REDUCTION OF QUATERNARY BENZOPHENANTHRIDINE ALKALOIDS BY NADH AND NADPH

Jan KOVÁŘ[a], Jaromír STEJSKAL[a], Hana PAULOVÁ[b] and Jiří SLAVÍK[b]

[a] *Department of Biochemistry, University of J. E. Purkyně, 611 37 Brno and*
[b] *Department of Medical Chemistry and Biochemistry, University of J. E. Purkyně, 662 43 Brno*

Received June 26th, 1985

Spectroscopic techniques have shown that quaternary benzophenanthridine alkaloids (sanguinarine (*Ia*), chelerythrine (*Ie*), sanguilutine (*If*), sanguirubine (*Ic*), chelirubine (*Ib*), and chelilutine (*Id*)) are reduced by NADH or NADPH to their dihydro forms. The formation of the oxidized form of a coenzyme was demonstrated by HPLC. The kinetics of the reaction of sanguinarine with NADH has been studied rather in detail; the reaction was found to be reversible, its stoichiometry was 1 : 1. Reactivity of the tested alkaloids toward the coenzymes was given by reactivity of the imine bond. This was graded like pK values for the formation of the corresponding pseudobases and correlated with the energy of the lowest unoccupied molecular orbital of these compounds. The possible biological and pharmacological importance of the studied reactions is discussed.

The quaternary benzophenanthridine alkaloids *Ia*–*If* occur in various species of the families *Papaveraceae*, *Fumariaceae*, and *Rutaceae*[1]. These substances affect the metabolic processes in bacteria or animals and can act as, *e.g.*, antibacterial, antitubular or antitumour substances[2–6]. The effects of these substances *in vivo* are closely related to their interactions with important macromolecular compounds of cells, especially with certain enzymes, such as Na/K – ATPase[7], choline esterases[8,9], and amino transferases[10]. Interactions of these substances with biopolymers depend on their chemical reactivity[11,12]. The substances can readily react with —SH groups of proteins and low-molecular-weight compounds, get converted (in moderately alkaline media) to pseudo-bases *II*, and can quite easily be reduced to the corresponding dihydro compounds *III*. All these reactions occur on the imine bond of the substances (Scheme 1), which is a natural site for attack by nucleophilic groups (OH^-, —SH, H^- or CN^-, ref.[12]).

The objective of this paper is to describe the reactions of these substances with NADH and NADPH, *i.e.*, with the reduced forms of nicotinamide adenine dinucleotide coenzymes present in considerable quantities in all cells. These reactions may be metabolically important in the parent plants and in the bacterial or animal cells, where these substances get as drugs.

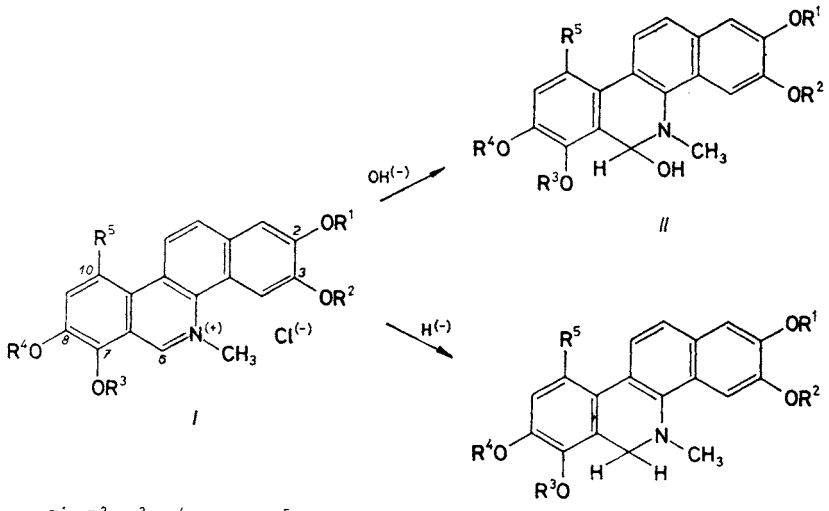
EXPERIMENTAL

Materials

Sanguinarine chloride, sanguirubine chloride, sanguilutine chloride, chelerythrine chloride, chelirubine chloride were isolated at the Department of Medical Chemistry and Biochemistry, Faculty of Medicine, University of J. E. Purkyně, Brno. The coenzymes were products of Boehringer (Mannheim, F.R.G.): NADH (disodium salt, grade II, 98%), NAD^+ (free acid, grade II, 98%), NADPH (tetrasodium salt, 98%), and $NADP^+$ (disodium salt, 98%). The buffers (0·1 mol l^{-1}) were prepared from distilled water for the following pH ranges: acetate (pH 4·0—5·5), Na-phosphate (pH 6·0—8·0), and Na-carbonate (pH 9·0—10·0).

Methods

Determination of p*K of the formation of pseudo-bases* (Scheme 1): These values were determined spectrophotometrically (Cary 118, Varian, U.S.A.) from the decrease in absorbance of the



a, $R^1 + R^2 = R^3 + R^4 = CH_2$; $R^5 = H$
b, $R^1 + R^2 = R^3 + R^4 = CH_2$; $R^5 = OCH_3$
c, $R^1 = R^2 = CH_3$; $R^3 + R^4 = CH_2$; $R^5 = OCH_3$
d, $R^1 + R^2 = CH_2$; $R^3 = R^4 = CH_3$; $R^5 = OCH_3$
e, $R^1 + R^2 = CH_2$; $R^3 = R^4 = CH_3$; $R^5 = H$
f, $R^1 = R^2 = R^3 = R^4 = CH_3$; $R^5 = OCH_3$

SCHEME 1

long-wave band of the quaternary form of an alkaloid with an increase in pH at 25°C. Ten pH values were used, the concentration of alkaloids being 50 μmol l^{-1}. The p*K* values were calculated, by means of a non-linear regression[13], from the equation:

$$A = (A_1 + A_2 \cdot 10^{(pH - pK)})/(1 + 10^{(pH - pK)}), \qquad (1)$$

wherein A designates the absorbance at a given pH, A_1 the absorbance of the quaternary form of an alkaloid, and A_2 the absorbance of a pseudo-base (in most cases the value of A_2 was close to zero).

The reaction rates were measured spectrometrically (Cary 118) at wave lengths of the long-wave peak of the quaternary forms of the alkaloids, and at 340 or 346 nm (decrease in NADH or NADPH). The formation rate of dihydrosanguinarine was measured fluorimetrically in a standard fluorimeter (Aminco–Bowman, U.S.A.) (λ_{exc} = 320 nm, λ_{em} = 435 nm), or in a stopped-flow apparatus (Aminco–Morrow, U.S.A.), equipped with a high-pressure Xe-lamp (λ_{exc} = 320 nm), using an emission cut-off filter for 415 nm.

High-performance liquid chromatography: the chromatograph consisted of a pump VCM 300 (Development Workshops of the Czechoslovak Academy of Sciences), a glass column (150 × × 4 mm) packed with Silasorb C_{18}, 5 μm (Laboratorní přístroje, Czechoslovakia), and a detector unit with an adjustable wave length (LKB, Sweden), set at 259 or 340 nm (absorption peaks of the coenzymes). The mobile phase for isocratic elution of the coenzymes was a 25 mmol l^{-1} Na-phosphate buffer, pH 6, with 3 vol. % of methanol, the flow-rate being 0·5 ml min^{-1}. The sample was applied from an injector Rheodyne (U.S.A.), having a 10 or 20-μl sample loop. The absorbance was recorded with an apparatus TZ 4 200 (Laboratorní přístroje, Czechoslovakia).

The redox potential of sanguinarine/dihydrosanguinarine was determined in relation to the redox potential of the pair $NADH^+/NADH$ from analysis of the equilibrium mixtures of sanguinarine (*Ia*), NADH and NAD^+ (pH 5·5, after standing for 2 or 3 h at 25°C). The difference between the redox potentials of the two pairs (ΔE^0) was calculated from the equation:

$$\Delta E^0 = 0{\cdot}058/n \,.\, \ln ([IIIa]/[Ia] \,.\, [NAD^+]/[NADH]). \qquad (2)$$

The concentration of sanguinarine (*Ia*) was determined spectrophotometrically, the concentration of dihydrosanguinarine (*IIIa*) fluorometrically, and that of NADH by means of HPLC; an excess of NAD^+ was used and the value of $n = 2$ was assumed.

The quantum-chemical calculations were performed by the topological method of Pancíř[14,15].

RESULTS AND DISCUSSION

The absorbance at c. 340 nm (characteristic of NADH) and at c. 470 nm (characteristic of the quaternary form of sanguinarine) gradually decreased after sanguinarine and NADH (in concentrations of c. 10–100 μmol l^{-1}) had been mixed in neutral or mildly acid aqueous buffers. The other alkaloids having similar structures (Scheme 1) behaved analogously. Like phenomena were observed if NADH was replaced by NADPH. At room temperature these reactions attained equilibria within 1–2 h (the absorbances no longer changed).

The reaction of sanguinarine with NADH was followed in greater detail; it has proved to be reduction of sanguinarine (*Ia*) to dihydrosanguinarine (*IIIa*, Scheme 1), accompanied by oxidation of NADH to NAD^+. The formation of *IIIa* was demonstrated by UV - Vis and fluorescence spectra, as described above. The disappearance of the band at 470 nm, the decrease of absorbances at 280 nm and 320 nm, and the moderate increase of absorbances at 300 nm were practically the same in the reduction of sanguinarine with NADH and/or $NaBH_4$ (to dihydrosanguinarine[16]). Also

the fluorescence emission spectrum of the product obtained by reaction of sanguinarine with NADH was identical with that of dihydrosanguinarine (*IIIa*), prepared by reduction of sanguinarine (*Ia*) with $NaBH_4$ (Fig. 1). The decreased fluorescence of NADH (emission peak at c. 460 nm) was almost negligible compared to the intense fluorescence of dihydrosanguinarine, *IIIa* (at equimolar concentrations of sanguinarine (*Ia*) and NADH at the start of the reaction it was less than one twentieth). The formation of NAD^+ from NADH in the presence of sanguinarine (*Ia*) was demonstrated by reverse-phase chromatography, in which sanguinarine (*Ia*) and dihydrosanguinarine (*IIIa*) were retained in the column, whereas NAD^+ and NADH were eluted and well separated (phosphate buffer, pH 6, with a little methanol). The peak of NADH ($k = 5{\cdot}2$) was much lower after the reaction with sanguinarine (*Ia*), whereas that of NAD^+ ($k = 4{\cdot}1$), negligible with pure NADH, was much higher after it. Quantitative analysis of spectroscopic and chromatographic data obtained after the reaction of sanguinarine and NADH at different ratios proved to accord with the assumed 1 : 1 stoichiometry of the reaction.

The reaction of sanguinarine with NADH was essentially reversible, because an addition of NAD^+ (c.1 mmol l^{-1}) to the mixture at the start of the reaction

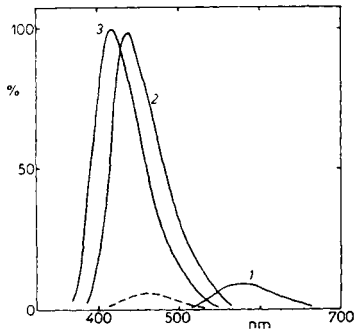


FIG. 1

Uncorrected fluorescence emission spectra of sanguinarine (*Ia*), dihydrosanguinarine (*IIIa*), and a pseudo-base of sanguinarine (*IIa*). The relative intensity of fluorescence (%) refers to $\lambda_{exc} = 320$ nm, 25°C, concentration of the compounds 10 μmol . l^{-1}; *1* at pH 5·5, *2* at pH 5·5 immediately after an addition of solid $NaBH_4$ or after 2-h standing in the presence of 20 μmol l^{-1} NADH, *3* at pH 8·5, (-----) spectrum of 20 μmol . l^{-1} NADH under the same conditions

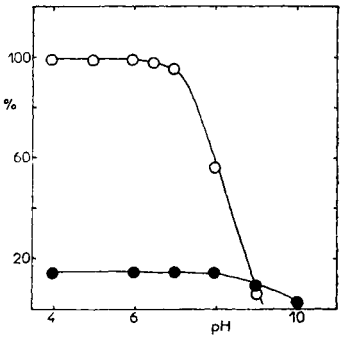


FIG. 2

Rates of reduction of sanguinarine (○) and chelerythrine (●) by NADH in relation to pH of the medium. The initial reaction rates were measured spectrophotometrically (λ = = 346 nm) at 25°C; the reduction rate of sanguinarine at pH 4—6 is taken as 100%

decreased the reaction rate. Also an addition of NAD^+ (c. 2 mmol l^{-1}) after the reaction of sanguinarine with NADH (after 2 hours' standing) partially reversed the reaction (increased concentrations of NADH and sanguinarine). With NADPH as the reducing agent the quantity of $NADP^+$ needed for reversing the reaction was greater. Further experiments were aimed at determining the approximate red-ox potential of the pair sanguinarine/dihydrosanguinarine, in relation to that of the pair NAD^+/ NADH, present in the mixture. A series of data was obtained from 12 experiments (at pH 5·5, 2 h at 25°C), the starting concentrations being: 20 and 40 μmol l^{-1} sanguinarine; 50, 100, and 200 μmol l^{-1} NADH, and 1, 2, and 3 mmol . . l^{-1} NAD^+. The concentrations of the substances in equilibrium solutions were determined as described in Methods. The value of ΔE^0 was calculated from Eq. (*2*) as 0·1 ± 0·03 V. This result suggests that the redox potential of sanguinarine (*Ia*) is comparable with those of flavine nucleotides, functioning in living organisms as natural acceptors of reduction equivalents from nicotinamide adenine dinucleotide coenzymes[17].

The rate of the reduction of sanguinarine with NADH (measured as decrease in concentration of NADH, spectrophotometrically at 346 nm, *i.e.*, in the isobestic point of the spectra of sanguinarine (*Ia*) and dihydrosanguinarine (*IIIa*)) was practically independent of pH in the range 4·0 to 6·5. Lower pH's were not tested, since NADH is unstable in acid solutions[18]. The reaction rate substantially decreased if pH exceeded 7·0 (Fig. 2). With chelerythrine (*Ie*, *cf.* Table I) the reaction rate did not start decreasing until pH was brought to higher values (Fig. 2). These results accord with the assumption that NADH reacts only with the quaternary form of the alkaloid, with no participation of protons or hydroxide ions (the p*K* values for the formation of pseudo-bases of the alkaloids are given in Table I). It appears likely that NADH and NADPH react like in the physiological reactions of these coenzymes (which are enzyme-catalysed), so that a hydride anion is directly transferred from the coenzyme to the quaternary form of the alkaloid.

The kinetics of the reduction of sanguinarine (*Ia*) with NADH was analysed in greater detail. Fig. 3 shows the dependence of the initial reduction rate on the concentration of NADH. This rate was determined by three methods. The decrease rate of *Ia* was followed spectrophotometrically at 470 nm, the decrease rate of NADH at 346 nm, the formation rate of *IIIa* was followed fluorometrically in a stopped-flow apparatus (the stopped-flow method makes it possible to determine the initial reaction rates more accurately, especially at higher concentrations of NADH). All the three methods gave practically the same results (Fig. 3); the decrease rates of *Ia* and NADH are consistent with the 1 : 1 stoichiometry in the reactions of these compounds (see above). The line in Fig. 3 proves that the reaction can be regarded as being of the pseudo-first order to NADH (in the presence of higher concentrations of *Ia*); the rate constant was 34 ± 2 mol l^{-1} s^{-1} at pH 5·5 and 25°C. The pseudo-first order of the reaction in respect to sanguinarine was demonstrated

in analogous experiments, when the initial reaction rate was measured in relation to concentration of *Ia*.

The temperature dependence of the rate constant of the reaction is shown in Fig. 4. The activation parameters obtained from the line are $\Delta H^{\neq} \approx 40$ kJ mol^{-1} and $\Delta S^{\neq} \approx -80$ J mol^{-1} K^{-1}. The relatively high negative value of the activation entropy is in keeping with the postulated direct transfer of a hydride anion from the coenzyme to the alkaloid. This reaction probably goes *via* a rather complex transition state, involving a molecule of the alkaloid and a molecule of the coenzyme. Direct transfer of H^- from 1,4-dihydropyridines to model sites of nucleophilic attack was corroborated even by quantum-chemical and thermodynamic calculations[20]. The model data on the transfer of H^- to a pyridinium cation, which shows resemblance to the reactive part of the compounds studied, also revealed a preferential formation of the so-called linear transition state, which is thermodynamically much preferable to the bent transition state, in which the aromatic rings are superimposed[20]. The comparatively low value of $\Delta H^{\neq}$ for the reaction investigated by ourselves suggests a linear transition state in this case, too. NADH and NADPH differ in reactivity with the benzophenanthridine alkaloids tested (Table I). The reduction by NADPH is faster than by NADH. This is in accordance with the more negative redox potential[17] of the pair $NADP^+$/NADPH, compared with the pair NAD^+/NADH. The alkaloids studied can be classified into two groups, according to reactivity with NADH or NADPH. The compounds with methylene-

TABLE I

Comparison of reactivity of quaternary benzophenanthridine alkaloids

Alkaloid	v_0(NADH)[a] %	v_0(NADPH)[a] %	pK[b]	Energy of LUMO MJ mol^{-1}
Sanguinarine (*Ia*)	100	121	8·05	−0·727
Chelirubine (*Ib*)	155	[c]	7·70	−0·728
Sanguirubine (*Ic*)	97	[c]	7·90	−0·727
Chelilutine (*Id*)	18	[c]	8·50	−0·708
Chelerythrine (*Ie*)	14	16	9·00	−0·705
Sanguilutine (*If*)	12	14	8·80	−0·705

[a] v_0 (NADH) and v_0 (NADPH) denote the relative initial reduction rates determined from the decreases of the long-wave absorption peaks of the alkaloids (430—500 nm) at pH 5·5 and 25°C, coenzyme concentration 0·2 mmol l^{-1} and alkaloid concentration 50 μmol l^{-1} (the rate with the pair sanguinarine–NADH is taken as 100%); the determined rates are charged with an error of c. 10%. [b] The values of pK were determined spectroscopically with an error of ±0·1 (they are close to the reported ones[19]). [c] Not measured.

dioxide groups at positions $C_{(7)}$ and $C_{(8)}$ are much more reactive than those with methoxyl groups at these positions (*cf. Ia–Ic vs Id–If* in Table I). The other substituents in the molecules of these natural alkaloids seem to be less influential, but again with the methylenedioxide groups at $C_{(2)}$ and $C_{(3)}$ the reduction was faster than with the methoxyl groups at these positions (*cf. Ia* and *Ib vs Ic* and *Id*, and *Ie vs If* in Table I). The reduction is also facilitated by a methoxyl at $C_{(10)}$ (*cf. Ia vs Ib*, and *Id vs Ie* in Table I). The reactivities of the studied compounds with NADH and NADPH are consistent with those on the imine bond (Scheme 1): the reduction rate steeply decreases with the increasing p*K* values of formation of the pseudo-base *II*, and with the increasing energy of the lowest unoccupied molecular orbital (LUMO) of the alkaloid; this value is essentially a measure of reactivity of a compound with a nucleophilic reactant. These data confirm the propounded mechanism of the reduction: the reduced nicotinamide ring of NADH or NADPH operates as a nucleophilic agent, directly attacking the $C_{(6)}$ atom of the benzophenanthridine alkaloid. An analogous reaction mechanism was described in the reduction of Δ^1-pyrroline-2-carboxylic acids with various 1,4-dihydropyridines[21].

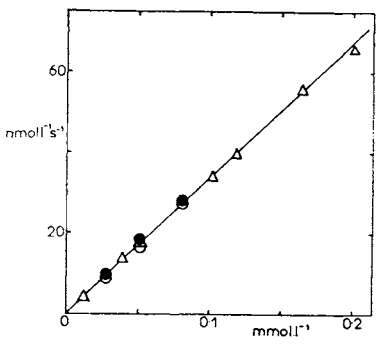


FIG. 3

Reduction rate of sanguinarine (nmol l^{-1} . s^{-1}) in relation to concentration of NADH (mmol l^{-1}). The data were obtained for 10 μmol l^{-1} sanguinarine (*Ia*) at pH 5·5 and 25°C; (○) initial rate of decrease of NADH (measured spectrophotometrically at 346 nm), (●) initial rate of decrease of the quaternary form of sanguinarine (measured spectrophotometrically at 470 nm), (△) initial rate of increase of dihydrosanguinarine (measured by the stopped-flow technique, the values are averages from four measurements)

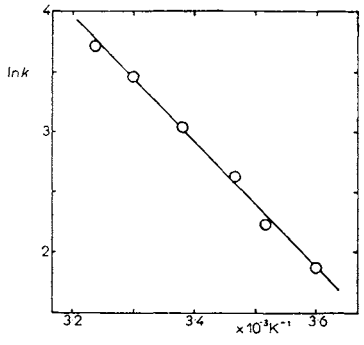


FIG. 4

Logarithm of the rate constant of reduction of sanguinarine (*Ia*) by NADH (ln *k*) in relation to reciprocal temperature (K^{-1}). The data were obtained by the stopped-flow method at pH 5·5, 10 μmol l^{-1} sanguinarine and 0·1 mmol l^{-1} NADH

The described experiments *in vitro* with quaternary benzophenanthridine alkaloids and NADH and/or NADPH suggest a possible role of these redox reactions in plants, where these substances occur. The significant content of dihydrosanguinarine in the roots of *Chelidonium majus* (0·045%), compared to the content of sanguinarine (0·138%, refs[22,23]) may be due to an *in vivo* reaction of sanguinarine with NADH or NADPH. The reactions described may operate even in the action of benzophenanthridine alkaloids on cells of other organisms. These reactions may, in principle, have two effects. The reaction of NADH or NADPH with the active and toxic quaternary forms of the alkaloids may be one of the mechanisms of detoxication of these substances (dihydrosanguinarine was really demonstrated as the detoxication product of sanguinarine in the fungus *Verticillium dahliae*[24]). Besides, the formation of the dihydro forms may enhance their permeation through biological membranes (the dihydro forms are much more hydrophobic than the quaternary forms). Better permeation through biological membranes, compared to the quaternary forms, had been observed with other hydrophobic derivatives of benzophenanthridine alkaloids, *viz.* their adducts with alcohols[5].

Acknowledgement for the quantum-chemical calculations are due to Dr P. Tvarůžek.

REFERENCES

1. Krane B. D., Moses O. F., Shamma M.: J. Nat. Prod. Lloydia *47*, 1 (1984).
2. Preininger V. in the book: *The Alkaloids* (R. H. F. Manske, Ed.), vol. 15, p. 207. New York, Academic Press 1975.
3. Kondo Y.: Heterocycles *4*, 197 (1976).
4. Odebiyi O. O., Sofowora E. A.: Planta Med. *36*, 204 (1979).
5. Mitscher L. A., Park Y. H., Clark D., Clark G. W.: J. Natl. Prod. *41*, 145 (1978).
6. Lenfeld J., Kroutil M., Maršálek E., Slavík J., Preininger V., Šimánek V.: Planta Med. *43*, 161 (1981).
7. Seifen E., Adams R. J., Riemer R. K.: Eur. J. Pharmacol. *60*, 373 (1979).
8. Ulrichová J., Walterová D., Preininger V., Slavík J., Lenfeld J., Cushman M., Šimánek V.: Planta Med. *48*, 11 (1983).
9. Ulrichová J., Walterová D., Preininger V., Šimánek V.: Planta Med. *48*, 174 (1983).
10. Walterová D., Ulrichová J., Preininger V., Šimánek V., Lenfeld J., Lasovský J.: J. Med. Chem. *24*, 1100 (1981).
11. Zee-Cheng K. Y., Shon-Jen Y., Cheng C. C.: J. Med. Chem. *21*, 199 (1978).
12. Caolo M. A., Stermitz F. R.: Heterocycles *12*, 11 (1979).
13. Marquardt D. W.: J. Soc. Ind. Appl. Math. *2*, 431 (1963).
14. Pancíř J.: This Journal *45*, 2453 (1980).
15. Pancíř J.: This Journal *45*, 2464 (1980).
16. Brossi H., Borer R.: Lloydia *28*, 199 (1965).
17. Lehninger A. L.: *Biochemistry*, 2nd Ed., p. 479. Worth Publications, New York 1975.
18. Velebný V., Kovář J.: Chem. Listy *73*, 389 (1979).
19. Ulrichová J.: *Thesis*. Palacký University, Olomouc 1985.
20. Verhoeven J. W.: *Nucleic Acids Symposium Series*, No 14, p. 1. IRL Press, Oxford 1984

21. Srinivasan R., Medary R. T., Fisher H. F., Norris D. J., Stewart R.: J. Am. Chem. Soc. *104*, 807 (1982).
22. Slavík J., Slavíková L.: Chem. Listy *48*, 1383 (1954).
23. Slavík J., Slavíková L.: This Journal *42*, 2686 (1977).
24. Howell C. R., Stipanovic R. D., Bell A. A.: Pesticide Biochem. Physiol. *2*, 364 (1972).

Translated by J. Salák.