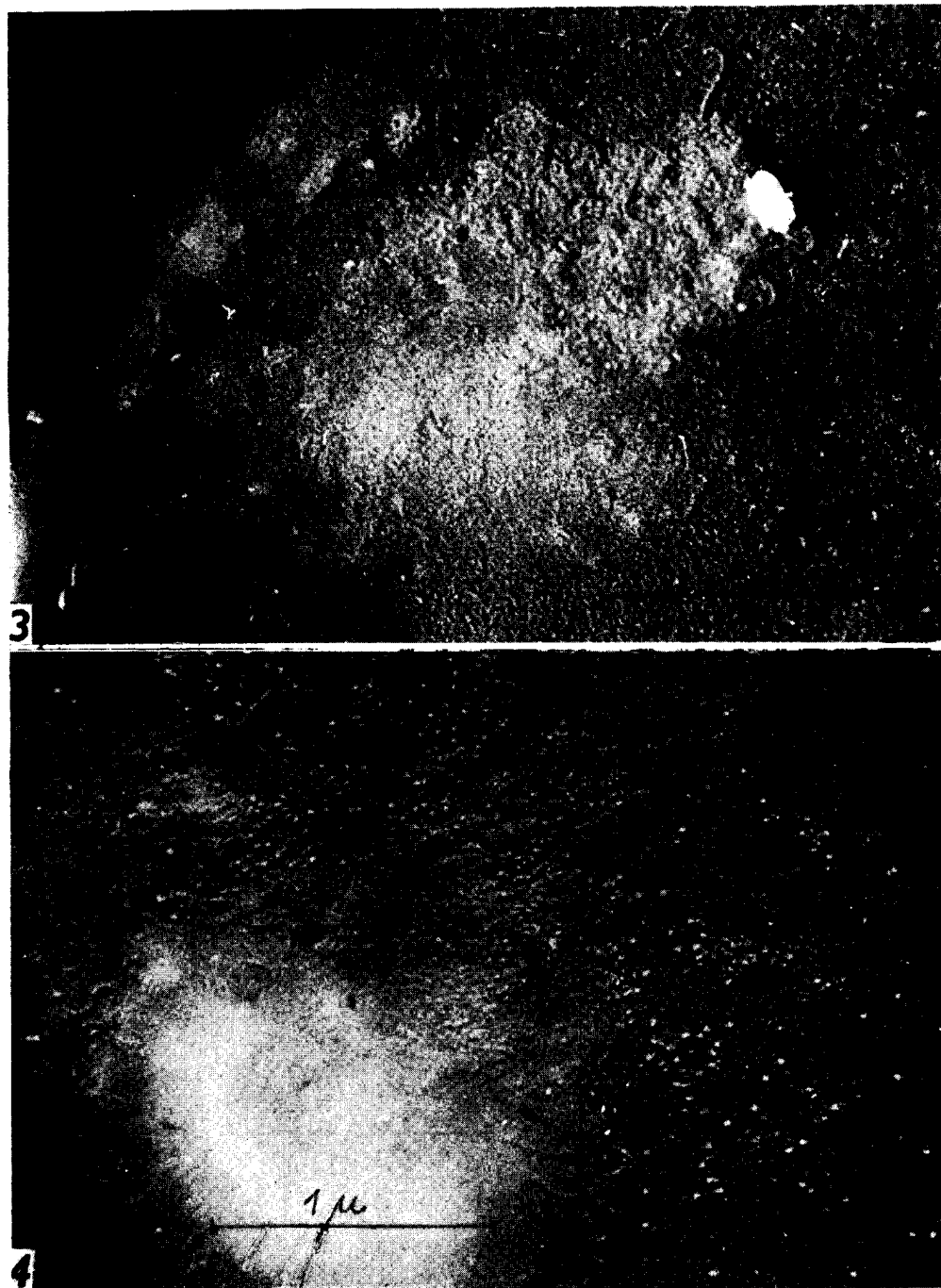
Рис. 3, 4.

М. Розенберг: Динамика распада лизогенных клеток под действием
ультрафиолетовых лучей.

Табл. XIX.
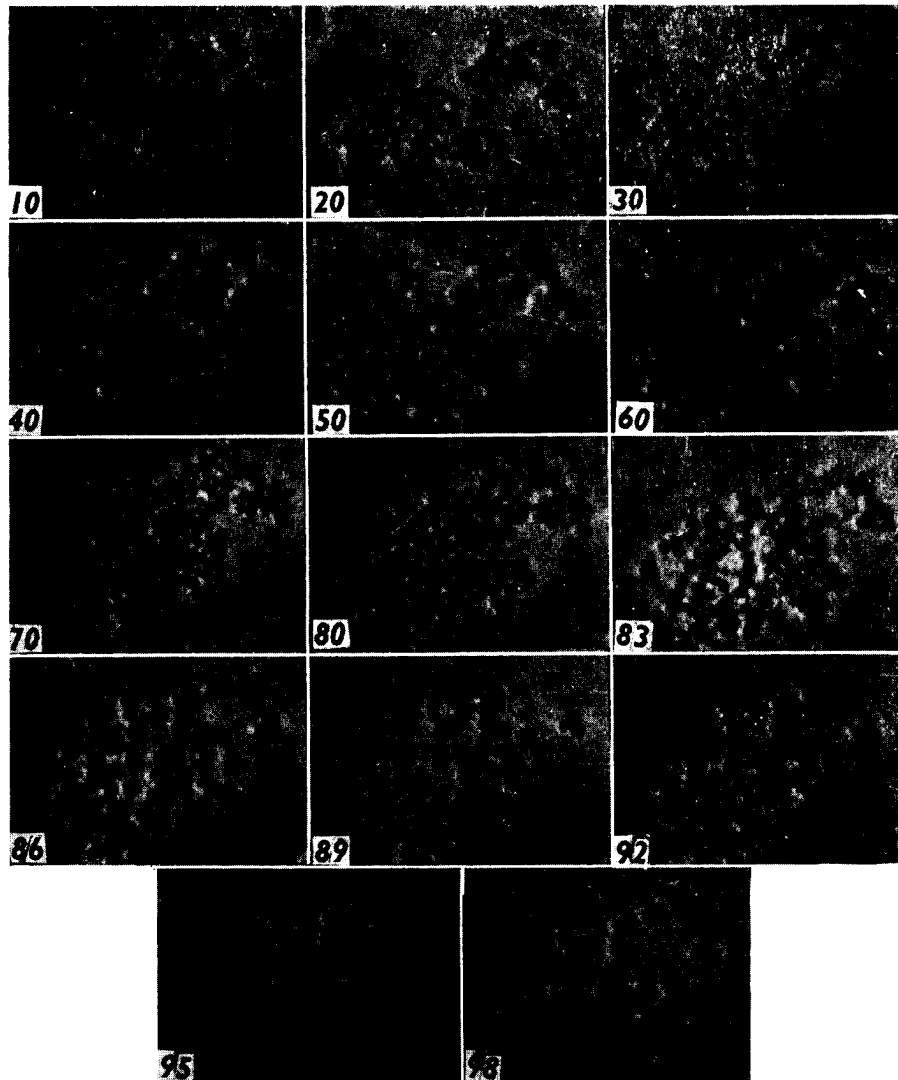
Рис. 3. Распад чувствительного штамма S 3 под действием бактериофага, выделяемого лизо-
генным штаммом LS 2.

M. Розенберг: Динамика распада лизогенных клеток под действием ультрафиолетовых лучей.
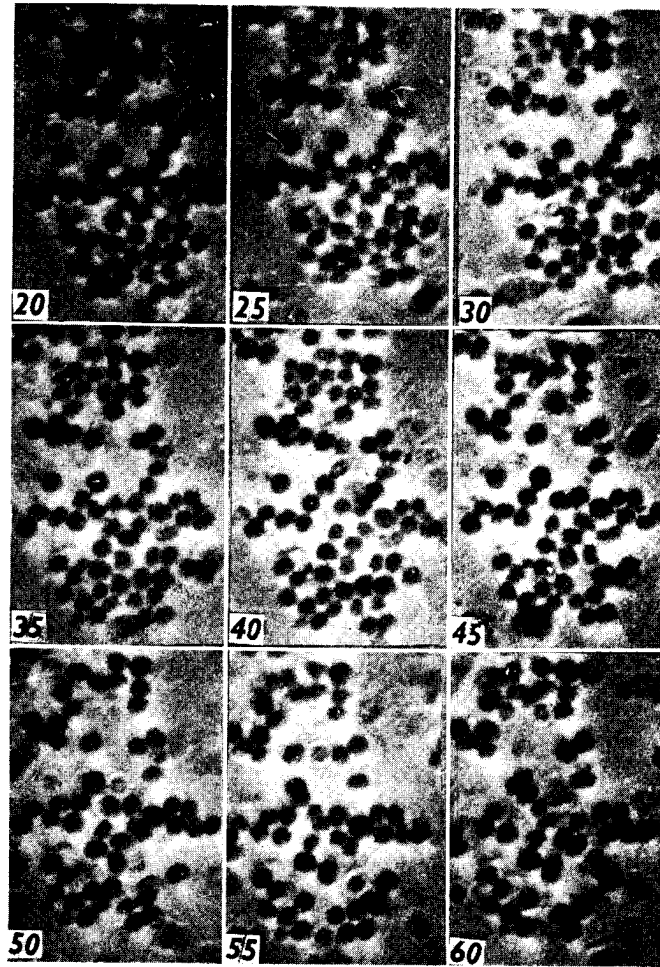
Табл. XX.

Рис. 4. Распад лизогенного штамма LS 2 после ультрафиолетового облучения.

M. Розенберг: Динамика распада лизогенных клеток под действием ультрафиолетовых лучей.
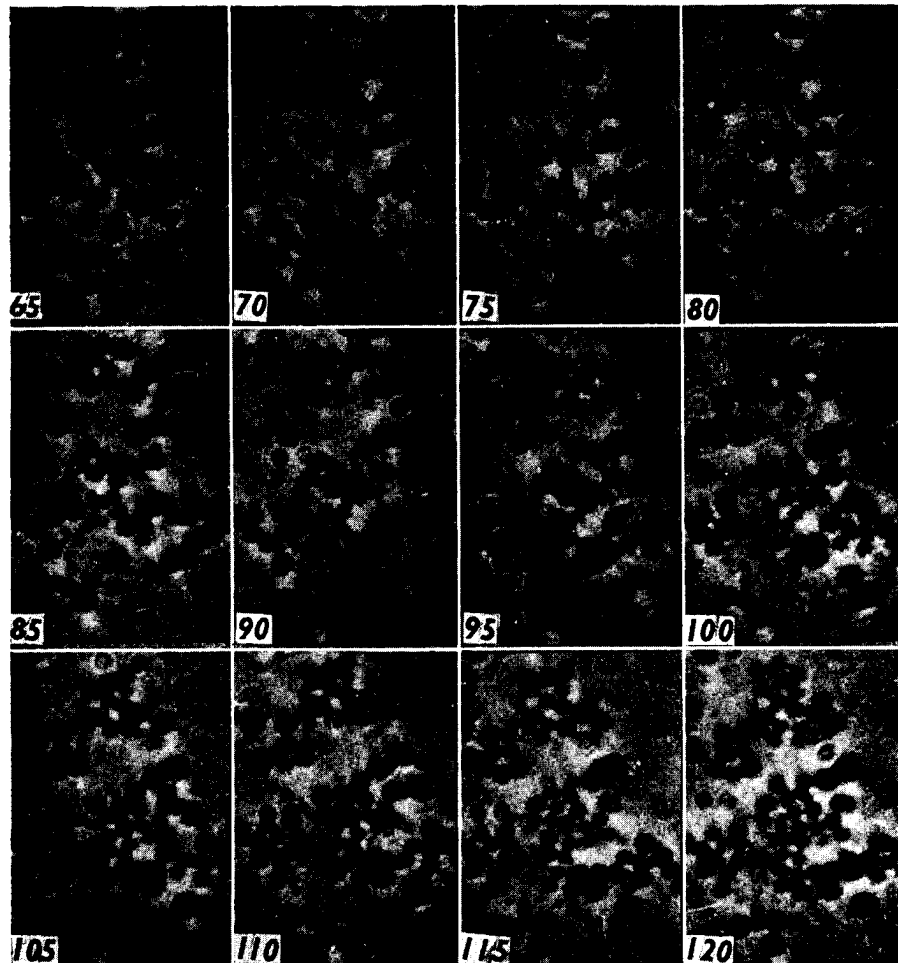
Табл. XX.

(Рис. 4.)

М. Войтишкова: К вопросу участия неоплодотворяющих живчиков   Табл. XXI.
в половом процессе.
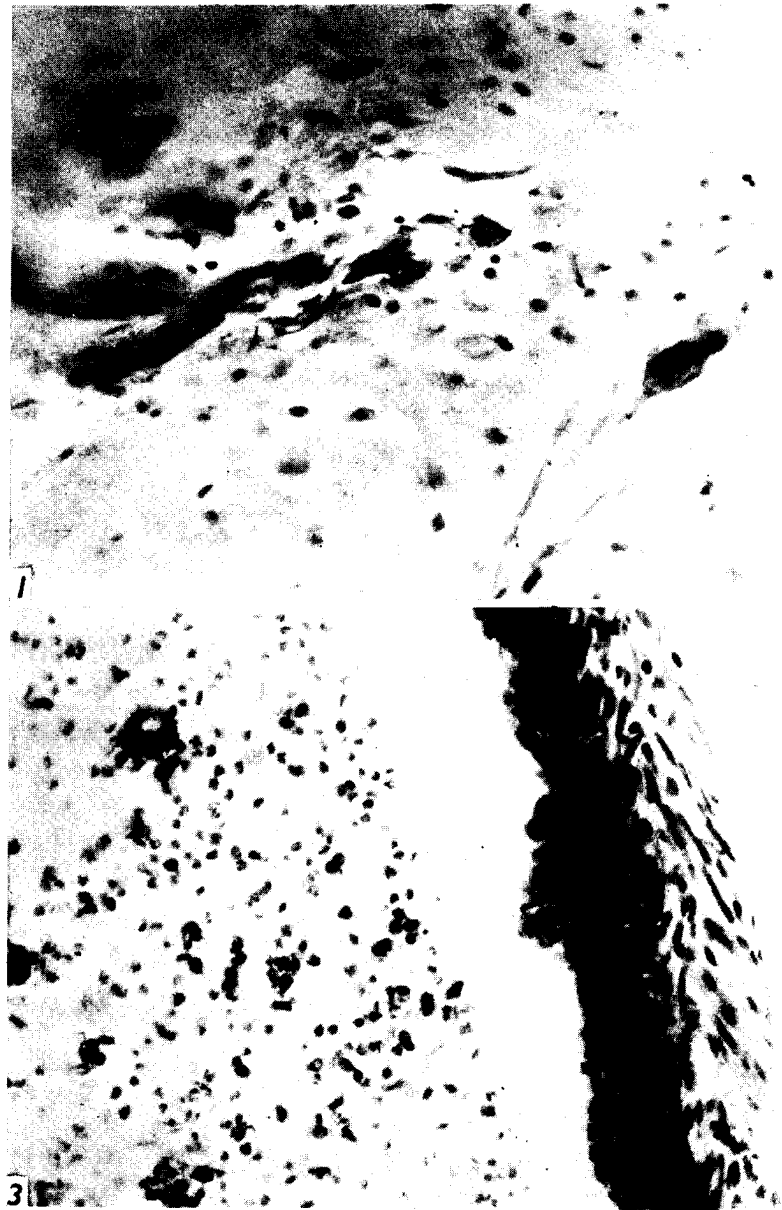
Рис. 1. Живчики в мышечной и соединительной ткани влагалища. Курица № 42, формол,
Harris, целлоидиновый срез, увеличение 400×.

Рис. 3. Множество фагоцитирующих клеток в просвете и в слизистой, отдельные живчики
в просвете матки у мыши. Формол, Harris, целлоидиновый срез, увеличение 400×.

М. Войтишкова: К вопросу участия неоплодотворяющих живчиков   Табл. XXII.
в половом процессе.
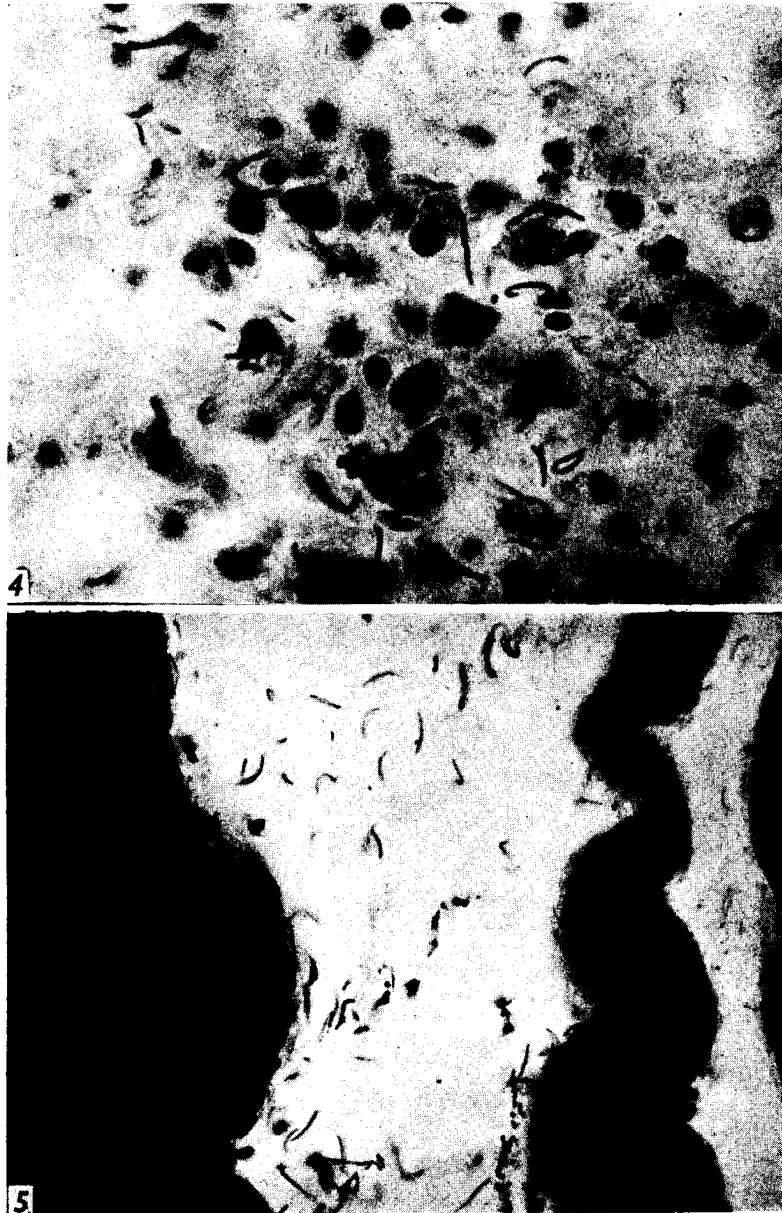
Рис. 4. Целлюлярная реакция через 24 часа. Заглотанные живчики и зернистость в полибластах. Формол, Harris, парафиновый срез, увеличение 1100×.

Рис. 5. Живчики в рыхлой соединительной ткани в мышце. Формол, Harris, целлоидиновый срез, увеличение 700×.

М. Войтишкова: К вопросу участия неоплодотворяющих живчиков   Табл. XXIII.
в половом процессе.
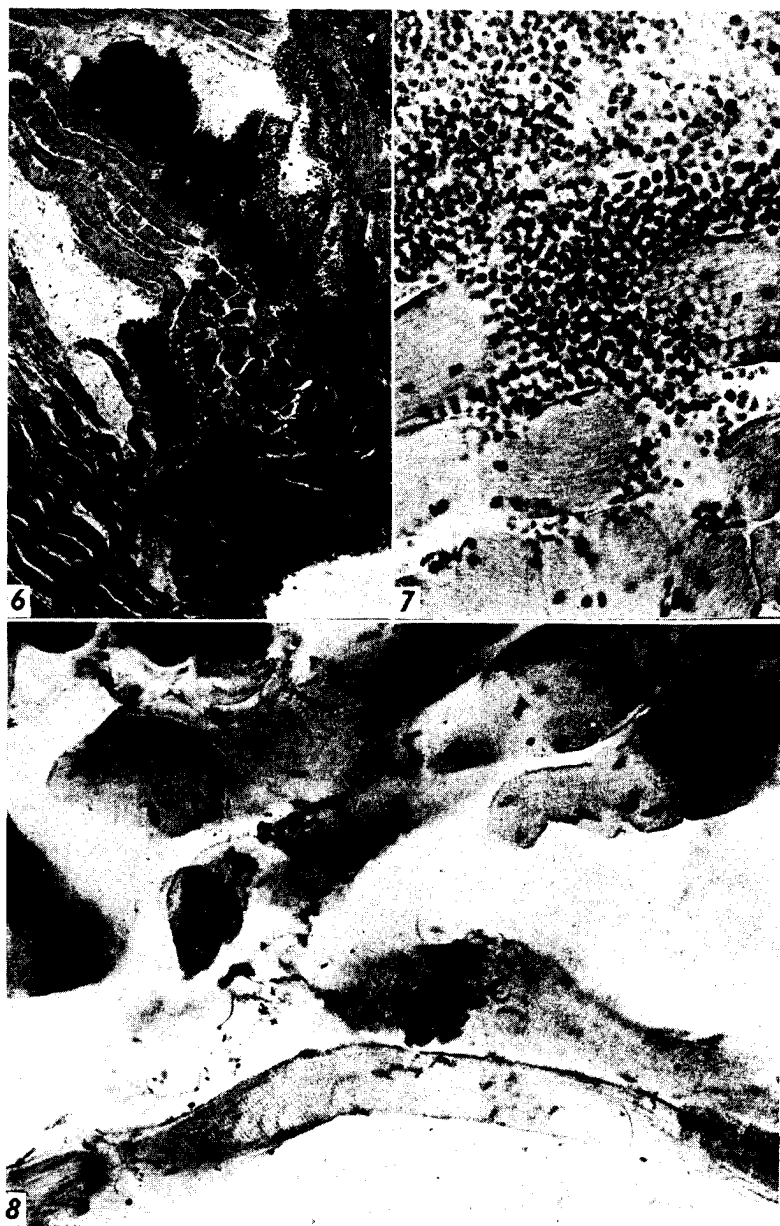
Рис. 6. Эритроциты кролика высоко в щелях между волокнами мышцы цыпленка. Формол, Harris, целлоидиновый срез, увеличение 70×.

Рис. 7. Эритроциты кролика, «проникающие» в волокна мышцы цыпленка. Формол, Harris, целлоидиновый срез, увеличение 400×.

Рис. 8. Живчики из фиксирующей жидкости, «проникшие» через рыхлую соединительную ткань в щелях между мышечными волокнами. Формол, Harris, целлоидиновый срез, увеличение 400×.

M. Burger and K. Beran: On the Transglucosidatory Activity of Enzymatic Preparations of the Fungus, Aspergillus niger.   Plate XXIV.
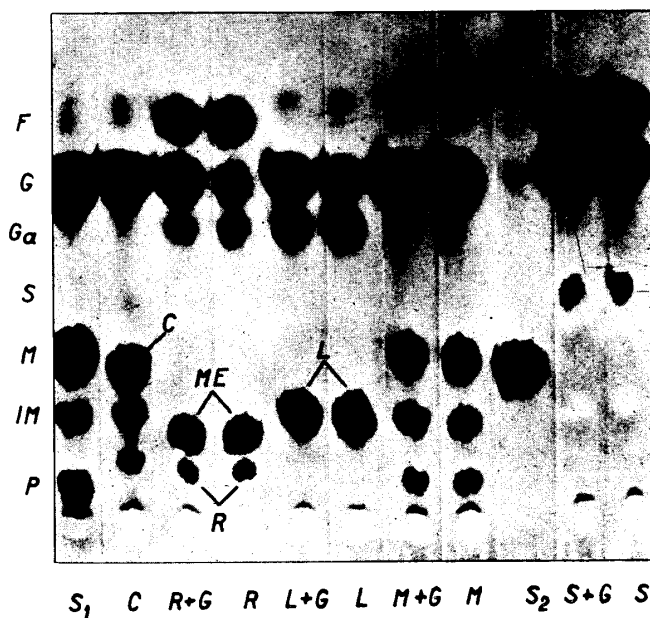
Fig. 1. Time of Incubation 75 minutes.   Fig. 2. Time of Incubation 18 hours.
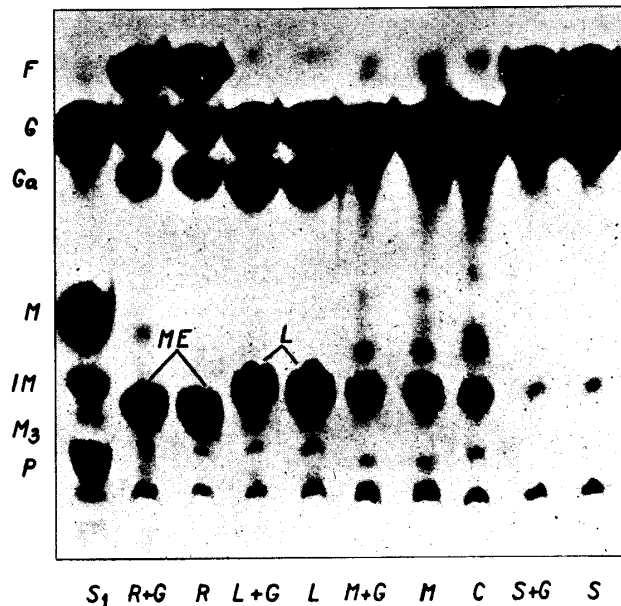
The Action of an Enzymatic Preparation of A. niger on Some Oligosaccharides

Incubated sugars: C = cellobiose, R = raffinose, R—G = raffinose with glucose, L = lactose, L—G = lactose with glucose, M = maltose, M—G = = maltose with glucose, S = saccharose, S—G = saccharose with glucose, S_2 = specimen of cellobiose. Sugar composition of the incubated mixtures: F = fructose, G = glucose, Ga = galactose, M = maltose, IM = isomaltose, M_3 = maltotriose, P = panose.

M. Burger and K. Beran: On the Transglucosidatory Activity of Enzymatic Preparations of the Fungus, Aspergillus niger. Plate XXV.
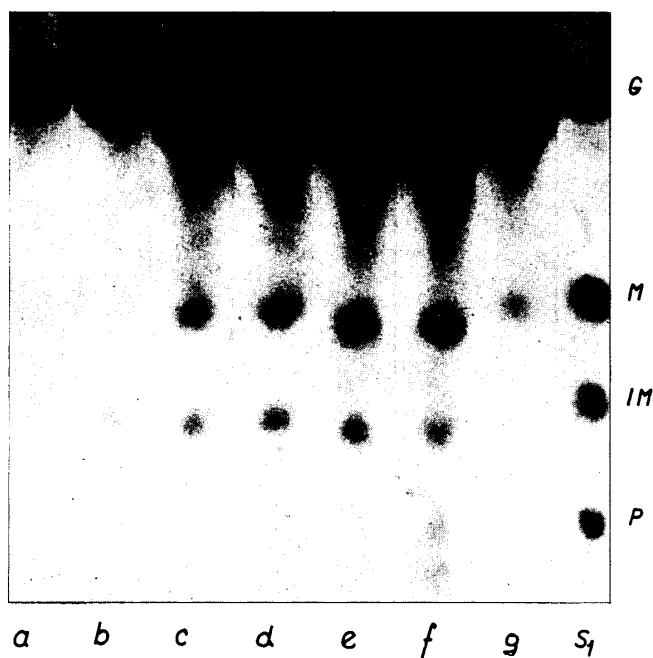
Fig. 6.
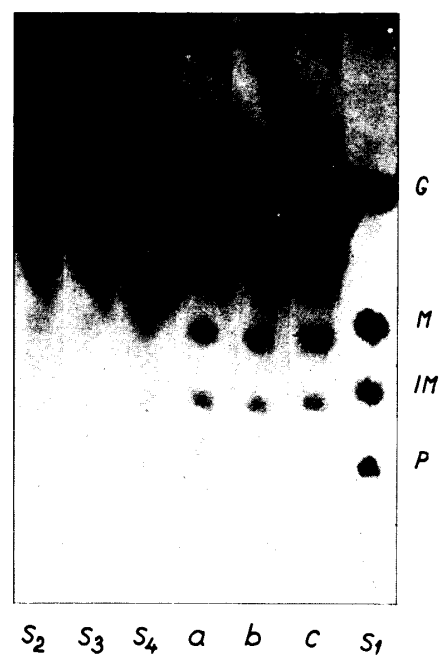
Fig 7

The Action of an Enzymatic Preparation of A. niger on Glucose

G = glucose, M = maltose, IM = isomaltose, P = panose. (Period of incubation — 18 hours). Fig. 6. a, b, c, d, e, f = 5, 10, 30, 40, 50 and 60% glucose. g = 15% glucose and 50% glycerin. Fig. 7. S_2, S_3, S_4 = 40, 50 and 60% non-incubated glucose solution, a, b, c = 40, 50 and 60% glucose solution incubated with enzymatic preparation.

J. Rozsypal und F. Mráz: Das Auftreten von Ölmohnbakteriosen in der Tschechoslowakei.

Bildtafel XXVI.
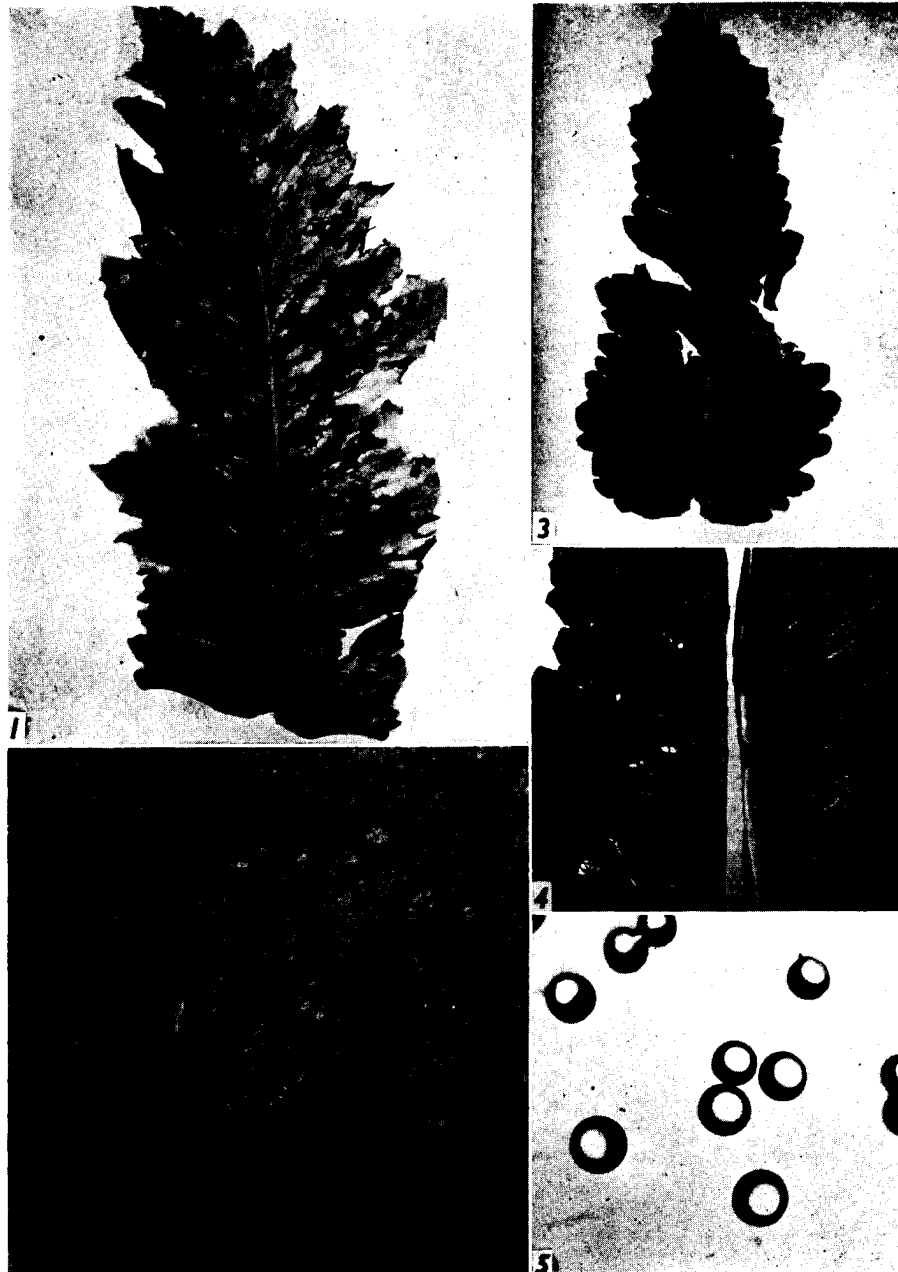
Die Blattfleckenbakteriose des Mohns

Abb. 1. Mohnblatt (*P. somniferum* L.) mit Blattfleckenbakteriose.
Abb. 2. Einzelne Blattflecken (vergrössert).
Abb. 3. Künstlich infiziertes Mohnblatt.
Abb. 4. Zwei Mohnblätter: links mit Blattfleckenbakteriose, rechts mit Helminthosporiose.
Abb. 5. Zweitägige Kolonien des Erregers der Blattfleckenbakteriose auf Fleischextrakt-Peptonagar (vergrössert).

J. Rozsypal und F. Mráz: Das Auftreten von Ölmohnbakteriosen in der
Tschechoslowakei.

Bildtafel XXVII.

Die Stengelbakteriose des Mohns

Abb. 6. An Stengelbakteriose erkrankte Pflanze mit beginnendem Welken am Gipfel.
Abb. 7. Durchschnitt eines Mohnstengels mit vorgeschrittener Bakteriose.
Abb. 8. Durch Stengelbakteriose vernichtete jüngere Mohnpflanzen.
Abb. 9. Ausfluss des Bakterienschleims an der Infektionsstelle am Wildmohnstengel.

J. Rozsypal und F. Mráz: Das Auftreten von Ölmohnbakteriosen in der Tschechoslowakei.   Bildtafel XXVIII.
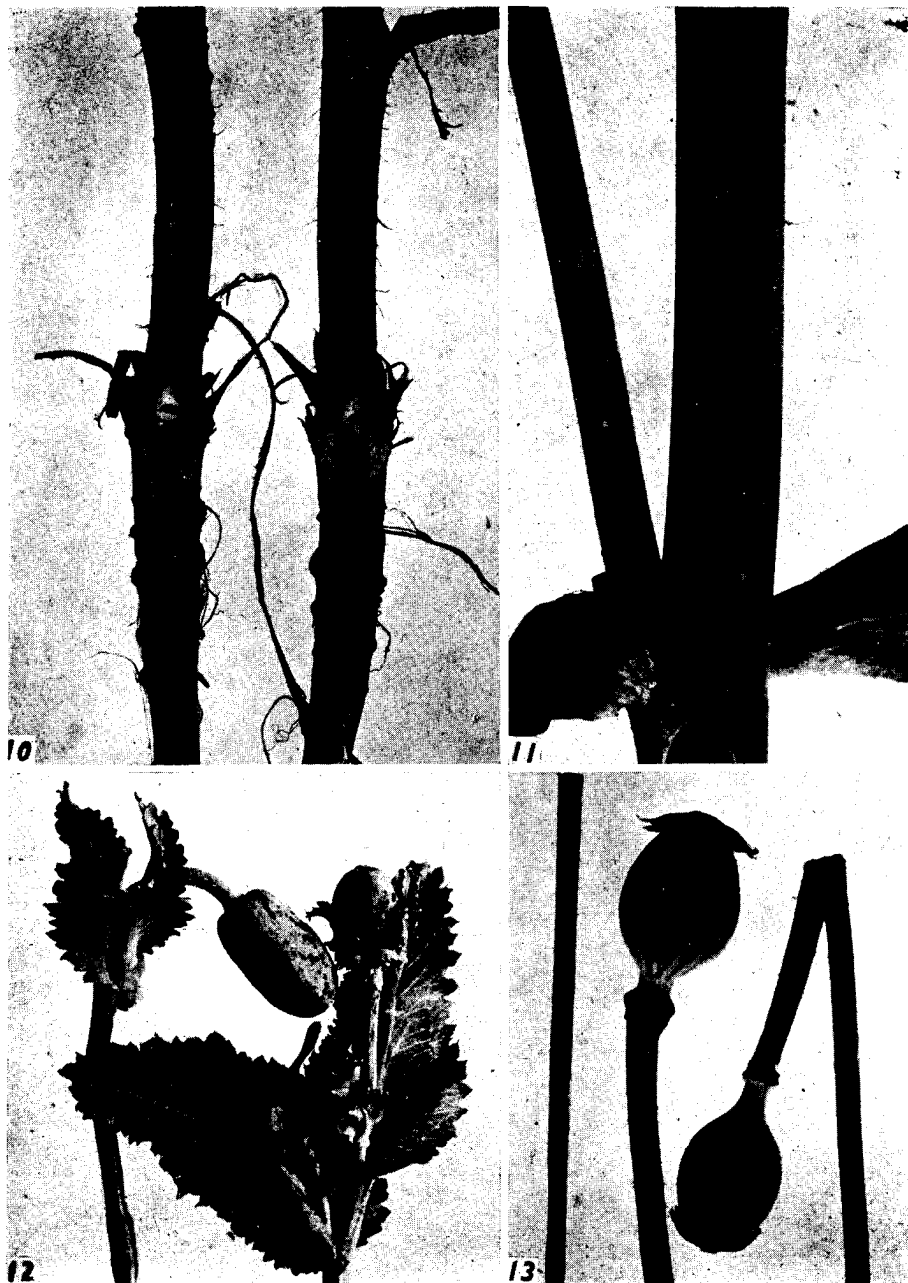
Abb. 10. Durchschnitt eines Wildmohnstengels mit vorgeschrittener Bakteriose.
Abb. 11. Mohnstengel mit Einstichen der Mohnstengelgallwespe und ihres Parasiten *Trichomalus fasciatus.*
Abb. 12. Von der Weichwanze *Calacoris norvegicus* Gmel. angestochene Mohnknospen.
Abb. 13. Durch *Calocoris norvegicus* übertragene Stengelbakteriose am Mohnkapselstiel.
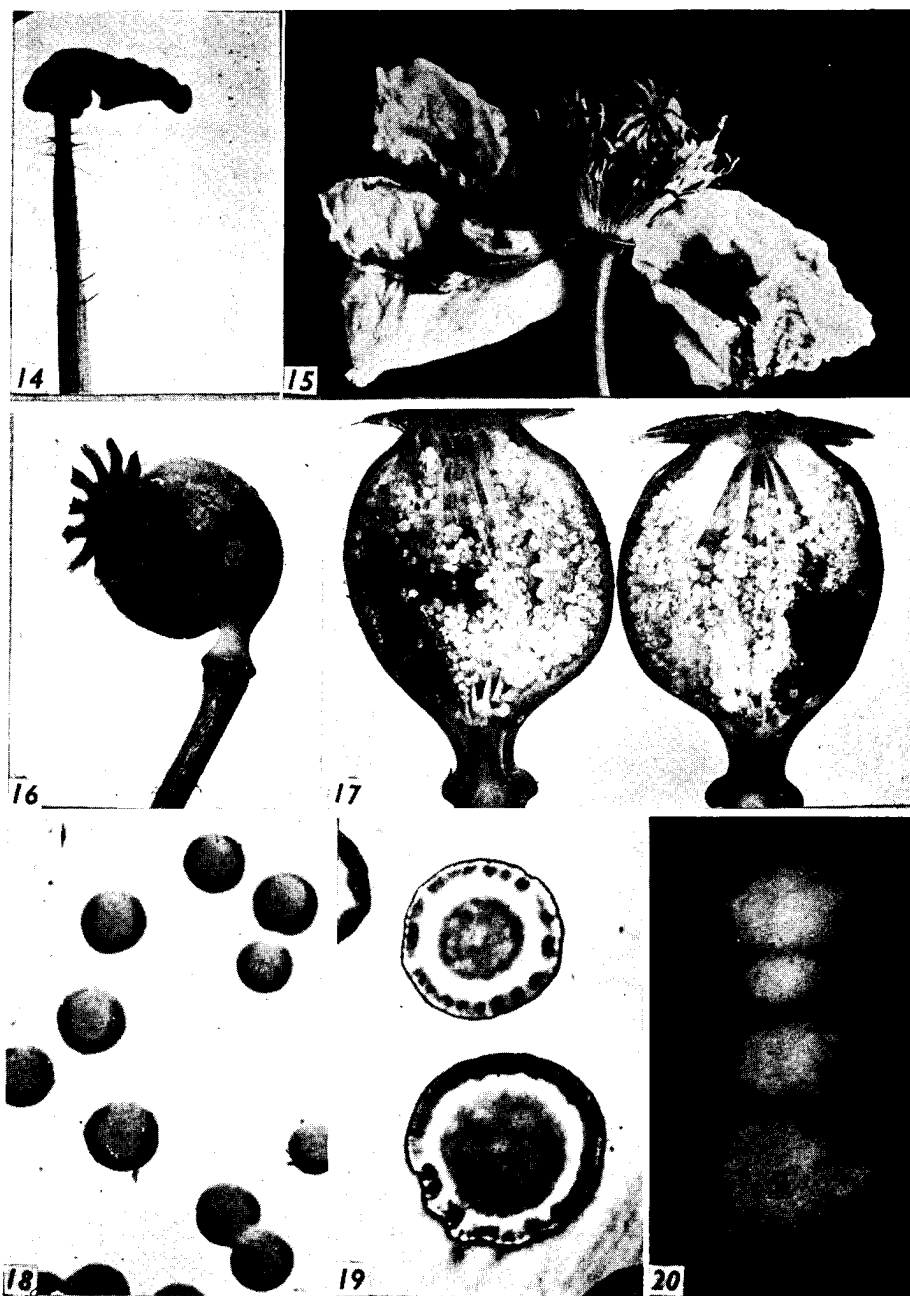
Abb. 14. Durch Stengelbakteriose vernichtete junge Knospe.
Abb. 15. Mohnblüte mit Nekrosen an Blütenblättern, Stielen der Staubgefässe und an den Narben nach
Knospeninfektion.
Abb. 16. Deformierte Mohnkapsel nach Infektion mit Stengelbakteriose.
Abb. 17. Infizierte Mohnkapsel während der Samenmilchreife.
Abb. 18. Kolonien des Erregers der Stengelbakteriose auf Fleischextrakt-Peptonagar im durchscheinen-
den Licht (5fache Vergrösserung).
Abb. 19. Ältere Kolonien im durchscheinenden Licht (6fach vergrössert).
Abb. 20. Ältere Kolonien auf Kartoffelagar (5fach vergrössert).

J. Svoboda and M. Hašek: Influencing the Transplantability of the Virus of Rous   Plate XXX.
Sarcoma by Immunological Approximation in Turkeys.

Fig. 1. Tumour on leg of turkey 8/865.
Fig. 2. Section through tumour on leg of turkey 8/719 in situ.
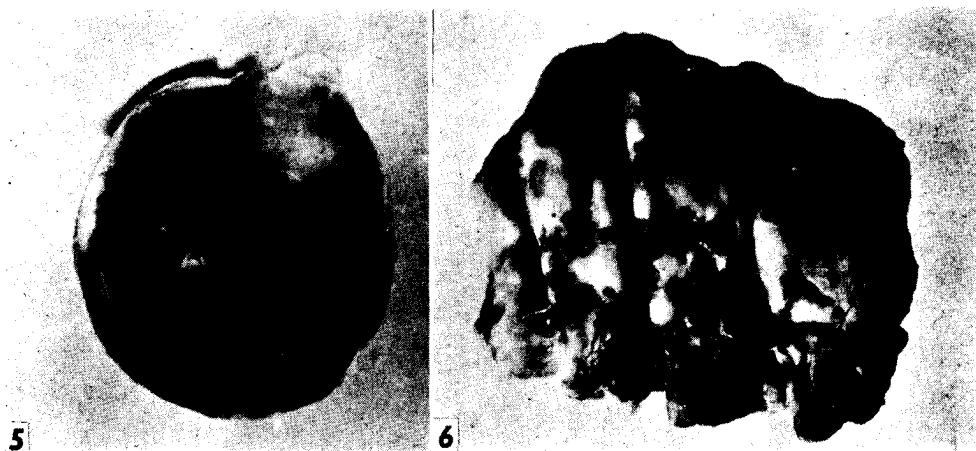
Fig. 5. Transverse section of spleen of turkey 8/402. White sarcoma nodes visible. Fixed with 10% formalin.
Fig. 6. Turkey 8/412—Lungs with metastases. Fixed with 10% formalin.

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Plate XXXI.
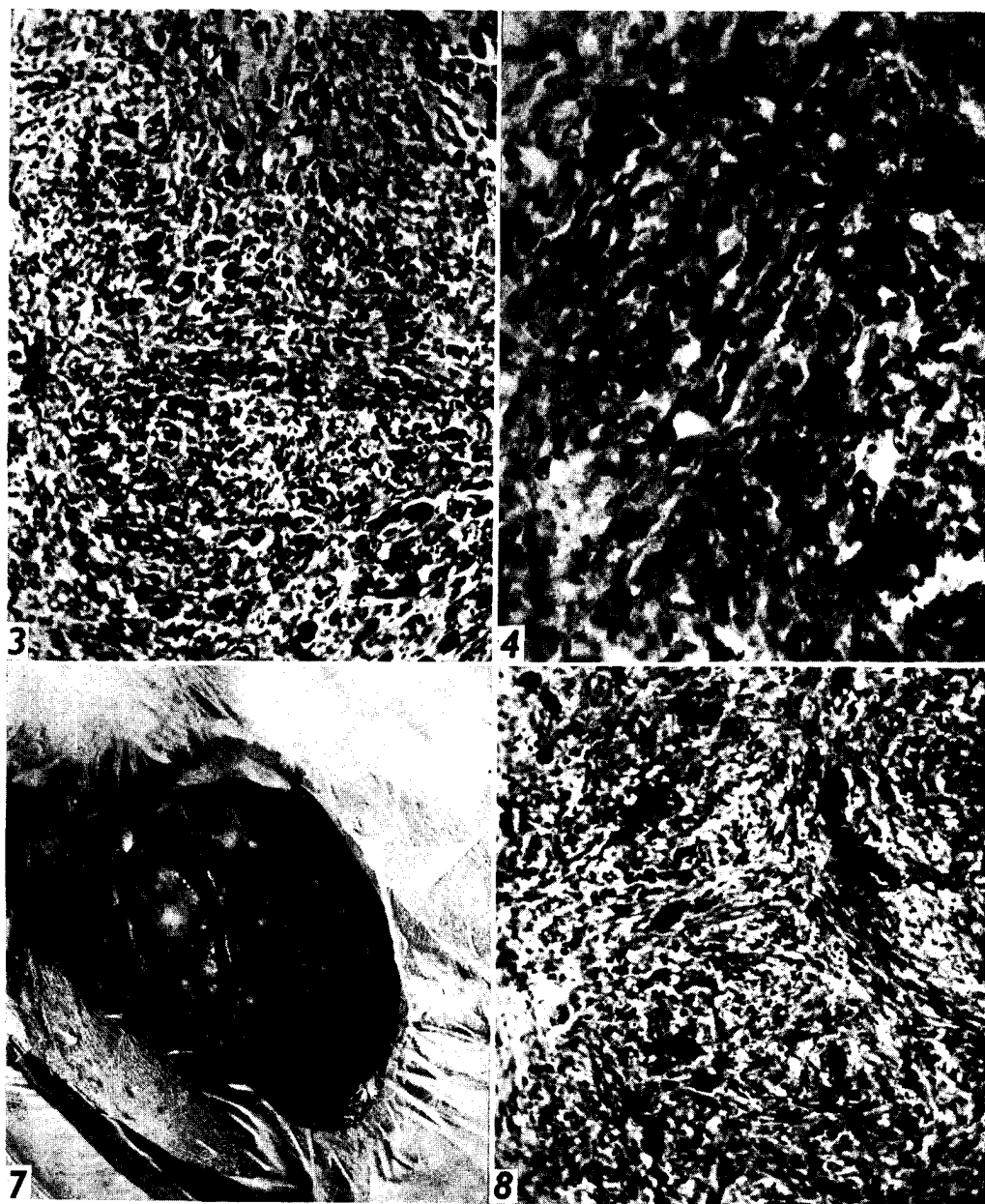
Fig. 3. Microscopic section of tumour on turkey 8/416. Tumor cells penetrating in linear formation into muscular tissue. Remains of muscle fibres still discernible. Magnification 200 : 1. Haem.-eosin.

Fig. 4. Turkey 8/412 — section of liver metastasis. Magnification 400 : 1. Haem.-eosin.

Fig. 7. Liver of turkey 8/865 after opening body cavity. Distinguishable metastases on surface of organ.

Fig. 8. Turkey 8/402 — section of metastasis in spleen. Tumorous elements retain fusiform shape. Magnification 200 : 1. Haem.-eosin.

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