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RESEARCH PROGRESS REPORT

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Warsaw, Poland

Name of principal investigator: Prof.dr H.Birecka

Project title: Metabolism of alkaloids in Lupinus and physiological role of these compounds

Project number: E21-CR-1

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SUMMARY

During recent years lupin alkaloids - as well as alkaloids of other species - have been investigated very intensely. The results obtained yielded much valuable informations about their chemical structure, patterns of biosynthesis and metabolism, quantitative changes during plant growth etc. However up till now the physiological role of these compounds in lupins /or the role of the processes leading to their synthesis or decomposition/ is unknown. Therefore further investigation in the field of: a/ identification of alkaloids occurring in plants often in traces, b/ their interconversions and metabolism in general and c/ their rate of biosynthesis during plant growth and the influence of different factors on this process can be of a great help.

These problems are important especially in the case of lupins[x] /fodder varieties/ because - among other things - the toxicity of various alkaloids is different /sparteine> lupanine> hydroxylupanine/.

In the reported experiments: 1/ transformations of alkaloids characteristic of bitter and fodder white lupin plants, 2/ identification of some alkaloids occurring in bitter plants and 3/ the role of aerial parts in the alkaloid synthesis during their greatest accumulation in the bitter plants were investigated.

ad 1/ Enzymatic extracts from bitter as well as fodder plants of white lupin are able to transform sparteine, lupanine, hydroxylupanine and angustifoline, i.e. alkaloids characteristic of the species investigated. The transformations are catalysed by dehydrogenases, present in extracts. The optima of temperature, pH, substrate concentration etc. for the activity of extracts from plants of both investigated varieties are analogous. The products of transformations of investigated alkaloids were the same in extracts from fodder and bitter plants, i.e. dehydroforms of the incubated bases. These facts show, that the extracted active enzymes from plants of both varieties are very similar. Some alkaloids which were not till now found in white lupin, like lupanine, 17-oxosparteine, 17-oxolupanine, did not undergo any changes in the extracts from plants of both varieties.

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x/ We are working mainly with white lupin because the variability of the populations investigated is not so great as in Lupinus luteus or angustifolius. Therefore - among other things - the differences in the plant weight, alkaloid content between the replications are not very great. We have carried out many experiments with Lupinus luteus, but because of great differences between the replications we could not draw any conclusions, statistically proved. Some other difficulties occur with Lupinus angustifolius. It seems to us that the new forms of L. luteus and angustifolius we have recently received are less variable.

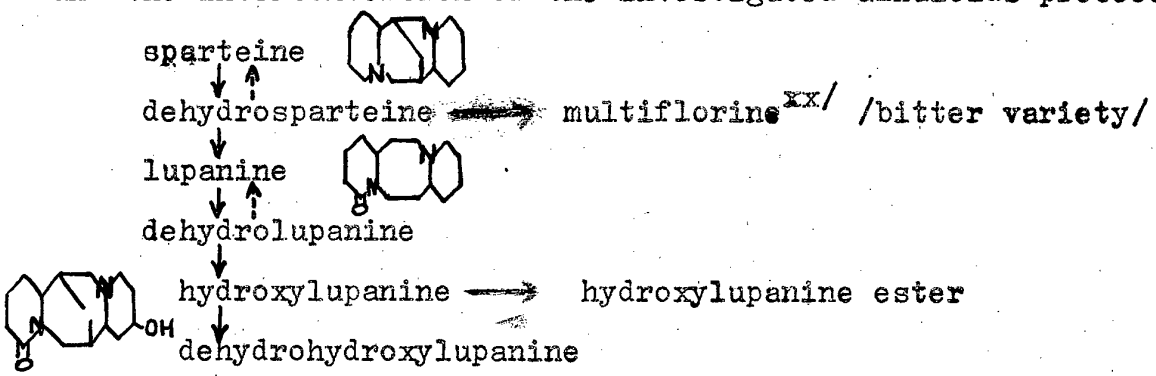
In order to understand better the patterns of alkaloid interconversion in white lupin labelled /radiactive/ sparteine, lupanine and their dehydroforms obtained in vitro /thanks to transformations by enzymatic extracts/were introduced into plants of bitter and fodder varieties. It must be added that the alkaloid spectrum of the two varieties /at least at the investigated stage of plant development/ differs somewhat from each other mainly in the fraction which chromatographically behaves like a base called multiflorine[x]; this alkaloid occurs in relatively large amount in bitter plants, but it could not be found in the fodder ones.

The detailed analysis of the investigated plants 2, 24 and 96 hours after injection - in addition to the results obtained in experiments with enzymatic extracts - permit the assumption that the general pattern of alkaloid transformation in white lupin is as follows:

alkaloid ⟶ dehydroform ⟶ another alkaloid

nonalkaloid compounds

and the interconversion of the investigated alkaloids proceeds:



This scheme does not include all alkaloids present in white lupin, alkaloids, which may play an important role in the interconversion processes.

According to some hypothesis a reverse direction of interconversion was postulated. One of the main argument of this hypothesis was the fact, that the occurence of sparteine in Lupinus albus /as well as in angustifolius/ was not fully proved.

ad 2/ Therefore we tried to examine the base occurring in vegetative parts of white lupin[xxx], which behaves chromatographically like sparteine. It was proved by Infrared spectrometry analyses that this base is identical with sparteine /not only in bitter but also in fodder white lupin plants/. Its occurrence in small amounts especially in bitter plants can be a result of a very high rate of transformation of this alkaloid. In addition

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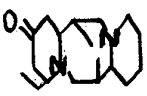
x/ 

xx/ it is possible that this is not the only way of multiflorine formation /in bitter plants/

xxx/ we could not find this base in seeds.

we showed that: a/ another alkaloid, present in bitter white lupin is hydroxylupanine ester identical with hydroxylupanine ester occurring in fodder plants, b/ hydroxylupanine in side shoots of bitter white lupin is identical with hydroxylupanine, isolated from seeds of Lupinus angustifolius /in our previous investigations we have proved that, hydroxylupanine in bitter white lupin seeds is identical with the hydroxylupanine from L.angustifolius/. However this hydroxylupanine seems to differ somewhat from the hydroxylupanine, isolated from vegetative organs of fodder plants of lupinus albus /investigations are going on/.

In our previous as well as in the reported investigations we have based our conclusions on data showing transformations of alkaloids introduced into plants. But these data could have been considered as not fully certain because they reflected mainly changes of compounds introduced and not exactly the changes of alkaloids formed in situ. Therefore we tried to obtain some informations about the interconversion of investigated alkaloids on the basis 1/ of alkaloid content changes in plants during their development and 2/ of the total and specific activity of some alkaloids in plants exposed to labelled /$C^{14}O_2$. The results obtained rather confirmed our assumption that the interconversion of investigated compounds proceeded from lower to higher levels of oxidation.

ad 3/ It was shown in our previous experiments that the greatest total alkaloid accumulation in bitter white lupin plants /similarly to fodder ones/ occurs during a relatively short time after the beginning of side shoot flowering. According to the results of the reported investigations a/ at this time the side shoots are mainly responsible for the alkaloid synthesis /70-90% of the alkaloid increment/, b/ at the later stages of growth the total alkaloid content in plants does not undergo any marked changes. This is in all probability the result of inhibition of synthesis as well as of decomposition of the investigated compounds, c/ the ageing process of leaves brings about a diminishing in the rate of alkaloid synthesis and d/ the pods are not able to synthesie alkaloids, the stem of the main shoot shows this capacity but to a very small degree.

These results draw our attention mainly to the side shoots of the investigated plants. The alkaloid precursors and products of their metabolism will be investigated with special reference to these plant parts. The results obtained may be of some importance especially for plant breeding work.

DETAILED REPORT

I. Enzymatic transformation of lupin alkaloids /Lupinus albus/.

Several years ago only two alkaloids characteristic in the bitter forms of lupinus albus and angustifolius were known /identified/, i.e. lupanine and hydroxylupanine. During the last years great progress was made in investigations on the alkaloid content in the two species. In seeds

of bitter white lupin - besides the two aforementioned bases - angustifoline, multiflorine, albine, hydroxymultiflorine, dehydrohydroxymultiflorine were identified; the latter three alkaloids occur in seeds only in traces.

At out laboratory: a/ in extracts from aerial parts of bitter white lupin multiflorine[x], hydroxylupanine ester[x], sparteine[xx] and an alkaloid, which in all probability is dehydrolupanine were identified; b/ in extracts from aerial parts of a fodder population of white lupin sparteine, lupanine, hydroxylupanine, hydroxylupanine ester, angustifoline were identified; the occurrence of three other alkaloids was established; the first one is probably dehydrolupanine, the second behaves chromatographically like multiflorine but differs from the latter in UV; the third one has a double bound C=C and can be reduced to sparteine, but it is not Δ-5-dehydrosparteine.

According to the hypothesis of Shöpf /1951/, Mothes /1955/, Hegnauer /1958/ the interconversion of lupin alkaloids proceeds from lower to higher levels of oxidation /e.g. lupanine $\longrightarrow$ hydroxylupanine . Wiewiórowski and Reifer /1961, 1962/ postulated a reverse direction in this interconversion /angustifoline ... $\longrightarrow$ hydroxylupanine ... $\longrightarrow$ lupanine ... ? $\longrightarrow$ sparteine/. It must be stressed that the toxicity of various alkaloids is different /sparteine > lupanine > hydroxylupanine .

Up till now lupanine has been considered as the main /dominant quantitatively/ alkaloid in bitter as well as fodder white lupin. If the hydroxylupanine ester is taken into account the total amount of hydroxylupanine in plants may sometimes even be equal to the content of lupanine /the total content of alkaloids is till now the main criterion for "sweetness"/.

Our previous investigations on alkaloid metabolism in fodder and bitter forms of lupinus albus indicated, that sparteine /injected into the plants/ could be converted - among other alkaloids - to lupanine, and lupanine to hydroxylupanine. But 1/ the identification of these compounds was based only on paper chromatography results; 2/ the intermediary /dehydro/ products could not be isolated. The main aims of the reported investigations were: a/ to obtain enzymatic extracts from bitter and sweet populations of white lupin, extracts which might be able to transform lupin alkaloids; b/ to isolate and identify the products of enzymatic transformation in vitro of some alkaloids characteristic of the investigated species, 3/ to investigate the patterns of transformation of sparteine and lupanine /and of the products of their transformation in vitro/ in plants of bitter and fodder white lupine, d/ to compare these two forms as regards the character of alkaloid interconversion in vitro and in vivo.

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x/ multiflorine - called by us previously a_x
hydroxylupanine ester - called by us a_z or /oxylupanine ? /;

xx/ previously identified only chromatographically. Sparteine and hydroxylupanine ester will be discussed latter.

Experimental procedures: A. Enzymatic extracts were obtained from the aerial parts of 3-4 weeks old plants of fodder /var. Przebędowska/ and bitter /var.Czechnicka/ populations of lupinus albus. Among various applied methods of extract purification the method of extraction of aceton powder with phosphate buffer followed by fractionated protein sedimentation with $/NH_4/_2SO_4$ was chosen for further experiments. These extracts showed after dialysis the highest enzymatic activity - 3.6 μM of transformed sparteine per 1 mg of protein per hour /extract obtained in other ways - 0.13 - 0.22 uM per 1 mg of protein per hour/. The rate of transformation of lupanine was about 30 and of hydroxylupanine about 60% higher. The results were the same for extracts from bitter as well as from fodder plants.

The influence of temperature, pH, of inhibitors and activators, of the concentration of substrates was investigated; temperature - from 0^0 to 60^0C, pH - from 5 to 9, the concentration /of sparteine, lupanine, hydroxylupanine/ from 0.1 to 13 uM in 1 ml; inhibitors and activators /sparteine or lupanine/: chlorides: Na^+, Ag^+, Ca^{++}, Mg^{++}, Mn^{++}, Co^{++}, Zn^{++}, Cu^{++}, Hg^{++} and Ba^{++}; NaF, CaF, H_2O_2, Na_2AsO_2, KCN, NaN_3 in conc. 10^{-5} to 10^{-2}M. The influence of ATP, ADP and DPN was also investigated.

Substrates: sparteine, lupanine, hydroxylupanine, angustifoline, multiflorine, 17-oxosparteine, 17-oxolupanine, hydroxylupanine ester and lupinine.

B. 1/ The transformation of sparteine, lupanine and hydroxylupanine by unpurified enzymatic extracts from bitter and fodder plants of lupinus albus. Hydroxylupanine was applied in labelled form /C^{14}/ after 16 hrs of incubation with buffered extracts, the solvent was evaporated, the residue dissolved in 75% ethanol and chromatographied /paper chromatography/ with n-butanol : HCl: toluen. The paper was cut in strips of 2 to 4 mm in width, eluated, the radioactivity and alkaloid content in each eluate was determined; 2/ unpurified enzymatic extracts were obtained from aerial parts /5 g/ of Phaseolus vulgaris, Vicia faba, Nicotiana glauca, Zea mays, Spinacea oleracea and Lupinus luteus - their activity /sparteine as substrate/ was compared with the activity of an analogous extract obtained from plants of fodder white lupin.

C. Identification of products of enzymatic transformation in vitro of some lupin alkaloids. Sparteine /300 mg/ was incubated with a purified enzymatic extract from bitter white lupin; the product of transformation after separation on a celulose column /aceton: 1n HCl/ was investigated chromatographically, in IR-spectrometer, crystallized as picrates /I/, m.p. determined; reduced with H_2 /PtO_2 in 2n HCl/. 10 mg of the base /picrates/ obtained from sparteine incubated with an enzymatic extract from fodder white lupin was added to 10 mg of picrates I and the depression of the m.p. was investigated. To 1 mg of T-labelled product of enzymatic transformation of sparteine /in extract from fodder plants/ 10 mg of unlabelled product, obtained after incubation with an extract from bitter form, was added. After crystallization /picrates/ the specific radioactivity was determined /crystalls and solution/.

A similar procedure was applied in the attempts of identification of products of enzymatic transformation in vitro of lupanine /labelled with C^{14}/, hydroxylupanine and angustifoline. Products of enzymatic transformations of lupanine, multiflorine, 17-oxosparteine, 17-oxolupanine and of hydroxylupanine ester were investigated only chromatographically and in UV.

D. Biosynthesis of C^{14} and T-labelled lupin alkaloids: 0.1 M water solution of 1-C^{14}-cadaverine /total activity 1 mc/ was introduced to 10 plants of the bitter variety /0.5 ml per plant/ at the stage of bud formation. In the same way 5 mg of H^3-sparteine /total activity 687.5 µc/ was introduced to other 10 plants. After 10 days the alkaloids were extracted, purified, separated /column chromatography/, and sublimated or distilled in vacuo at 10^{-3}T.

Lupanine - twice crystallized from n-hexane; to 1 mg of C^{14}-lupanine 19 mg unlabelled lupanine was added, twice crystallized, specific radioactivity determined.

Hydroxylupanine fraction - in order to eliminate dehydrolupanine, this fraction was sublimated four times at 145°C and 10^{-3}T; before sublimation 100 µg was reduced with H_2 and the dehydrolupanine content was determined /hydroxylupanine gives hydroxysparteine, dehydrolupanine --> sparteine/.

Multiflorine fraction /which may contain dehydrosparteine/was distilled between 145 to 166°C at 10^{-3}T; the specific radioactivity of particular fractions /5°C differences in distillation temperature/ was determined.

Angustifoline: twice distilled to 170°C at 10^{-3}T.

Hydroxylupanine ester - twice distilled to 165°C

Sparteine - /only T-labelled/ - after elution from the celulose column - was diluted with 15 mg of unlabelled sparteine. After distillation at 98°C radioactivity determined. From T-sparteine after incubation with an enzymatic extract from fodder plants T-dehydrosparteine was obtained. In the same way C^{14}-dehydrolupanine was also prepared.
2 π gas flow counter /from the firm Frieseke and Hoepfner/ was used. Its efficiency for C^{14} was 35 and for T - 2.2%.

E. Transformation of sparteine and lupanine in vivo.
1/ Identification of dehydrosparteine and dehydrolupanine in plants after injection of sparteine and lupanine.
a/ Dehydrosparteine. 50 plants of the fodder population received 15 mg of sparteine /per plant/ as sulphate
After 14 days alkaloids were extracted. The fraction of Rf of dehydrosparteine was chromatographied /on celulose column/ with aceton: 1n HCl; the alkaloid was distilled at 120°C and 10^{-3}T and compared in IR spectrometer, chromatographically and chemically with the product of enzymatic transformation in vitro.

A part of the fraction was crystallized as picrates with T-dehydrosparteine /1:1/ and the specific activity was determined.

b/ Dehydrolupanine. 2 mg of C^{14}-labelled lupanine were introduced into 3 plants of the fodder variety. The plants were killed /-60°C/ after 2 hours. Alkaloids extracted, chromatographically separated. The fraction of Rf of dehydrolupanine

/the same Rf as the Rf of hydroxylupanine/ was eluted, radioactivity measured; reduction with H_2/2n HCl and PtO_2/. Products of the reduction - after addition of a small amount of unlabelled sparteine - separated chromatographically and their radioactivity determined.

2/ Products of transformation of T-sparteine, C^{14}lupanine and their dehydroforms in plants. Into 49 days old plants of the bitter and fodder population of Lupinus albus H^3-sparteine, C^{14} lupanine and their labelled dehydroforms obtained enzymatically in vitro were introduced. The amount of injected alkaloid varied between 0.1 to 0.5 mg per plant in the fodder and 0.3 - 3 mg in the bitter variety.

The plants were analyzed after 2, 24, 96 hrs. The alkaloids extracted, separated on paper, eluted, radioactivity and content determined. The fraction of Rf of dehydrosparteine /the same Rf as the Rf of multiflorine in bitter and a_x in fodder var./ transferred into chloroform, heated for 3 min to b.p. After evaporation of chloroform the alkaloids were sublimated to 145°, activity and quantity determined. The differences between the results before and after sublimation indicate the amount and the activity of dehydrosparteine. The fractions of radioactive lupanine /T-labelled/ was investigated by means of isotope dilution with unlabelled lupanine. The fractions of Rf of dehydrolupanine from plants, which were injected with C^{14}-alkaloid, was reduced with H_2.

F. 1/ Synthesis of 1-C^{14}-cadaverine

1-C^{14}-cadaverine was obtained /at micro-scale/ as follows

I $\quad BaC^{14}O_3 \xrightarrow[K]{NH_4Cl} KC^{14}N \xrightarrow{H_3PO_4} HC^{14}N \xrightarrow{CH_3ONa} NaC^{14}N$

II $\quad NaC^{14}N + Br.CH_2.CH_2.CH_2.CN \longrightarrow NaBr + C^{14}NCH_2.CH_2.CH_2.CN$

III $\quad C^{14}N.CH_2.CH_2.CH_2.CN \xrightarrow[PtO_2]{H_2} NH_2.C^{14}H_2.CH_2.CH_2.CH_2.CH_2NH_2$

the obtained cadaverine was purified; crystallyzed; the m.pt. and activity determined.

2/ Preparation of H^3 - sparteine.

25 mg, of unlabelled sparteine /chloride/ reacted with 0.5 ml THO /with Adams red.catal./ at 100°C for 48 hrs. THO having been distilled, 1 ml H_2O was added and afterwards distilled, this procedure was repeated four times. The residue was dissolved in 5 ml 0.5 n NaOH, filtrated through glass fiber and heated at 95°C for 10 min. The solution was cooled to 4°C, sparteine extracted with chloroform, which afterwards was distilled off. 5 ml 0.5 n HCl was added and again heated. H^3-sparteine was extracted with chloroform ---> evaporated; 5 ml 0.5 n NaOH added and the described above procedure was repeated five times, the radioactivity of each chloroform extract having been determined. T-sparteine was twice crystallized as picrates, afterwards converted into chlorides. To 0.5 mg of labelled sparteine 25 mg of unlabelled sparteine was added, crystallized and the radioactivity determined.

Results: I.Enzymatic transformations of alkaloids in vitro.

The optimum temperature for the enzymatic activity of extracts from both investigated populations lies for sparteine between 30-45°C, for lupanine and hydroxylupanine 25-40°C. At 57°C the activity accounted only for 25% of its maximum.

The optimum of pH ranges between 6.6 - 7.7 for the three investigated substrates, the extracts from bitter plants reacted to the changes of pH similarly as the extracts from the fodder ones. Under optimal conditions of temperature and pH the influence of alkaloid concentrations was investigated. The optimum of sparteine concentration was 2 - 4.5 of lupanine conc. 2 - 7.4 of hydroxylupanine conc. 3 - 8 µM/ml - in both kinds of enzymatic extracts. In further investigations /quantitative in character/ 3 µM/ml of particular alkaloids was used.

Inhibitors and activators: Na^{+} and Ca^{++} - no influence on the enzymatic activity Mg^{+++}, Hg^{++} and Zn^{++}-increased the activity about 20%; Co^{++}, CU^{++}, Ba^{++}, Ag^{+} in conc. 10^{-3}M - inhibition about 40%, in conc. 10^{-2} - 100%. Versene had no influence, $NaAsO_2$ /10^{-3}/ - did not cause any inhibition, H_2O_2 at any concentrations did not influence the activity of extracts. ATP at conc. 2.10^{-4} to 3.10^{-3} M - increased the activity about 30%, but at 10^{-2}M caused a marked inhibition. ADP - no influence; DPN at $2.5 \cdot 10^{-3}$M increased the activity about 25%. The addition of activators or inhibitors to extracts previously boiled did not cause any changes in the substrates applied. The results obtained with both kinds of extracts were the same.

Table 1 and 2 represent the results obtained with purified and unpurified extracts from fodder and bitter white lupins:

Table 1

µM of the transformed substrate in 1 ml of enzymatic extracts per hour

Extracts from plants	before dialysis		after dialysis	
	sparteine	lupanine	sparteine	lupanine
fodder	0.8	1.6	0.6	1.2
bitter	0.06	0.09	0.5	1.0

The unpurified extracts from bitter lupin showed a much lower activity than analogous extracts from fodder lupin. Dialysis of "bitter" extracts increased their activity to the level, observed in the "fodder" extracts.

Table 2

The influence of juice from fresh bitter plants on the activity of extracts from fodder plants /substrate:sparteine/

ml of juice added	activity of the extract/%/ after addition of	
	fresh juice	boiled juice
0.0	100	100
0.1	100	100
0.2	100	90
0.3	85	82
0.4	80	68
0.5	70	50

The activity of extracts from fodder plants was lowered not only by the fresh but also by the boiled juice obtained from bitter plants. The addition of a mixture of alkaloids /characteristic of the bitter population/ to the investigated extract did not have any influence on its activity.

In the case of sparteine and lupanine the total amount of alkaloid introduced + the product of its transformation in unpurified as well as in purified extracts was the same as the amount applied. However in the case of hydroxylupanine the total amount of both bases after 16 hrs of incubation with unpurified extracts was about 50% lower than the amount applied /in purified extracts there was no difference/. Additional tests with C^{14}-hydroxylupanine showed that about 50% of the radioactivity applied could be found in compounds which did not react with Dragendorff's reagent.

The activity of extracts from fodder white lupin plant /100%/ was compared with the activity of extracts from other species /substrate: sparteine/: Nicotiana glauca - 0, Zea mays - 0, Phaseolus vulgaris - 15, Vicia faba - 20, Spinacea oleracea - 40, Lupinus luteus /fodder/ - 60%.

Fractionation of proteins of the extract from fodder white lupin on a hydroxylapatite column showed that only fractions eluted with buffer solution from 0.14 to 0.22 M are active. The maximum of activity was manifested by the fraction eluted with 0.16 M /11.5 uM of transformed substrate per 1 mg of protein per hour/.

The identification of products of alkaloid transformations in vitro gave the following results:

1/ sparteine - in extracts from both forms of white lupin - yielded dehydrosparteine /the only product/, m.p./picrates/ 204-206°C; this dehydroform however is not D^5-dehydrosparteine /m.p.-picrates - 189 - 190°C/.

2/ lupanine - yielded /in both extracts/ dehydrolupanine /only/, but it is not D^{11}-dehydrolupanine or dehydrolupanine found by Rink and Schäfer /1954/ in seeds of Lupinus perennis.

3/ hydroxylupanine - yielded dehydroxylupanine.

4/ angustifoline --> dehydroangustifoline.

Lupinine, 17-oxosparteine, 17-oxolupanine, hydroxylupanine ester and multiflorine /the latter investigated in extracts only from fodder plants/ - did not change during incubation with enzymatic extracts.

II. Transformation of alkaloids in vivo.

The degree of incorporation of C^{14}-cadaverine into alkaloids in plants of bitter lupin after 10 days accounted only for 0.7%, while the incorporation of T-sparteine amounted to 50%. In both cases - all isolated alkaloids were radioactive, the distribution of the radioactivity between the investigated bases was similar, and the quantitative proportions between them were in general analogous to the proportions for radioactivity, except for the multiflorine, whose participation in the total alkaloids activity was higher than in the total alkaloid content. These facts show - among other things - that T-sparteine after chemical removal of easily exchangeable hydrogen - can be used with good

results for labelling other lupin alkaloids in spite of the fact that hydrogen atoms bound with the sparteine skeleton are "attacked" by enzymes more easily than carbon in this skeleton.

An intense transformation of sparteine into other alkaloids was found also in plants of the fodder variety.

The isolated fraction, whose Rf value was the same as the Rf of dehydrosparteine after distillation to 120°C yielded an oily substance. The chemical analysis, paper chromatography and IR spectrum showed that it was dehydrosparteine identical with the dehydrosparteine obtained enzymatically in vitro. The fraction, in which besides hydroxylupanine dehydro--xylupanine may also be present, was isolated from plants, which received C^{14}-lupanine and two hours after injection were killed.

Table 3

Radioactivity of the fraction investigates c p m

Plants	Before reduction: fraction Rf of hydroxylupanine and dehydrolupanine	After reduction: fraction Rf of sparteine	After reduction: fraction Rf hydroxysparteine
1	2 300	2 100	40
2	1 450	1 520	0
3	2 880	2 640	0

The results obtained after reduction with H_2 /tab.3/ indicate that the product of lupanine transformation in vivo is dehydrolupanine. This conclusion was confirmed by other analysis.

Results obtained in the experiment with bitter lupin plants 2, 24 and 96 hours after injection of alkaloids:

1/ During the time under investigation the plants did not change their total radioactivity. A part of the activity could be found in nonalkaloid compounds. This part was always higher in plants, which received dehydroforms of the investigated bases /35 and 29% as compared to 11 and 12 per cent, after 96 hours/.

2/ All injected alkaloids almost immediately were transformed partially into other alkaloids. The degree of transformation of dehydroforms especially of dehydrolupanine was much higher as compared to the saturated bases. The rate of transformation was increasing during the time under investigation. After 96 hrs: a/ the injected sparteine was transformed into: dehydrosparteine - 12%, lupanine - 9%, hydrolupanine + hydroxylupanine ester - 3% and multiflorine - 3%; b/ the injected dehydrosparteine: lupanine - 17%, hydroxylupanine + its ester - 10%, multiflorine - 10%. 8% the radioactivity applied could be found also in sparteine; c/ the injected lupanine: dehydrolupanine - 4%, hydroxylupanine + its ester - 18%, multiflorine[x] - 6%, sparteine did not show any radioactivity; d/ dehydrolupanine: hydroxylupanine + its ester - 33%, multiflorine - 10%; 10% of the radioactivity applied could be found in lupanine./About the same value was obtained 2 hrs after injection/; no radiactive sparteine was found.

In experiment with fodder lupin plants:
1/ the total radioctivity of plants during 96 hrs did not change. The radioactivity found in non-alkaloid compounds:

x/ not very well purified

after a/ sparteine - 30%, b/ dehydrosparteine - 44%, c/ lupanine - 20%, d/ dehydrolupanine - 38%.

2/ the transformations of injected alkaloids into other bases investigated after 96 hrs: a/ sparteine: dehydrosparteine - 21%, lupanine - 2%, hydroxylupanine + its ester - 1%; a_x - Rf value the same as the Rf of multiflorine - 1%; b/ dehydrosparteine: lupanine - 4%, hydroxylupanine + its ester - 3%, a_x - 0.3%. 7% could be found in sparteine /the same value was obtained 2 hrs after injection and did not change afterwards; c/ lupanine:dehydrolupanine - 8%, hydroxylupanine + its ester - 9%; a_x - 0.3% /sparteine did not show any radiactivity/. d/ dehydrolupanine : hydroxylupanine + its ester - 37%; a_x - 1.4%, 9% was found in lupanine. No radioactive sparteine was found.

DISCUSSION

The results concerning the purified enzymatic extracts from bitter and fodder plants of Lupinus albus show that they are very similar in their reaction to changes in temperature, pH, the kind of substrates, their concentrations and to inhibitors and activators investigated. The similarity of these extracts was also revealed in the fact, that their enzymatic activity led to the formation of only one product, which did not undergo further transformation; this product was always a dehydroform of the alkaloid applied. Thus it can be assumed that enzymes, which caused these transformations, were dehydrogenases of analogous characteristics in both investigated forms of white lupin.

The investigated extracts from both kinds of plants showed some differences in their activity depending on the substrate used. But this fact is not yet proof that the extracts contained several dehydrogenases. It is known, that some dehydrogenases of a very little specificity may show different activity depending on the kind of the substrate applied. The enzymatic activity of extracts from other species indicates, that dehydrogenases, which have affinity to the investigated compounds are rather widespread. The behaviour of hydroxylupanine in unpurified extracts indicate that this alkaloid as compared to sparteine and lupanine is much more labile /this was confirmed in our other investigations/.

The results obtained in vivo show that the interconversion of alkaloids proceeds mainly from a lower to a higher level of oxidation. The facts of transformation of dehydrosparteine into sparteine and of dehydrolupanine into lupanine show, that a reverse direction of interconversion is possible; these facts however may be an "artefact" resulting from the technique applied /injection, translocation on one hand - localisation of transformations in intact plants on the other hand/.

It is possible that the low content of alkaloids in fodder white lupin results not only from a very low rate of synthesis from nonalkaloid precursors /this was established in our previous works/ but also from the low rate of interconversion of the alkaloids themselves. It must be added that the transformation of introduced alkaloids into nonalkaloid compounds in fodder plants was greater than in the bitter ones /but the differences in isotope dilution could also play a role in this phenomenon/.

The results obtained in vivo and in vitro permit the assumption that the general pattern of alkaloid transformation in white lupin is as follows:

alkaloid /introduced/ --> dehydroform --> another alkaloid
↓
nonalkaloid compounds

The interconversion of the investigated alkaloids:

sparteine
↓
dehydrosparteine --> multiflorine /bitter variety/
↓
lupanine
↓
dehydrolupanine
↓
hydroxylupanine --> hydroxylupanine ester
↓
dehydrohydroxylupanine

CONCLUSIONS

1/ Enzymatic extracts from bitter as well as fodder populations of white lupin are able to transform sparteine, lupanine, hydroxylupanine and angustifoline i.e. alkaloids characteristic of the species investigated. The transformations are catalyzed by dehydrogenases present in the extracts. The best method of enzymatic extract preparation was protein sedimentation from aceton powder with /$NH_4/_2SO_4$ /33-66% of saturation/.

2/ The highest enzymatic activity in purified extracts from fodder plants was found in the protein fraction, eluted from a hydroxylapatite column with 0.16 - 0.18 M phosphate buffer. The amount of this fraction accounted for less than 3% of the total protein content in the extracts investigated.

3/ The optima of temperature, pH and of substrate concentration /sparteine, lupanine and hydroxylupanine/ for extracts from plants of both investigated varieties are analogous.

4/ Sparteine, lupanine, hydroxylupanine and angustifoline were transformed by purified enzymatic extracts - from bitter as well as fodder plants - into their dehydroforms. In unpurified extracts hydroxylupanine only underwent transformations also to other compounds, which do not react with Dragendorff's reagent.

5/ Sparteine and lupanine after injection into plants of bitter and fodder lupinus albus are transformed into other alkaloids: sparteine is transformed mainly into dehydrosparteine, lupanine and hydroxylupanine; lupanine : into dehydrolupanine and hydroxylupanine; dehydrosparteine and dehydrolupanine after injection show w similar pattern of transformation, but they can be partially converted also into sparteine and lupanine respectively. Lupanine and dehydrolupanine were not transformed in any case into sparteine.

6/ One of the products of sparteine and dehydrosparteine transformation - after injection into bitter plants - is also multiflorine.

7/ The character and the rate of transformations of sparteine, lupanine and their dehydroforms indicate, that the interconversion of these alkaloids in plants of bitter as well as fodder white lupin proceeds mainly from lower to higher levels of oxidation.

On the basis of the results obtained in the described experiments it seems worthwhile to continue investigations 1/ on the dehydrogenases from bitter and fodder plants, 2/ on transformations of other alkaloids /besides sparteine and lupanine/ characteristic of the species investigated, 3/ on the alkaloid a_x in fodder lupin. /We must interrupt the investigations mentioned in p.1 for 8-10 months, because Mr Nalborczyk went abroad/.

II. The synthesis of alkaloids in bitter white lupine.

Our previous investigations /1958-59/ on bitter white lupine /var.Czechnicka/ showed, that the greatest increase of the alkaloid in plants occurred during a relatively short time, in the period of side shoot flowering and pod formation on the main shoot. During the subsequent period of growth the alkaloid content did not change. Very similar facts were observed in the fodder variety /Przebędowski/.

The aims of the reported investigations were:
a/ to determine the role of particular aerial parts in the biosynthesis of alkaloids at the time of the greatest alkaloid increment /the insignificant role of roots in this process was shown in our earlier investigations/;
b/ to identify two alkaloids whose chromatographic behaviour was similar to 1/ sparteine, 2/ oxolupanine /called previously by us a_z or /oxylupanine ?/;
c/ to examine the total and specific radioactivity of particular alkaloids in order to understand their interconversion better.

In 1960 pot experiments were carried out: on the 54-th day of growth /beginning of side shoot flowering/ following treatments were applied: 1/ control; 2/ 5 upper leaves removed; 3/ side shoot leaves removed; 4/ pods from the main shoot removed. On the same day a part of the plants investigated /from treat 1, 3 and 4/ were exposed to $C^{14}O_2$ for one and two weeks in a plexiglass chamber /750 l vol./. The plants in the greenhouse were harvested several times at weekly intervals. According to the results obtained the contributions of side shoots to the accumulation of alkaloids amounted to 57-70%. The radiactivity of lupanine accounted for about 80% of the total alkaloid activity. But in this experiment the removal of particular plant parts brought about a reaction of the remaining vegetative organs, consisting in a more intense growth and in a greater rate of alkaloid synthesis. The greatest alkaloid accumulation, in control plants was observed not between 56 and 70 days of growth /as in the experiment 1958/ but 10-14 days later. Therefore in experiments 1961 and 1962 the technique was changed.

Experimental procedure: 1/ experiments 1961 seeds of bitter white lupin were sown in pots on april 24. On the 62nd day of growth the plants were divided into several groups. In the

first group some plants were deprived of 1/ side shoots or 2/ pods. They were harvested together with controls after 7 days. On the day of the harvest the same treatments were applied in the second group. The next harvest was completed also after 7 days. On the 76th day of plant growth the experiment was repeated in the same way. The last harvest was completed at full maturity. On the 66th and 73th day of growth analogous treatments were applied and the plants were exposed to $C^{14}O_2$. After 24 hrs they were killed /-60°C and analysed. In the first experiment with labelled carbon excised stems and pods were also investigated.

2/ experiment 1962: bitter white lupin was sown on February 23. On the 63rd day of growth plants were exposed to $C^{14}O_2$, for 3 hrs; than a part of them was harvested; from the remaining plants pods and stems were cut off and exposed to $C^{14}O_2$ for 21 hrs.

In the plants harvested in the greenhouse total alkaloid content only was determined. Alkaloids extracted from the radioactive plants were separated on a celulose column /n-butanol: 0.1n HCl/ and distilled /or sublimated/ at 10^{-3}T; total and specific radioactivity was determined.

From side shoots of lupin investigated in 1960 alkaloids were extracted and separated on a celulose column. Fractions corresponding chromatographically to sparteine hydroxylupanine and 17-oxolupanine were isolated, purified, distilled in vacuo and analysed in IR-spectrometer.

RESULTS AND DISCUSSION

I. Identification of three alkaloid fractions

a/ The amount of the fraction whose Rf value was similar to the Rf of sparteine accounted for only about 0.5% of the total content in the investigated side shoots; after distillation at 90-95°C it yielded an oily substance, whose IR absorption spectrum was identical with the IR spectrum of sparteine prod. CEFARM and with the IR-spectrum of this base, published by Leonard and Beyler /1950/.

b/ The alkaloid fraction, whose Rf value was similar to the Rf of hydroxylupanine[x/] was sublimated at 130-140°C. Its IR-spectrum showed the same maxima of absorption /-OH at 3500 cm^{-1}, -C=O group in lactam at 1630 cm^{-1}/ and the same vibrations in the "finger print" region as hydroxylupanine isolated from seeds of bitter lupinus angustifolius /the hydroxylupanine from the fodder variety differs somewhat in the "finger print".

c/ The IR absorption spectrum of the purified alkaloid fraction whose Rf was similar to the Rf of 17-oxolupanine, was analogous to the IR spectrum of an alkaloid isolated from vegetative organs of fodder plants of lupinus albus and identified as hydroxylupanine ester. The investigated fraction after hydrolysis in HCl yielded hydroxylupanine.

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x/ This fraction contains - in very small amounts - a base which in all probability is dehydrolupanine, but the purification procedure applied causes its decomposition.

II. According to the results presented in tab.4, the alkaloid increment during the first week amounted to 37% of the content, found on the 62nd day of plant growth. During the subsequent period /till full maturity/ there were no significant changes in the total content of the investigated bases. After the first week of investigation the reaction of the plants to pod removal can be clearly seen /a more intense growth of side shoots/. But the same treatment applied a week later did not cause any marked changes. The removal of side shoots in the first period brought about a very small alkaloid increment whereas the removal of pods had no influence on the total increase of these compounds.

The results of the first experiment with $C^{14}O_2$ /table 5/ show that: 1/ the contribution of side shoots to the total carbon assimilated was about 70%, 2/ the plants deprived of pods were less radioactive than the controls /the same phenomenon was observed in the experiment 1960 and in an additional experiment in 1962; the difference - in comparison with the control - in the amount of C^{14} translocated to the roots could not have been considered as the main cause of the difference in the total radioactivity of the aerial parts/; 3/ the amount of CO_2 assimilated only by pods accounted for 1.5% and assimilated by the stem - 0.3% of the total.

Similar results concerning the side shoot contribution to the total CO_2 assimilation were obtained in the experiment carried out one week later /tab.6/. However it must be added, that the rate of photosynthesis was less /CO_2 content and activity, conditions of temperature and light were very similar in both experiments/.

The total alkaloid activity amounted in the first experiment to about 0.29%, in the second one to about 0.03 per cent of the total plant activity. If the difference in the rate of photosynthesis is taken into account, thus the alkaloid synthesis in the second experiment can be considered as very low, less than 10% of the synthesis rate found one week earlier.

The side shoots were the main organs, in which alkaloid synthesis occurred during the period under investigation; in the first week their contribution to the C^{14} alkaloid accumulation was about 90%. Although the total alkaloid activity in plants deprived of pods was less than in the controls, but it amounted also to about 0.30 per cent of the total plant activity. According to the results obtained in the experiment 1962 /table 7/ it may be concluded that: 1/ pods are not able to synthesize alkaloids /from nonalkaloid compounds/ at least at the investigated stage of their development, 2/ the stem of main shoot show this capacity, but the participation of alkaloids - formed after 21 hrs - in the total stem activity /about 0.03 per cent/ is very low in comparison with their participation in stems of intacts plants /tables 2 and 3/. Therefore it can be assumed that in the latter the most part of C^{14}alkaloid found in stems resulted from translocation from other organs, mainly from the side shoots.

The alkaloid content in particular plant parts of 62 and 69 days old plants /table 1/ and the distribution of alkaloid activity among these parts /table 3/ show, that the new formed

alkaloids /at least those derived from the products of current photosynthesis/ had been translocated during 24 hrs from side shoots and leaves of the main shoot at a greater rate than the alkaloids earlier accumulated in these plant organs.

The radioactivity of side shoot alkaloids in particular in plants deprived of pods can give some informations about the relationships between the alkaloids investigated: 1/ the specific /per mg/ activity of lupanine was 3-5 times higher than that of hydroxylupanine /whose content accounted only for 1/3 of the lupanine content/ and multiflorine. In parts that show a small capacity of alkaloid synthesis, e.g. leaves of the main shoot or in parts whose C^{14} alkaloids derived mainly from other organs e.g. the stem the specific activity of the hydroxylupanine ester /which included also the activity of the bound organic acid/ was very high and sometimes even greater than the specific activity of lupanine.

It is possible that a part of hydroxylupanine was bound in the stem with an organic acid of a relatively high radioactivity.

The results concerning lupanine and hydroxylupanine /content and activity/ indicate that the interconversion of these two alkaloids - under conditions, where no injection of an alkaloid takes place - would proceed from the lower to the higher level of oxidation. The reverse direction would have been probable only in the case when the rate of C^{14} -hydroxylupanine transformation into lupanine had been so high that the specific activity of hydroxylupanine in the side shoots could not have been significant. It is difficult to accept this possibility, when the quantitative relationships between the two alkaloids /not only in this experiment but also in our previous investigations/ are borne in mind.

CONCLUSIONS /including the results of the first experiment/.

1/ The greatest alkaloid accumulation in bitter white lupin occurs during two to four weeks after the beginning of side shoot flowering /this fact confirms our previously obtained results/.

2/ At this time the side shoots are mainly responsible for the alkaloid synthesis. At the first stage of accumulation their contribution accounted for 70 and in the later period for about 90 per cent of the alkaloid increment.

3/ At the late stages of growth the total alkaloid content in plants does not undergo any marked changes. This is in all probability the result of inhibition of synthesis as well as of decomposition of these compounds.

4/ The ageing process of leaves on the main as well as on the side shoots brings about a diminishing in the rate of alkaloid synthesis.

5/ The pods were not able to syntheze alkaloids, the stem of the main shoot showed this capacity but to a very small degree.

6/ At the early stage of pod development the translocation of new formed alkaloids from leaves of the main shoot as well as from side shoots is greater than the translocation of alkaloids earlier accumulated.

7/ On the basis of the infra red spectra of investigated alkaloids a/ the occurrence of sparteine in side shoots /in very small amount/ could be established, b/ the alkaloid of the Rf value similar to the Rf value of 17-oxolupanine was identified as an ester of hydroxylupanine and an organic acid, c/ the hydroxylupanine occurring in the side shoots was shown to be identical with the hydroxylupanine from seeds of Lupinus angustifolius.

The interconversion between lupanine and hydroxylupanine seems to proceed from the lower to the higher level of oxidation.

It seems worthwhile to repeat the experiment with older bitter lupin plants, when the total alkaloid content does not change, and to examine the rate of C^{14} incorporation into alkaloids /an analogous course of alkaloid accumulation - but different as regards the total amounts - was observed previously in fodder white lupin/.

III. The described experiments drew our attention mainly to the side shoots of the investigated plants. That s why in the experiments with bitter and fodder homozygotes of lupinus at the beginning of side shoots flowering the pods were removed from the main shoots and the plants were allowed to grow for another week in the greenhouse. Afterwards they were put into a plexiglass chamber and exposed to $C^{14}O_2$ for 4 hours. A part of them was killed /-60oC/ immediately and the remaining plants were exposed for 20 hrs to $C^{12}O_2$. Then they were killed and have been analysized. We are now determining the alkaloid and amino-acid content as well as their specific activity. We pay great attention to arginine.

IV. The plants /bitter and fodder homozygotes/ grown in the greenhouse were harvested several times during the vegetation period, till full maturity. The weather conditions were unusual this year and perhaps this is the main reason why a very early fall of leaves from the main as well as from the side shoots was observed. We are not content with the size and number of seeds we have obtained /they are to be investigated next year/. The harvested plants are analysed /alkaloids and nitrogen/.

RESULTS

The dry weight and alkaloid content /per plant/

Table 4

Plant parts	Age of plants /days/ 62 control	69 control	69 plants deprived: side shoots	69 plants deprived: pods	76 control	76 plants deprived: side shoots	76 plants deprived: pods
	dry weight - g d.m. per plant						
main shoot	3.01	3.06	3.08	3.03	3.17	2.86	3.16
side shoots	1.62	1.70	-	2.28	1.80	-	1.85
pods x/	0.66	1.62	1.12	-	1.96	2.00	-
total	5.29	6.38	4.20	5.31	6.93 xx/	4.86	5.01
	alkaloid content mg per plant						
main shoot	10.3	11.3	10.4	11.2	11.4	6.6	12.3
side shoots	18.1	14.3	-	32.9	10.9	-	14.2
pods	7.6	23.7	11.6	-	30.0	29.3	-
total	36.0	49.3	22.0	44.1	52.3	35.9	26.5

x/ of the main shoots

xx/ the total dry weight at the stage of full maturity was 7.46 g per plant.

Radioactivity[x] of 67 days old plants and alkaloids after 24 hr exposure to $C^{14}O_2$ /per plant/

Table 5

Treatment	plant parts	total alkaloid content mg	total plant activity 10^3c m p	Alkaloid[xx] activity c p m: specific activity la	hl	mf	hl ester	total activity la	hl	mf	hl ester	total	alkaloid activity % of total plant activ.
Control	main shoot leaves	2.2	665	70	66	105	250	24	71	18	70	203	0.03
	stem	8.6	499	448	560	625	446	2186	529	575	526	3616	0.72
	side shoots	15.4	1442	607	125	116	223	6282	179	108	598	7167	0.50
	pods	21.6	2267	156	165	150	79	1736	577	572	196	3081	0.16
	total	47.8	4873					10288	1316	1073	1390	14067	0.29
Side shoot removed	main shoot leaves	2.0	746	93	90	121	294	107	26	23	91	257	0.03
	stem	8.2	251	62	63	405	219	308	72	174	256	810	0.32
	pods	20.3	538	19	19	10	51	186	73	30	147	436	0.08
	total	30.5	1535					601	181	227	494	1503	0.10
Pods removed	main shoot leaves	2.1	758	180	172	246	228	243	69	26	64	402	0.05
	stem	9.0	787	238	229	427	626	1076	343	393	1070	2882	0.37
	side shoots	18.0	2125	638	215	231	163	6762	662	446	398	3268	0.39
	total	29.1	3670					8081	1074	865	1532	11552	0.31
Main stem excised			15										
Main stem /on roots/			12										
Pods excised			72										

x/ In all experiments total plant radioactivity was determined with a Geiger-Miller counter /mica window, 1.1 mg/cm Ø 25 mm/ the alkaloid activity in a 2 π gas-flow counter /firm/ Friesecke und Höpfner/ whose efficiency was 3 times higher, the data obtained for alkaloids were divided by 3.

xx/ la - lupanine, hl - hydroxylupanine, mf - multiflorine, hl -ester-hydroxylupanine ester /this last fraction contains a minimal amount of an alkaloid of a similar Rf/, the base -ay- which occurs only in traces was not taken into account, angustifoline -found in relatively very small amount could not have been separated from lupanine therefore its specific radioactivity was not determined.

Radioactivity of 74 days old plants and of their alkaloids after 24 hrs exposure to $C^{14}O_2$ /per plant/

Table 6

Treatment	plant parts	total alkaloid content	total plant activity 10^3 c p m	alkaloid activity c p m		alkaloid activity % of the total plant activity
				specific	total	
Control	main shoot					
	leaves	0.96	453	148	142	0.03
	stem	9.03	197	46	415	0.21
	side shoots	14.37	697	26	374	0.05
	pods	28.50	1179	8	228	0.02
	total	52.86	2526		1159	0.04
Side shoots removed	main shoot					
	leaves	0.85	392	152	137	0.03
	stem	8.98	62	9	81	0.13
	pods	27.73	375	2	55	0.01
	total	37.56	829		273	0.03
Pods removed	main stem					
	leaves	0.90	729	202	182	0.03
	stem	13.20	543	53	700	0.13
	side shoots	12.63	882	35	442	0.05
	total	26.73	2154		1324	0.06

Radioactivity of alkaloids in the stem and pods of the main shoot /per plant/

Table 7

Time of harvesting	plant parts	fresh weight g	alkaloid content mg	plant activity 10³ c p m	alkaloid activity c p m	
					specific	total
After 3 hrs exposure to $C^{14}O_2$	main shoot					
	leaves + side shoots	10.18		1320		
	stem	4.75	2.93	282	9	26
	pods	10.65	11.78	183	0	0
	total	25.58		1785		
After subsequent 21 hrs exposure to $C^{12}O_2$	excised					
	stem	4.89	2.88	263	40	115
	pods	11.10	12.49	192	0	0