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BIOCHEMICAL ASPECTS OF THE BIOSYNTHESIS OF OPIUM ALKALOIDS*

ANTONÍN JINDRA, PETER KOVÁCS, ZITA PITTNEROVÁ and MIKULÁŠ PŠENÁK

Department of Biochemistry, Faculty of Pharmacy, Comenius University, Bratislava, Czechoslovakia

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Abstract—Enzyme systems contained in poppy plants (*Papaver somniferum* L.) at different stages of development catalyse hydroxylations, oxidative deaminations, transaminations and decarboxylations of precursors of opium alkaloids. Activities of corresponding enzymes were demonstrated by both *in vivo* and *in vitro* experiments. Hydroxylation of phenylalanine could not be demonstrated, but an active phenolase was present catalysing the hydroxylation of tyrosine and the oxidation of 3,4-dihydroxyphenylalanine. These reactions were inhibited by sodium diethyldithiocarbamate and activated by L-ascorbic acid. A direct relation was observed between phenoloxidase activity and the content of the main opium alkaloids (thebaine, codeine, morphine) in poppy plants. Deamination of different amino acids, including aromatic amino acids, was demonstrated by *in vitro* experiments in the presence of catechol: corresponding oxo-derivatives were formed. These substances may also arise in *P. somniferum* plants by transaminations as was shown by *in vitro* experiments in the presence of α-ketoglutaric acid and pyridoxal phosphate. Aromatic amino acids were also decarboxylated by enzyme systems of *P. somniferum*; in these reactions corresponding amines were formed. The results described showed the importance of enzymic catalysis in the transformation of basic precursors in the first phase of biosynthesis of opium alkaloids where the 1-benzylisoquinoline structure is formed.

INTRODUCTION

THIS paper discusses results obtained by examination of certain enzyme systems in poppy plants (*Papaver somniferum* L. "Váhovecký") connected with the metabolism of the presumed precursors of opium alkaloids, as shown by experiments using labelled substances. As in these latter experiments, the study of enzyme activities involved in the biosynthesis of alkaloids leads to several difficulties; negative results may not be decisive proof that the activities studied are not important and on the contrary positive results are not absolute evidence that under normal conditions the activity is related to the biosynthesis of the alkaloids.[1–3] It is important to remember that *in vitro* experiments inevitably mean that damage has been done to the organization of plant tissues, and this may be followed either by the inactivation of normally active enzymes or by the activation of enzyme systems which are inactive under normal conditions.

The aromatic amino acids phenylalanine, tyrosine and 3,4-dihydroxyphenylalanine play a principal role in the biosynthesis of opium alkaloids[4–7] and their formation and transformation are connected with the activities of transaminases, decarboxylases, deaminases

* Dedicated to Professor Dr., Dr.h.c., Dr.h.c. K. Mothes at the occasion of the anniversary of his 65th birthday.

[1] K. MOTHES, *Hauptversammlung und wissentschaftliche Tagung der Pharmazeut. Gessel.* Leipzig (1961).
[2] A. R. BATTERSBY, R. BINKS and J. T. HARPER, *J. Chem. Soc.* 3534 (1962).
[3] E. A. H. ROBERTS, *Adv. Enzymol.* **2**, 119 (1942).
[4] E. LEETE, *Science* **147**, 1000 (1965).
[5] K. MOTHES and H. R. SCHÜTTE, *Angew. Chem.* **75**, 265, 357 (1963).
[6] A. R. BATTERSBY, *Proc. Chem. Soc.* 189 (1963).
[7] D. H. R. BARTON, *Proc. Chem. Soc.* 293 (1963).

and phenoloxidases in the plant. These enzymes are involved not only in the fundamental metabolism of these amino acids but also in reactions resulting in the formation of precursors or intermediates important in the biosynthesis of opium alkaloids (Fig. 1). Of all the assumed precursors of such intermediates only a few have been demonstrated in *P. somniferum* plants: phenylalanine and tyrosine,[8,9] phenylpyruvic and *p*-hydroxyphenylpyruvic acids,[10] reticuline[12] and salutaridine.[11] It may be supposed that phenylalanine and tyrosine originate from sugars via the shikimic acid pathway, but other reactions governing their presence are also important.[13] Their release from proteins as a result of the activity of proteolytic enzymes and their incorporation into these macromolecules is obviously of some interest.

When studying the biochemical aspects of the biosynthesis of opium alkaloids it is necessary to elucidate not only the relations between the basic precursors and end-products, but also the exact sequence of reactions leading to the latter. This may be deduced first of all

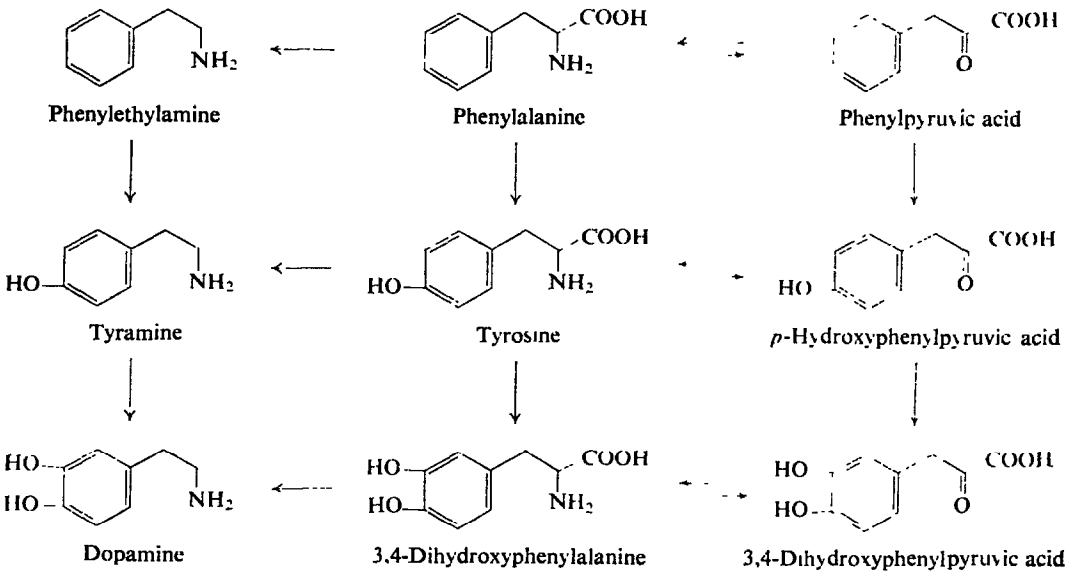


FIG. 1. INTERRELATIONSHIPS BETWEEN AROMATIC AMINO ACIDS AND RELATED COMPOUNDS.

by an examination of enzymic reactions catalysing the transformations in question.[5,14–16,18] The localization and distribution of individual enzymes and their substrates in the plant is also of great importance.[1–3,17] Further progress in such studies should add weight to the

[8] P. KOVÁCS, Habilitation Treatise, Comenius University, Bratislava (1964).
[9] G. KLEINSCHMIDT, *Planta Med.* **8**, 114 (1960).
[10] A. JINDRA, Z. ŠÍPAL and V. HUDECOVÁ, *Experientia* **20**, 371 (1964).
[11] D. H. R. BARTON, G. W. KIRBY, W. STEGLICH, G. M. THOMAS, A. R. BATTERSBY, T. A. DOBSON and H. RAMUZ, *J. Chem. Soc.* 2423 (1965).
[12] A. R. BATTERSBY, R. BINKS, R. J. FRANCIS, D. J. MCCALDIN and H. RAMUZ, *J. Chem. Soc.* 3600 (1964).
[13] D. D. DAVIES, J. GIOVANELLI and T. A. P. REES, *Plant Biochemistry*. Blackwell, Oxford (1964).
[14] E. LEETE, In *Biogenesis of Natural Compounds* (Edited by P. BERNFELD), p. 763. Pergamon Press, Oxford (1963).
[15] A. J. BIRCH, *Proc. Chem. Soc.* 3 (1962).
[16] G. KLEINSCHMIDT, *Abhandl. Deut. Akad. Wiss. Berlin Kl. Chem. Geol. Biol.* No. 4, 265 (1963).
[17] N. A. BURGES, In *Enzyme Chemistry of Phenolic Compounds* (Edited by J. B. PRIDHAM), p. 1. Pergamon Press, Oxford (1961).
[18] K. HASSE, *3rd Int. Symp. Biochem. Physiol. Alkaloide*, Halle/Saale 1965, Rahmenvorträge, p. 22.

above-mentioned schemes of opium alkaloid biosynthesis, which most probably represent the process in living plants.[4, 5, 14] Of course it must be realized that not all reactions in plants are enzymatically catalysed, some of them may proceed spontaneously.

RESULTS AND DISCUSSION

Transaminase Activity

Transamination in systems of both pyruvate to alanine and α-ketoglutarate to glutamic acid was demonstrated qualitatively in germinating seeds of poppy plants with endogenous amino acids as donors of amino groups. These reactions were followed by detecting the corresponding amino acids by paper chromatography. The transamination with α-ketoglutaric acid as acceptor was shown to be far stronger. These preliminary experiments formed a basis for evaluation of transaminase activities in *P. somniferum* by quantitative *in vitro* experiments.

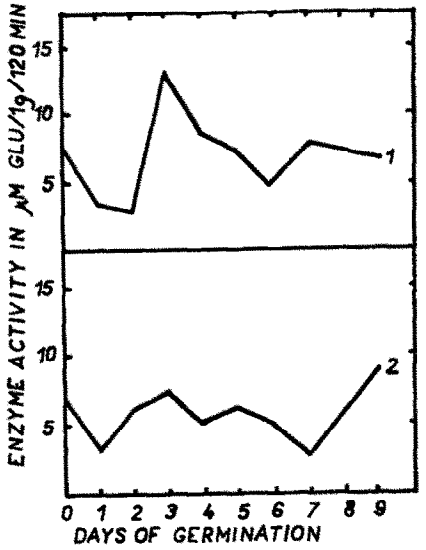


FIG. 2. TRANSAMINASE ACTIVITIES IN GERMINATING SEEDS OF *Papaver somniferum* L.
(1) With phenylalanine, (2) with tyrosine.

The results shown in Fig. 2 demonstrate that the activities are present in seedlings of *P. somniferum* and vary during the germination period. The activity of phenylalanine–α-ketoglutaric acid aminotransferase reached its maximum at the third day of germination and then slowly decreased. The activity of tyrosine–α-ketoglutaric acid aminotransferase showed a similar course, but in the later stages gradually increased again. The activities of the transaminase systems in different phases of development of poppy plants are shown in Table 1. The results show that transamination of phenylalanine, tyrosine and 3,4-dihydroxyphenylalanine can occur during the whole period of growth. The activity of the enzyme catalysing the transamination in the system 3,4-dihydroxyphenylalanine–α-ketoglutarate was also examined on the fifth day of germination and was shown to be 2·6 μmoles of glutamate/g/120 min. The reactions were confirmed by the identification of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid (as corresponding quinoxalinols) which were formed from phenylalanine and tyrosine used as donors of the amino groups.

TABLE 1. TRANSAMINASE ACTIVITIES IN ORGANS OF *P. somniferum* L. PLANTS

	Enzyme activity*						
	Stage of development III_2		Stage of development IV_1		Stage of development V		
Substrate	Leaves	Roots	Leaves	Roots	Leaves	Roots	Capsules
Phenylalanine	0·6	0·9	1·0	0·3	3·3	0·9	1·3
Tyrosine	1·8	1·2	2·5	0·4	2·5	1·2	1·5
DOPA	0·8	0·8	0·9	0·2	3·3	1 0	1 4

* μmoles glutamate formed/g/120 min from α-ketoglutarate.

The reactions studied are reversible; this was proved in a system where glutamic acid was used with phenylpyruvic and *p*-hydroxyphenylpyruvic acids as acceptors. The rate of formation of phenylalanine was found to be 4·2 μmoles/g/120 min, and of tyrosine 2·0 μmoles/g/120 min.

These experiments demonstrate the presence of active transaminases in *P. somniferum* plants catalysing the transformation of aromatic amino acids (phenylalanine, tyrosine 3,4-dihydroxyphenylalanine) to the corresponding keto acids, and show the possible mechanism of the formation of direct precursors of 1-benzylisoquinolines. The enzymic preparations inactivated by heat were all ineffective.

Decarboxylase Activity

The results obtained in an examination of decarboxylase activities in seedlings of poppy plants are given in Table 2. The activities against the aromatic amino acids (phenylalanine, tyrosine, 3,4-dihydroxyphenylalanine) are lower than against glutamic acid.[19] The corresponding amines formed in the reactions with tyrosine and glutamic acid as substrates were identified using paper chromatography. Heating the enzyme results in a complete inactivation. The addition of hydroxylamine at 8×10^{-5} M[44] resulted in a 50 per cent inhibition.

TABLE 2. DECARBOXYLASE ACTIVITY IN GERMINATING SEEDS OF *P. somniferum* L.

	Enzyme activity (μl CO_2/g/hr) Days of germination								
Substrates	1	3	5	7	9	10	12	13	
Phenylalanine	—	19·8	—	—	24·0	6·2	12·7	9·4	31·0
Tyrosine	12·0	—	28·4	5·7	44·7	30·2	17 6	25·8	4·3
DOPA	—	14·5	5·4	—	26·0	*	*	*	*
Glutamic acid	77·6	120·2	202·8	172·9	143·2	136·5	145·3	67·3	—

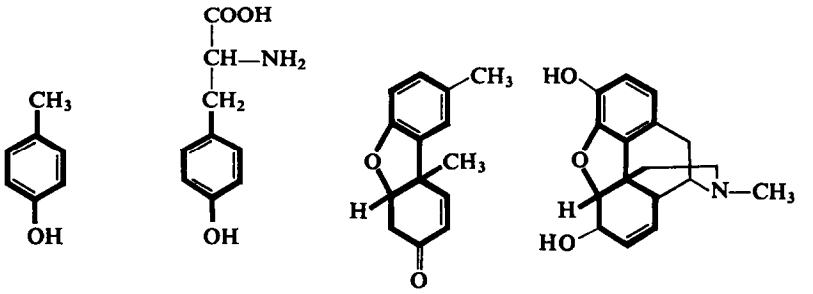
* Not analysed.

[19] R. P. SMITH, G. E. CWALINA and E. RAMSTAD, *J. Am. Pharm. Assoc. Sci. Ed.* **48**, 103 (1959).

On the basis of these results decarboxylases may be assumed to be present and catalyse the transformation of the aromatic amino acids to the corresponding amines which are important precursors of opium alkaloids.

Phenoloxidase Activity

The importance of oxidative reactions catalysed by systems of phenoloxidases was stressed many years ago by Robinson[20] and Schöpf and Thierfelder,[21] when discussing biosynthetic processes concerning opium alkaloids; these authors showed for the first time the significance of the oxidation and condensation of phenolic compounds in the formation of opium alkaloids from their precursors. This hypothesis has been confirmed by the work of Pummerer *et al.*,[22,23] Barton and Cohen,[24] Erdtman and Wachtmeister,[25] Ollis[26] and Pridham.[27] Reactive intermediates formed by the oxidation of phenolic compounds may be transformed, under certain conditions, to substances containing several types of bonds: —C—C—, —C—O—, —C—N—: e.g. the intermediates resulting by oxidation of *p*-cresol are transformed to Pummerer's ketone in addition to other substances. The relationship in structures of *p*-cresol (1), tyrosine (2), Pummerer's ketone (3) and morphine (4) shows the probable importance of such reactions in the biosynthesis of the latter compound.



(1) (2) (3) (4)

The best-known phenolic precursors in the biosynthesis of opium alkaloids, i.e. tyrosine, 3,4-dihydroxyphenylalanine and reticuline, may be transformed by similar oxidative reactions. We attempted therefore to show the phenoloxidase activity in different parts of *P. somniferum* plants by *in vitro* experiments using the substances in question as substrates.

The results obtained in experiments with germinating seeds are shown in Fig. 3. It is interesting to note the large increase of *o*-diphenoloxidase activity during the second and third day of germination. The appearance of the main opium alkaloids (thebaine, codeine, morphine) in germinating seeds at the same stage of development was demonstrated in parallel experiments using thin-layer chromatography.[28]

[20] R. Robinson and S. Sugasawa, *J. Chem. Soc.* 3163 (1931); 789 (1932); 280 (1933).
[21] C. Schöpf and K. Thierfelder, *Liebigs Ann. Chem.* **497**, 22 (1932).
[22] R. Pummerer, D. Melamed and H. Puttfracken, *Ber. Deut. Chem. Ges.* **55**, 3116 (1922).
[23] R. Pummerer, H. Puttfracken and P. Schopflocher, *Ber. Deut. Chem. Ges.* **58**, 1808 (1925).
[24] D. H. R. Barton and T. Cohen, *Festschrift A. Stoll*, p. 117. Birkhäuser, Basel (1957).
[25] H. Erdtman and C. A. Wachmeister, *Festschrift A. Stoll*, p. 144. Birkhäuser, Basel (1957).
[26] W. D. Ollis, *Recent Developments in the Chemistry of Natural Phenolic Compounds.* Pergamon Press, Oxford (1961).
[27] J. B. Pridham, *Enzyme Chemistry of Phenolic Compounds.* Pergamon Press, Oxford (1963).
[28] D. Neubauer and K. Mothes, *Planta Med.* **9**, 466 (1961).

The results given in Figs. 4 and 5 were obtained when the activities of the enzyme in both leaves (Fig. 4) and roots (Fig. 5) of *P. somniferum* at different stages of development were examined.[29] The activities are obviously present during the whole period of growth.[30,31] In

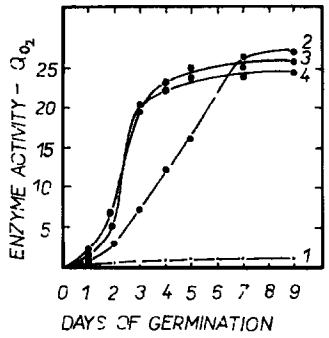


FIG. 3. PHENOLOXIDASE ACTIVITIES IN GERMINATING SEEDS OF *Papaver somniferum* L.
(1) Tyrosine, (2) *p*-cresol, (3) DOPA, (4) catechol.

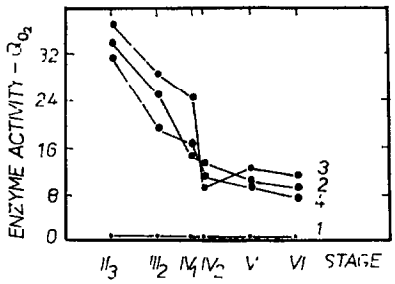


FIG. 4. PHENOLOXIDASE ACTIVITIES IN LEAVES OF *Papaver somniferum* L. PLANTS.
(1) Tyrosine, (2) *p*-cresol, (3) DOPA, (4) catechol.

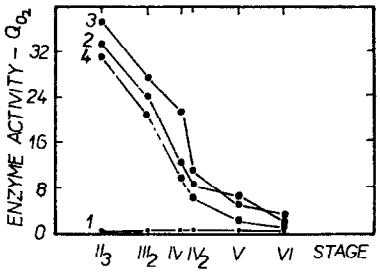


FIG. 5. PHENOLOXIDASE ACTIVITIES IN ROOTS OF *Papaver somniferum* L. PLANTS.
(1) Tyrosine, (2) *p*-cresol, (3) DOPA, (4) catechol.

the developed leaves phase (II_3) the oxidation of *p*-cresol, catechol and 3,4-dihydroxyphenylalanine was relatively very strong, but older plants showed low enzyme activities (Figs. 4

[29] S. SÁRKÁNY and B. DÁNOS, *Acta Botan. Acad. Sci. Hung.* **3**, 293 (1957).
[30] P. KOVÁCS, M. PŠENÁK and A. JINDRA, *Herba Hung.* **2**, 145 (1963).
[31] P. KOVÁCS, M. PŠENÁK and A. JINDRA, *Čsl. Farmácia* **13**, 179 (1964).

and 5). Only in the flowering phase and during the growth of capsules (V) did the polyphenoloxidase activity show a sudden increase. Tyrosine oxidase activity was very low in all parts during the whole period examined.

In side roots, the enzyme activities were examined only in phases IV_2, V and VI. In the phase IV_2 *p*-cresol oxidase and catechol oxidase were more active (Q_{O_2} 22·7 and 18·3 respectively) in comparison to 3,4-dihydroxyphenylalanine oxidase activity, (Q_{O_2} 10·5); tyrosine oxidase even in these parts was practically undetectable. In older plants (phases V and VI) *p*-cresol oxidase and catechol oxidase activities dropped considerably (to Q_{O_2} 14·7 and 11·6, and Q_{O_2} 7·2 and 7·4 respectively); on the other hand the activity of the enzyme with 3,4-dihydroxyphenylalanine increased (Q_{O_2} 14·4 and 13·1 respectively). In the capsules the 3,4-dihydroxyphenylalanine oxidase activity was also higher (in phase IV_2 Q_{O_2} was 13·6 and in V 18·9). In the rapid growth phase activities with all substrates were nearly the same, but decreased very rapidly towards the end. In the ripening phase (VI) the Q_{O_2} values for individual substrates were: for 3,4-dihydroxyphenylalanine 6·1, *p*-cresol 4·4 and catechol 5·3. Even in the capsules the activity of tyrosine oxidase was always very low. It should be noted that 3,4-dihydroxyphenylalanine oxidase activity was found in side roots at all stages.

TABLE 3. INFLUENCE OF INHIBITOR AND ACTIVATOR ON PHENOLOXIDASE ACTIVITIES

Substrate	Sodium diethyldithiocarbamate % Inhibition	L-Ascorbate % Activation
Catechol	81	11
p-Cresol	100	70

The results obtained show the possible participation of phenoloxidases in the formation of derivatives of norlaudanosoline as a result of oxidative condensation of 3,4-dihydroxyphenylalanine or its biochemical equivalents. The influence of phenoloxidases may thus lead to the formation of opium alkaloids. The inhibitory effect of sodium diethyldithiocarbamate and the effect of L-ascorbate, expressed in per cent of inhibition and activation respectively, calculated from differences of Q_{O_2} values obtained in experiments with and without inhibitor of activator added, are given in Table 3. At the concentrations used both substances strongly affected the activity of phenoloxidase, particularly against *p*-cresol. The inactivated enzyme preparations were completely ineffective in experiments with both model or natural substrates.

Secondary oxidative deamination of amino acids as result of the action of phenoloxidases has been demonstrated in higher plants;[32, 33] the products of this reaction are ammonia and corresponding ketones or aldehydes. The results given in Table 4 were obtained with enzyme preparation from poppy plants and catechol; if these were absent no oxygen uptake took place and no ammonia was formed during incubation of the amino acids in question (3,4-dihydroxyphenylalanine being an exception). The most intensive deamination (under the conditions used) was when using glycine, 3,4-dihydroxyphenylalanine or phenylalanine, the

[32] W. C. JAMES, E. A. H. ROBERTS, H. BEEVERS and P. C. DE KOCK, *Biochem. J.* **43**, 626 (1948).
[33] V. R. POPOV, *Biokhimiya* **21**, 380 (1956).

last two being important in the biosynthesis of the opium alkaloids. The products of the oxidative deamination of phenylalanine, tyrosine and 3,4-dihydroxyphenylalanine (phenylpyruvic, *p*-hydroxyphenylpyruvic and 3,4-dihydroxyphenylpyruvic acids) are important intermediates in this biosynthesis. These experiments show that these derivatives may be formed in poppy plants by oxidative deamination[34] in addition to the transamination reactions described earlier.

One of the direct effects of phenoloxidase is the *o*-hydroxylation of monophenols to the corresponding *o*-diphenols. From the point of view of the biosynthesis of opium alkaloids, the most interesting reaction is the transformation of tyrosine to 3,4-dihydroxyphenylalanine. This reaction was demonstrated by *in vitro* experiments by determination of decreasing amounts of tyrosine in the incubation mixture and by identification of the 3,4-dihydroxyphenylalanine formed as shown in Figs. 6 and 7. During the incubation, both in the presence and in the absence of L-ascorbic acid, a decrease in amounts of tyrosine could be demonstrated (see Fig. 7); at the same time the appearance of the spots corresponding to 3,4-dihydroxyphenylalanine was observed. The identification of 3,4-dihydroxyphenylalanine was confirmed by re-chromatography of the eluted substance.

TABLE 4. SECONDARY OXIDATIVE DEAMINATION OF SOME AMINO ACIDS BY CATECHOLASE FROM *P. somniferum* L.

Substrate	Amounts of deaminated substrates (in μg/g dry material)
Glycine	625
Alanine	140
Glutamic acid	215
Phenylalanine	325
Tyrosine	175
3,4-Dihydroxyphenylalanine with catechol added	360
3,4-Dihydroxyphenylalanine without catechol added	285

In experiments with increased amounts of L-ascorbic acid ($3{\cdot}6 \times 10^{-2}$ M final concentration) other ninhydrin-positive spots were detected on chromatograms; the corresponding substances could not be identified; nevertheless some of them probably correspond to previous results.[35]

3,4-Dihydroxyphenylalanine, which is a key-substance of all schemes suggested for the formation of opium alkaloids in plants could not, however, be demonstrated in a free form in the plant material in question.[36] It might, however, be present in a glycosidic form, as found in other higher plants, and thus protected for certain steps involved in the biosynthesis of the alkaloids.[37]

The effect of inhibitors and activators on plants in *in vivo* experiments was then studied. The influence of these substances on enzyme activity was studied along with parallel determinations of the main opium alkaloids—thebaine, codeine and morphine.

[34] P. KOVÁCS and A. JINDRA, *Naturwissenschaften* **52**, 395 (1965).
[35] S. LISSITZKY and M. ROLLAND, *Nature* **193**, 881 (1962); *Biochim. Biophys. Acta* **56**, 83 (1962).
[36] P. KOVÁCS and A. JINDRA, *Experientia* **21**, 18 (1965).
[37] R. S. ANDREWS and J. B. PRIDHAM, *Nature* **205**, 1213 (1965).

The inhibitor (sodium diethyldithiocarbamate) or the activator (L-ascorbate) were applied to germinating seeds. Both *p*-cresol and catechol oxidase activities were strongly influenced

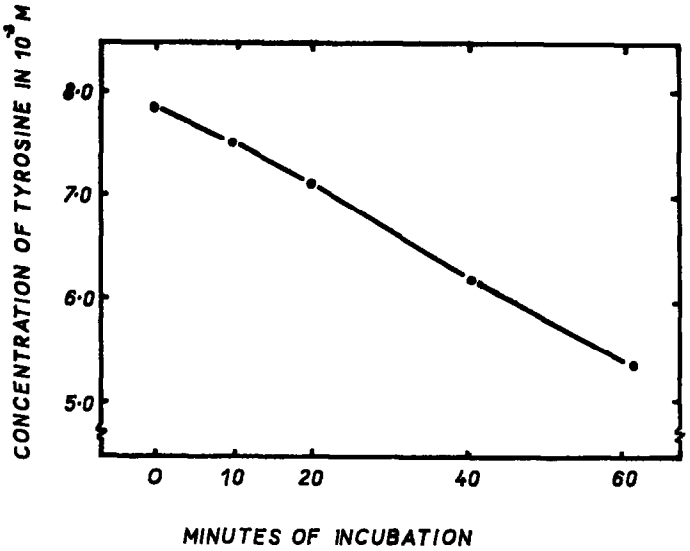


FIG. 6. DECREASE IN TYROSINE CONCENTRATION WITH TYROSINE OXIDASE.

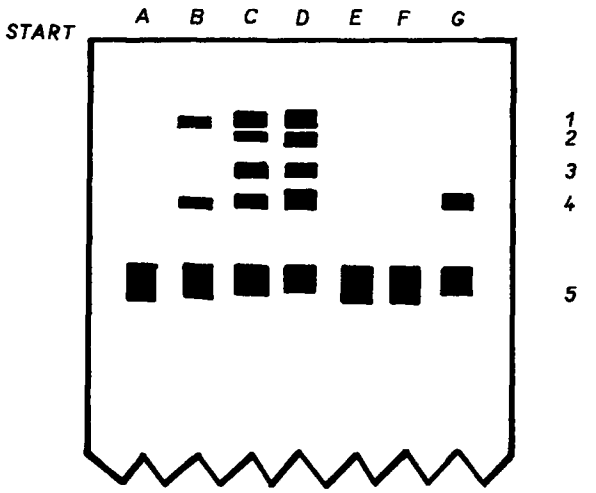


FIG. 7. PAPER CHROMATOGRAPHIC PATTERN OF THE FORMATION OF 3,4-DIHYDROXYPHENYLALANINE. (1), (2), (3) Unidentified products; (4) 3,4-dihydroxyphenylalanine; (5) tyrosine and L-ascorbic acid. Samples taken at (A) 0 min, (B) 20 min, (C) 40 min, (D) 60 min. Sample with inactivated enzyme; (E) at 0 min, (F) after 60 min; (G) standards.

as shown in Table 5. It is interesting to note that the content of examined alkaloids also varied as shown in Fig. 8. The results obtained showed that the effect of diethyldithiocarbamate produced a decrease in content of morphine and thebaine; the content of codeine was not significantly influenced. L-Ascorbate caused a noticeable increase in the content of

all alkaloids analysed. For analysis the same weights of plant material were used (the inhibitor and the activator influenced the morphology of the germinating seeds). The activities of phenol-oxidases appear to be directly related to the amounts of thebaine and morphine synthesized in the *P. somniferum* plants.

TABLE 5. INFLUENCE OF INHIBITOR AND ACTIVATOR ON PHENOLOXIDASE ACTIVITIES IN GERMINANT SEEDS OF *P. somniferum* L. PLANTS

Substrates	Activities of phenoloxidases in Q_{O_2} units		
	Control experiments	Inhibited (sodium diethyldithiocarbamate)	Activated (L-ascorbate)
p-Cresol	16·4	6·2	21·1
Catechol	24·0	9·7	30·5

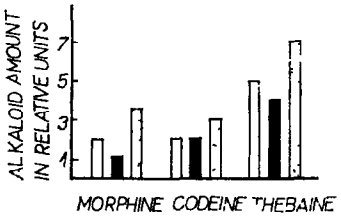


FIG. 8. INFLUENCE OF INHIBITOR AND ACTIVATOR ON ALKALOID CONTENT IN GERMINATING SEEDS OF *Papaver somniferum*.

White: control; black: inhibited by sodium diethyldithiocarbaminate; dotted: activited by L-ascorbate.

EXPERIMENTAL

Plant Material

All the experiments were carried out with plants of *Papaver somniferum* L. "Váhovecký". Individual enzyme activities were studied in seedlings and in fully grown plants.

Germinating seeds. The seeds (1–2 g) were germinated in Petri dishes (14 cm) on moistened filter paper, Whatman No. 1, at 18–20°. The paper was moistened daily. The seeds were used for experiments on each day up to the 9th.

Poppy plants in field-culture. The poppy plants were grown under normal conditions of soil and climate in the Botanical Garden of the University in Bratislava. During the vegetation period samples were taken in each of the main phases of development as specified by Sárkány and coworkers:[29] the phase of developed leaves (II_3), the final stage of rosette (III_1, III_2), during the rapid growth phase (IV_1, IV_2), in the flowering phase and growth of capsules (V) and in the ripening phase (VI). Plants were collected at the same hour of the day (at 8–9 a.m.). Average samples from twenty plants were used and the following parts separated: main roots, side roots, stalks, leaves (from the upper third of the plant) and capsules. (Only at the first stage, II_3, were the whole plants used.) The plant material was cooled immediately to −5° and homogenized in a glass apparatus at 0 for 4 min in a suitable buffer solution. In some cases acetone powders were prepared from the plant material in the usual way[38] (at −20°). The determination of the dry matter content was carried out at 105°.

[38] P. KOVÁCS and Z. NAGYOVÁ, *Čsl. Farmácia* **12**, 32 (1963).

Determination of Enzyme Activities

Transaminases. The presence of these enzymes were first proved qualitatively by *in vivo* experiments; it was shown that in germinant seeds pyruvic acid and α-ketoglutaric acid were efficient acceptors of amino groups from endogenous amino acids. The acids, as sodium salts (3 ml, 0·1 M), were applied to 5-day-old seeds (0·3 g) by *in vacuo* infiltration (4 min),[39] and the seeds then left on wet filter paper for 150 min. Distilled water was used in control experiments. Afterwards the germinant seeds were washed several times by distilled water and the content of free amino acids was determined. The seeds were homogenized with several portions of 4 ml of 80% ethanol. The combined extracts were evaporated to dryness *in vacuo* and the residue extracted several times by 10% isopropanol.[40] The amino acids were analysed by paper chromatography (Whatman No. 1 with *n*-butanol–acetic acid–water (4:1:5);[41] detection: 0·5% solution of ninhydrin in acetone with 10% pyridine; fixation of chromatograms using the solution of $Cu(NO_3)_2$ in acetone[42]). Alanine and glutamic acid were analysed against suitable standards.

The reversible activity of transaminases was further studied by *in vitro* experiments. α-Ketoglutaric acid and an amino acid (phenylalanine, tyrosine, 3,4-dihydroxyphenylalanine) were incubated at 40° with a homogenate of germinating seeds prepared from 2 g of plant material and 12·5 ml of a 0·2 M phosphate buffer (pH 8·0) and dialysed against the same buffer solution for 24 or 48 hr at 0–2°. The incubation mixtures contained 3 ml of plant homogenate, 0·5 ml of a solution of amino acid (20 μmoles), 0·5 ml of a solution of α-ketoglutaric acid (20 μmoles) neutralized by addition of 0·1 N NaOH to pH 6·5–7·0 and 0·5 ml of a solution of pyridoxal phosphate (1 μmole). As a control experiments were carried out without the amino acid.

The rate of transamination reaction between α-ketoglutaric acid and the aromatic amino acids used was studied by determination of the amount of glutamic acid formed and by characterization of the aromatic α-keto acids formed. At suitable intervals 0·1 ml of the incubation mixture was taken and separated by paper chromatography as described above except when examining 3,4-dihydroxyphenylalanine when *n*-propanol–water (3:1) was used.[38] After detection by ninhydrin the spots corresponding to glutamic acid were cut out, and eluted with 4 ml of methanol and the intensity measured photometrically at 495 nm, and compared with a standard curve prepared in the same way. The results are given in μmoles of glutamic acid formed after 120 min incubation. The transamination was not observed heat-denaturated preparations (10 min at 100°) were used. The aromatic α-keto acids formed during the incubation were identified by paper chromatography of condensation products of these substances with *o*-phenylenediamine.[43]

The reversibility of the transaminase activity was studied in the system formed by glutamic acid and an aromatic α-keto acid (phenylpyruvic or *p*-hydroxyphenylpyruvic acid). The experiments were carried out with homogenates of 8-day germinated seeds. The incubation mixtures contained: 3 ml of plant homogenate, 0·5 ml of the solution of the corresponding α-keto acid (40 μmoles), 0·5 ml of the solution of glutamic acid (20 μmoles) and 0·5 ml of the solution of pyridoxal phosphate (1 μmole). The conditions of incubation were the same as described above.

[39] V. L. KRETOVICH and O. L. POLYANOVSKIY, *Biokhimiya* **24**, 995 (1959).
[40] L. GRACZA, *Acta Pharm. Hung.* **35**, 20 (1965).
[41] S. M. PARTRIDGE, *Biochem. J.* **42**, 238 (1948).
[42] T. TURSKÝ, *Biológia* **12**, 118 (1957).
[43] K. H. NIELSEN, *J. Chromatog.* **10**, 463 (1963).

The rate of transamination between the aromatic α-keto acids and glutamic acid was followed by identification and determination of the aromatic amino acids formed as described before. The results are given in μmoles of phenylalanine or tyrosine formed respectively.

Decarboxylases. The activity of phenylalanine, tyrosine, 3,4-dihydroxyphenylalanine and glutamic acid decarboxylases were determined using a modified manometric method.[44] The evolution of CO_2 was measured in the atmosphere of nitrogen at 37° during 60 min. The homogenates were prepared in the way described above using 4 g of fresh plant material and 20 ml of a 0·15 M phosphate buffer solution, pH 5·6. The mixture contained 2 ml of plant homogenate, 0·3 ml of a 25 mM solution of corresponding amino acid and 0·2 ml of 0·04 mM pyridoxal phosphate. For comparison, experiments were carried out without the corresponding amino acids. The amount of evolved CO_2 without exogenous substrates was subtracted from that found in experiments with added substrate. The results are given in μl CO_2/g fresh plant material/hr.

Preparations denaturated by heat (10 min at 100°) were completely without activity either with or without added substrates. Hydroxylamine in 8×10^{-5} M concentration[44] was used in inhibition experiments.

The amines resulting from the decarboxylation of the amino acids were identified by paper chromatography as before only the samples with tyrosine and glutamic acid were used.

Phenoloxidases. Phenoloxidases were demonstrated using both monophenolic and *o*-diphenolic substrates (tyrosine, 3,4-dihydroxyphenylalanine, *p*-cresol and catechol) by the manometric method.[30] The consumption of O_2 was measured in the atmosphere of air at 26° during 60 min. The homogenates were prepared from 4 g of fresh plant material and 14·0 ml of 0·2 M phosphate buffer solution, pH 6·5. The activities of phenoloxidases are given in standard Q_{O_2} units (μl O_2/mg of dry substance/hr).

The activities of phenoloxidases were also examined in the presence of sodium diethyldithiocarbamate (final concentration, 3×10^{-4} M) and L-ascorbate (final concentration, 5×10^{-2} M).[45] (The solutions were always freshly prepared.) The effect of heat denaturation (10 min at 100°) was also examined.

Acetone powders of leaves from plants in the development phase II_3 caused the deamination of certain amino acids (glycine, phenylalanine, tyrosine, 3,4-dihydroxyphenylalanine). For this reaction the presence of the catechol system was necessary. Enzymic preparations (acetone powders) were prepared from homogenates of leaves as described elsewhere.[34] The incubation mixtures contained 2·5 ml of enzyme preparation, 0·5 ml of a solution of catechol (final concentration, 0·002 M), 0·5 ml of the solution of the different amino acids (final concentration, 0·02 M) and 0·2 ml of 2 N KOH. The individual samples were incubated at 30° for 60 min in Warburg vessels in an atmosphere of air and the consumption of O_2 was measured. The ammonia released at the end of the incubation was determined by the Conway microdiffusion technique.[46]

The hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine catalysed by tyrosine oxidase was demonstrated by *in vitro* experiments. As enzyme preparations, solutions of acetone powders from plants in phase II_3 were used (1·2 g of acetone powder was dissolved in 15·0 ml of 0·2 M phosphate buffer, pH 7·05), dialysed at 0–2° for 24 hr against the same buffer. The incubation mixture contained 5 ml of enzyme preparation, 1·0 ml of tyrosine

[44] S. P. COLOWICK and N. O. KAPLAN (Eds.), *Methods in Enzymology*, Vol. 2, p. 190. Academic Press, New York (1955).
[45] P. KOVÁCS, Dissertation, Charles University, Prague (1961).
[46] B. KEIL and Z. ŠORMOVÁ, *Laboratorní technika biochemie*, p. 256. ČSAV, Praha (1959).

(final concentration, $7{\cdot}8 \times 10^{-3}$ M) and 0·5 ml of water. In other experiments 0·5 ml of a solution of L-ascorbic acid as an activator (final concentration, $1{\cdot}8 \times 10^{-3}$ M and $3{\cdot}6 \times 10^{-3}$ M respectively) were used.

The incubation was carried out at 30° for 60 min. At different time-intervals the samples of incubation mixtures were analysed both by determination of tyrosine and by chromatographic identification of 3,4-dihydroxyphenylalanine formed.[36]

3,4-Dihydroxyphenylalanine formed during a 40-min incubation was characterized by chromatography as described above; the 80% ethanolic eluate of the spots was concentrated in nitrogen and aliquots were re-chromatographied in the usual way using Partridge's solvent[41] and a mixture of *n*-propanol–water (3:1)[38] respectively.

Analysis of Opium Alkaloids

The main alkaloids (thebaine, codeine and morphine) contained in germinating seeds and plants were identified by thin-layer chromatography.

The alkaloids were extracted from plant material (3 g of plant material ground with purified sea sand) by shaking them with 60 ml of 1 N HCl for 1 hr.[47] After filtration the solution was made alkaline with ammonia to pH 8·0–8·5 and immediately extracted three times with chloroform. The combined extracts were washed with water, dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in a known volume of chloroform and the aliquots analysed by thin-layer chromatography.[28] For detection Dragendorff's reagent was used. Semiquantitative determination of alkaloids was carried out by comparing the size of the spots of known amounts of alkaloids. The results are given in relative units.

Influence of Activators and Inhibitors on Phenoloxidase Activity and Opium Alkaloid Content in in vivo *Experiments*

The seeds were germinated as before, the filter paper being moistened with 9 ml of the solution of inhibitor or activator respectively. After 2 min excess solution was decanted. In the inhibition experiments the paper was moistened with a solution of sodium diethyldithiocarbamate trihydrate (20 mg in 9·0 ml H_2O); the second day only distilled water was used and the third day the inhibitor (10 mg in 9·0 ml H_2O). The treatment was continued on alternate days till the seventh day, when the seeds were washed three times with distilled water and used for determination of enzyme activities and alkaloid content.

In the activation experiments, germination was carried out as above using 50 mg of L-ascorbic acid in 9·0 ml of distilled water on the first day, and a solution of 40 mg in the same volume every other day.

[47] A. JINDRA, M. FELKOVÁ, V. JIRÁČEK and J. BÖSWART, *Planta Med.* **12**, 305 (1964).
[48] I. M. HAIS and K. MACEK, *Handbuch der Papierchromatographie*, Band I. Fischer Verlag, Jena (1958).