Biochemical Pharmacology, Vol. 16, pp. 2369–2385. Pergamon Press Ltd. 1967. Printed in Great Britain

THE *IN VITRO* INHIBITION OF THE *N*-DEMETHYLATION OF PETHIDINE BY PHENELZINE (PHENETHYLHYDRAZINE)

B. CLARK

Department of Pharmacology, The Medical School, University of Newcastle upon Tyne

(*Received* 15 *May* 1967; *accepted* 21 *August* 1967)

Abstract—Some properties of the pethidine *N*-demethylating system of hepatic microsomes are described. The competitive inhibition of this system by phenelzine is also reported. Evidence is given that it is the terminal reaction of the system which is particularly sensitive to phenelzine.

Pethidine caused a significant increase in the rate of NADPH oxidation in microsomes from rabbit liver. Some properties of this pethidine—stimulated activity are reported. The pethidine—stimulated activity was inhibited by phenelzine.

Microsomal NADPH-cytochrome c reductase activity was only slightly inhibited by phenelzine in amounts which caused complete inhibition of *N*-demethylation.

N-DEMETHYLATION is an important pathway for the metabolism of a number of drugs including pethidine.[1–9] The enzyme system which catalyses the *N*-demethylation of pethidine occurs in man,[10–12] rabbit,[6] guinea pig,[6] and rat[1, 6, 8] and requires NADPH and molecular oxygen for activity.[6] Norpethidine and formaldehyde are formed.[6] In common with many other drug metabolizing enzymes pethidine *N*-demethylase occurs in highest concentration in the microsomal fraction from liver[6, 8], but a low activity has also been detected in rat brain preparations.[1]

Phenelzine (phenethylhydrazine), a potent inhibitor of monoamine oxidase may cause a dangerous degree of potentiation of the effects of pethidine in man[13–17] and in animals.[18–20] Papp and Benaim,[21] London and Milne,[22] and Brownlee and Williams,[18] suggested without any direct evidence that this potentiation might be due to the inhibition of the metabolism of the analgesic by the monoamine oxidase inhibitor. It is known, however, that other hydrazine derivatives inhibit some drug metabolizing enzymes. Iproniazid[4, 23] and Nialamide[23] inhibit the *in vitro* metabolism of hexobarbitone. Iproniazid also inhibits the *in vitro* metabolism of ethylmorphine,[24] pentobarbitone,[25] aminopyrine, amphetamine and acetanilid.[4]

This paper reports part of a study of the effects of monoamine oxidase inhibitors on the metabolism of pethidine. A preliminary account has been published.[26]

MATERIALS AND METHODS

Pethidinic acid. This was prepared by refluxing pethidine HCl (1 mMole) dissolved in 1 ml water with 25 ml of 0·1 N NaOH for 1·5 hr. After cooling, the alkaline solution was washed three times with 4 vol. of benzene to remove any unchanged pethidine, filtered and neutralized with HCl. $BaCl_2$ (1·2 mMole) was added and the solution cooled in ice. The white precipitate was filtered off after 15 min, washed with a few

ml of ice cold water and dried over P_4O_{10}. The yield was 90 mg and the m.p. was 260° (decomp.). The product gave a positive Dragendorff reaction (a detection test for alkaloids) and the u.v. absorption spectrum was similar to that of pethidine. The free acid was freshly prepared by stirring with Dowex 50WX8 (H^+ form). Titration showed the presence of two ionizing groups with pK_a values of approximately 5·3 and 8·6.

Other materials. Pethidine HCl and Phenelzine H_2SO_4 ("Nardil") were kindly donated by Roche Products Ltd., Welwyn Garden City, England, and William R. Warner & Co. Ltd., Eastleigh, England, respectively. Generous gifts of SKF 525A from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, England, and imipramine HCl ("Tofranil") from Geigy (U.K.) Ltd., Pharmaceuticals Division, Manchester, England, were also received. Morphine sulphate was obtained from the British Drug Houses Ltd., Poole, England. Disodium glucose 6-phosphate was obtained either from the Sigma Chemical Co. Ltd., St. Louis, Missouri, U.S.A., or from the British Drug Houses Ltd., Poole, England. $NADP^+$, NAD^+, NADPH, and crystalline glucose 6-phosphate dehydrogenase (EC.1.1.1.49) and alcohol dehydrogenase (EC.1.1.1.1.) were supplied by Boehringer und Soehne, Mannheim, W. Germany. Cytochrome c was obtained from the Sigma Chemical Co. Ltd. Other materials were either of 'AnalaR' grade or Laboratory reagents of high purity.

Unstarved male rats of the Wistar strain (about 300 g) were used.

Enzyme preparations

(a) Preparation of subcellular fractions for distribution studies. Rat liver was homogenized in 10 vol. of ice-cold 0·3 M sucrose (homogenate) and centrifuged for 10,000 *g* min. The sediment (nuclear fraction) was resuspended in 0·3 M sucrose. The supernatant was centrifuged for 115,000 *g* min and the sediment (mitochondrial fraction) was resuspended in 0·3 M sucrose. The supernatant (115,000 *g* min supernatant) was finally centrifuged for 4,500,000 *g* min to yield a microsomal pellet and a particle-free supernatant. Samples of each fraction were retained for determination of protein content and *N*-demethylase activity. (b) For routine measurements of *N*-demethylase activity either a 'microsomes plus supernatant' fraction (preparation M + S) or a microsomal suspension (preparation M) was used. These were prepared as follows; rat liver was homogenized in 4 vol. of ice-cold 0·15 M KCl and centrifuged for 115,000 *g* min. The sediment was discarded. The supernatant containing 'microsomes' and the soluble proteins of the cell (preparation M + S) was used as the source of enzyme activity in many experiments but in others a microsomal fraction (preparation M) was isolated from preparation M + S by centrifuging for 4,500,000 *g* min. The microsomal pellet was resuspended in 0·3 M sucrose. The microsomal fraction was not washed. Fresh preparations (M + S and M) or ones which had been frozen for only a few days were used. In some experiments, rabbit liver subcellular fractions were used. These were prepared in the same manner as rat liver fractions.

Determination of protein. Protein was determined by the method of Lowry, Roseborough, Farr and Randall, as modified by Miller.[27]

Determination of pethidine esterase activity. Pethidine is hydrolysed by an esterase present in rat liver to pethidinic acid and ethanol.[28] Esterase activity was determined by measuring the amount of ethanol formed, by an enzymic method. The incubation mixture contained 60 μmole of sodium phosphate, pH 7·3, 0·2 ml of rat microsomal

suspension (M) containing about 20–30 mg protein per ml, pethidine (usually 10 μmole, or in the amounts described in the Results section), and water to a final volume of 1 ml. The tubes were incubated at 37° for 60 min. Preliminary experiments showed that the reaction was linear with time for 60 min. The reaction was terminated by mixing in 0·05 ml of 72% perchloric acid to each tube. After standing in ice for 10 min, the tubes were briefly centrifuged and 1 ml of KH_2PO_4 (1·1 M, pH adjusted to 8·2 with KOH) was added to each tube and mixed with the supernatant without disturbing the first sediment. The tubes were again centrifuged and the clear supernatants taken for alcohol determinations. To 1 ml of the supernatant was added 2 ml of pyrophosphate buffer [20 g $Na_4P_2O_7$. 10 H_2O, 5 g semicarbazide HCl and 1 g glycine were dissolved in glass distilled water. NaOH (20 ml of 2 N) was added and the pH was adjusted with NaOH to 9·7. The final volume was made up to 300 ml], followed by 0·01 ml of NAD^+ (120 mg dissolved in 1 ml water), and 50 units of alcohol dehydrogenase. The extinction at 340 mμ was measured after incubating the tubes at room temperature for 30 min.

Determination of N-*demethylation* of narcotic analgesics and imipramine.* The *N*-demethylation of pethidine, morphine and imipramine results in the production of norpethidine, normorphine and desmethylimipramine respectively and formaldehyde. The method used for the determination of the rate of *N*-demethylation relies on the estimation of formaldehyde formed and is a modification of that described by Clouet.[1] The incubation mixture contained sodium phosphate buffer, pH 7·3 (150 μmole), $MgCl_2$ (35 μmole), semicarbazide (25 μmole) [semicarbazide HCl neutralized with NaOH and diluted to 0·1 M], nicotinamide (50 μmole), and pethidine HCl or morphine H_2SO_4 (5 μmole, or in the amounts described in the Results section). Either NADPH (1 μmole) or an NADPH generating system was also added. The NADPH generating system consisted of glucose 6-phosphate, 10 μmole; $NADP^+$, 0·25 μmole; and glucose 6-phosphate dehydrogenase which was either already present in the preparation M + S or added (7 units) when microsomal suspension (M) was used. Preparation M + S (0·05 ml, 30–60 mg protein per ml) or preparation M (0·05 ml, 20–40 mg protein/ml) was also added to the incubation mixture together with inhibitors and other additions as described in the Results section. The final incubation volume was 1·5 ml. The tubes were incubated at 37° for 60 min or for the times and at the temperatures indicated in the Results section. Preliminary experiments showed that the reaction was linear with time up to 90 min and with up to 5·7 mg protein. The reaction was terminated by the addition of 0·5 ml of 7% $ZnSO_4$. 7 H_2O to each tube. After mixing, 0·5 ml of $Ba(OH)_2$ (concentration adjusted so that 0·5 ml of $Ba(OH)_2$ solution neutralized 0·45 ml of 7% $ZnSO_4$. 7 H_2O) was added and the contents of the tubes thoroughly mixed. Samples (1·5 ml) of the clear supernatants obtained after centrifuging were heated at 60° for 30 min with 0·6 ml aliquots of Nash reagent[29] [15 g ammonium acetate and 0·2 ml of redistilled acetyl acetone in 50 ml water]. After cooling, the extinction at 415 mμ was measured using cuvettes with a 1 cm light path length.

Measurement of microsomal swelling. The method of Arcos and Arcos[30] was employed except that 0·02 M Tris-HCl buffer, pH 8·1 containing 0·055 M KCl was

* The *N*-demethylation of narcotic drugs requires a complex of enzymes the terminal one being the actual *N*-demethylase (see Discussion). *N*-demethylation here refers to the activity of the whole system.

used in place of barbitone buffer. Phenelzine was added as indicated in the Results section.

Determination of microsomal NADPH-cytochrome c reductase activity. The method consisted of measuring the reduction of cytochrome c by NADPH at room temperature (17°) by following the increase in extinction at 550 mμ in a cuvette with a 1 cm light path length. The reaction mixture contained 0·02 ml of diluted preparation M + S (about 1 mg protein) from rat liver, NADPH (1 μmole), cytochrome c (0·004 μmole), 0·2 M sodium phosphate buffer, pH 7·3, (360 μmole) and water to give a final volume of 2·0 ml. Phenelzine or SKF 525A was added as described in the Results section. The reaction was started by the addition of NADPH and was linear for 5 min. The increase in extinction at 550 mμ over the first 3 min was taken as a measure of enzymatic activity.

Measurement of NADPH oxidation. The decrease in NADPH concentration was followed fluorimetrically (excitation 365 mμ, emission 460 mμ) at room temperature using a Zeiss PMQ11 spectrofluorimeter. The reaction mixture consisted of 1·6 ml of 0·2 M sodium phosphate buffer, pH 7·3, NADPH (0·2 μmole, or in the amount described in the Results section), and 0·05 ml of microsomal suspension (Rat or rabbit microsomal preparations M, 30–60 mg protein/ml). Other additions were made as described in the Results section. The final volume was 2 ml. The reaction was started by the addition of enzyme and was usually linear for at least 15 min. The decrease in fluorescence in the first 10 min was taken as a measure of enzyme activity.

Determination of microsomal non-specific esterase activity. The activity of this enzyme was determined by following the release of *p*-nitrophenol from *p*-nitrophenylacetate. The reaction was carried out at room temperature in cuvettes with a 1 cm light path. The reaction mixture consisted of 1 ml of 0·2 M sodium phosphate buffer, pH 7·3, 1 ml of substrate (6·3 mg *p*-nitrophenylacetate dissolved in 1 ml ethanol and diluted to 100 ml with water), rat microsomal preparation M (approximately 2 μg protein) and other additions in a final volume of 3 ml. Phenelzine was added in the amounts indicated in the Results section. The reaction was started by the addition of enzymes and was linear for at least 10 min. ΔE_{400} mμ over the first 10 min was taken as a measure of enzyme activity after correcting for non-enzymic hydrolysis.

Determination of microsomal non-specific alkaline phosphatase activity. This was measured by following the formation of *p*-nitrophenol from *p*-nitrophenylphosphate at room temperature in cuvettes with a 1 cm light path. The reaction mixture consisted of Tris-HCl buffer, pH 8·6 (100 μmole), $MgCl_2$ (20 μmole), *p*-nitrophenyl phosphate (20 μmole), microsomal preparation M (0·75 mg protein) from rat liver, and other additions in a final volume of 2 ml. Phenelzine was added as described in the Results section. The increase in extinction at 400 mμ over the initial 10 min was taken as a measure of enzymic activity after first correcting for non-enzymic hydrolysis. The reaction was linear for at least 18 min.

Determination of microsomal glucose 6-phosphatase activity. This was carried out as described by Porteous and Clark.[31] 1·5 mg of microsomal protein (preparation M from rat liver) were added to each test, together with phenelzine as described in the Results section.

RESULTS

Pethidine esterase

The K_m for the rat liver enzyme was 1·65 mM (average of two experiments) and

the specific activity of one of the M + S preparations used was 24 μmole ethanol formed/hr/g microsomal protein. Phenelzine in concentrations up to 50 mM gave no inhibition.

Subcellular distribution of pethidine N-*demethylating activity in rat liver*

Expressing the activity of the homogenate as 100 per cent, the relative percentage activities of the subcellular fractions isolated were; nuclear fraction (13·5%), mitochondrial fraction (15·0%), microsomal fraction (76%), and particle-free supernatant fraction (1·9%). Recovery of activity was 106 per cent. Particle-free supernatant (0·1 ml) was added to each incubation mixture as a source of glucose 6-phosphate dehydrogenase. Expressing the homogenate as 100 per cent the relative protein contents of the fractions were; nuclear fraction (37%), mitochondrial fraction (27%), microsomal fraction (11%), and particle-free supernatant fraction (26%). Recovery of protein was 101 per cent. Fig. 1 shows the relative sp. act. of each fraction against

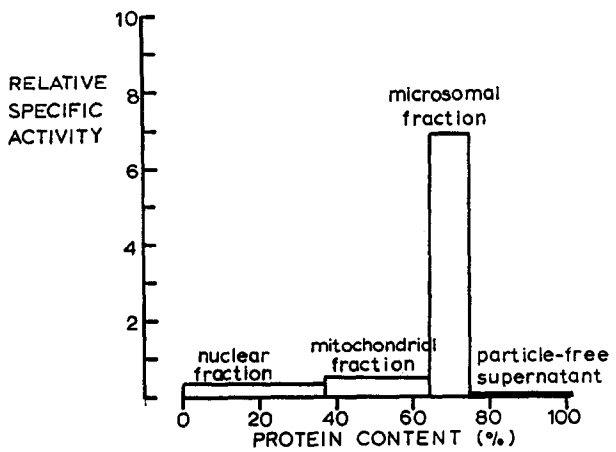

FIG. 1. Subcellular distribution of Pethidine *N*-demethylase. The ordinate indicates relative sp. act [activity (%)/protein (%)] of the isolated subcellular fractions; the abscissa indicates the protein content of the fractions. Experimental details are given in the Materials and Methods section.

the protein content of that fraction. It can be seen that the microsomal fraction had the highest relative sp. act. as well as the greatest proportion of the total activity.

Some properties of the pethidine N-*demethylating system of rat liver*

K_m. The K_m of the system for pethidine (over the range 0–6·67 mM) at pH 7·3 was 0·35 ± 0·14 mM (S.D. of 12 determinations in which either preparations M + S or M were used) and K_m for NADPH (range 0–1·33 mM) at pH 7·3 was 0·42 mM (average of three results, 0·43, 0·27, 0·56 mM of experiments using preparation M. Preliminary results show that the apparent K_m value falls from 2·0 mM at pH 6·8 to 0·3 mM at pH 7·0. This pH range corresponds with the point at which pethidine base just begins to form (pK 8·7) in significant amounts. Since one would expect the free base to be more lipid soluble the concentration in the lipid-like material of the membranes might rise sharply over this pH range, thus accounting for the fall in the apparent K_m. The K_m value does not alter over the range pH 7·0–7·8 which suggests that the

microsomal membranes are already saturated by the time pH 7·0 is reached. This result may also indicate that it is the unionized pethidine molecule which reacts with the *N*-demethylase.

pH. The pH optimum was 7·2 with preparation M + S. This was determined from a plot of V_{max} (determined for each pH value) against pH and is shown in Fig. 2.

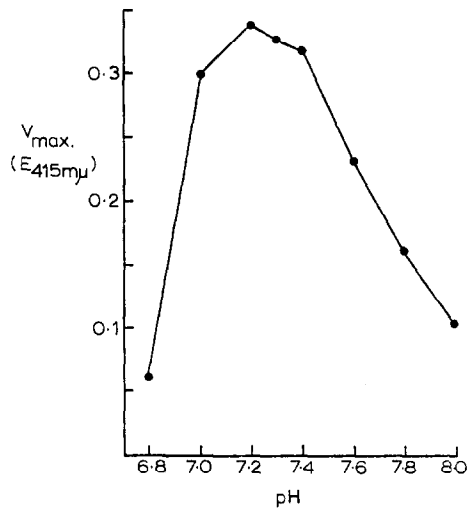

FIG. 2. Variation in pethidine *N*-demethylating activity with pH. Pethidine concentration curves (five points) were done at each pH value to determine V_{max}. Experimental details are given in the Materials and Methods section.

Energy of activation. The apparent energy of activation determined by an Arrhenius plot was about 17,000 cal/mole over the range 23–37°. Omitting semi-carbazide from the incubation mixture caused a 50 per cent decrease in the final extinction at 415 mμ.

Factors affecting activity. The activity of preparation M + S was stable to storage at −10° for several days, but declined by 12 per cent after 60 min and by 50 per cent after 180 min preincubation of 37°. A sp. act. of 48 μmole formaldehyde liberated/hr/g microsomal protein was obtained for the same preparation which gave a sp. act. for pethidine esterase of 24 μmole/hr/g microsomal protein.

Inhibition of the pethidine N-*demethylating system of rat liver by phenelzine.* Fig. 3 shows the inhibition of the *N*-demethylation of pethidine by phenelzine in a rat liver M + S preparation. 50 Per cent inhibition was given by 0·2 mM phenelzine (0·15 mM in another experiment) and inhibition was virtually complete with 1 mM phenelzine. The competitive nature of the inhibition is shown by Figs 4A and 4B. The apparent K_i for phenelzine assuming purely competitive inhibition was variable ranging from 0·011 to 0·152 mM (0·065 ± 0·048 mM, S.D. of 11 determinations), but it is recognized that inhibitor uptake by microsomes in the presence of a two phase system may cause atypical kinetics.

The apparent K_i value increased with increasing microsomal protein added when the inhibitor concentration was kept constant. Under these conditions K_m varied only slightly. The inhibition of pethidine *N*-demethylating activity by phenelzine can be reversed by the addition to the incubation mixture of inactive microsomal

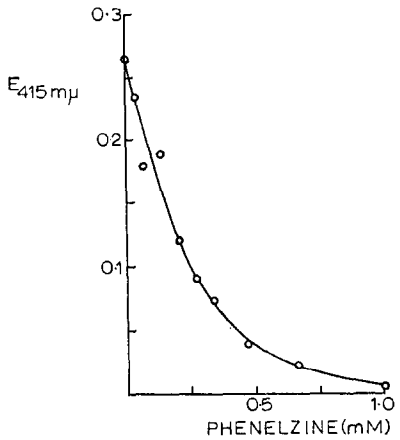

FIG. 3. Inhibition of *N*-demethylation of pethidine by increasing phenelzine concentration. Pethidin concentration was 3·33 mM. Experimental details are given in the Materials and Methods section.

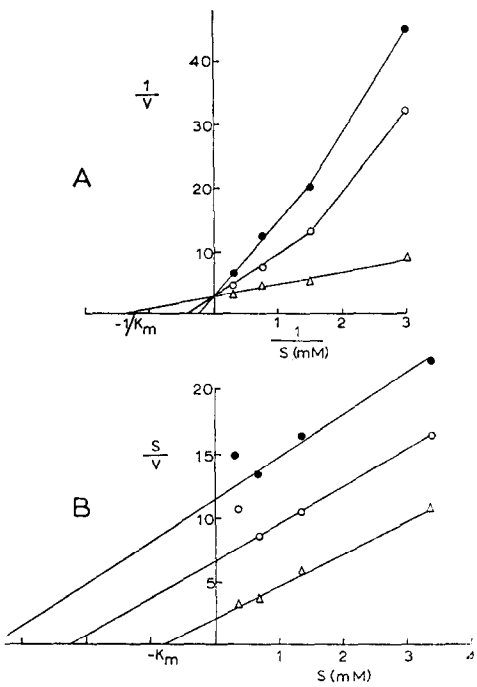

FIG. 4. Inhibition of pethidine *N*-demethylating activity by phenelzine. 1/v Against 1/S plots (A) and S/v against S plots (B) are shown for pethidine alone (△) and in the presence of two concentrations of phenelzine, 0·066 mM (○) and 0·133 mM (●).

S is the concentration of pethidine present in incubation mixtures containing a constant amount of rat liver 'microsomes' (preparation M) and NADPH. Other details are given in the Materials and Methods section.

protein. Fig. 5 shows the effect on the degree of inhibition by adding increasing amounts of a microsomal suspension prepared from guinea pig liver, which in this case had negligible *N*-demethylating activity, to the incubation mixtures containing a constant amount of active rat microsomal suspension (1·2 mg protein). Phenelzine, which was added in a final concentration of 0·33 mM, is known to survive under these conditions because reversal of inhibition can also be obtained when 'inactive' rat microsomal suspension is used. This result excludes the possibility that reversal of inhibition in the presence of an active microsomal suspension is due to metabolism of phenelzine. It is not known whether guinea-pig microsomes metabolize phenelzine by reactions other than *N*-demethylation.

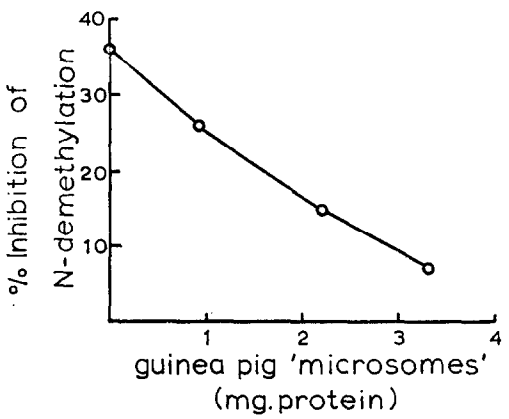

FIG. 5. Decrease in percentage inhibition by phenelzine of pethidine *N*-demethylating activity of 'microsomes' from rat liver with increasing amounts of inactive protein added. Guinea pig microsomal protein was added to incubation mixtures containing constant amounts of rat liver 'microsomes' (1·2 mg protein) in the presence and absence of phenelzine (0·33 mM).

The inhibition by phenelzine with respect to NADPH seemed to be mainly non-competitive. Fig. 6 shows S/v against S plots for NADPH with and without phenelzine in the presence of a constant pethidine concentration (3·33 mM). The phenelzine concentration was 0·133 mM and microsomal suspension (preparation M) from rat liver was added. Ideally the intercept of the two lines should be on the x axis. Assuming that the inhibition was purely non-competitive then the K_i value for phenelzine was 0·04 mM.

The same degree of inhibition by phenelzine was obtained when either NADPH or an NADPH generating system was used indicating that the inhibitor did not affect the formation of NADPH from $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase. Since phenelzine sulphate was used, sulphate ions were tested but gave no inhibition.

Rabbit liver. The *N*-demethylation of pethidine by microsomes from rabbit liver (K_m for pethidine 0·16 mM) was also sensitive to phenelzine. 50 Per cent inhibition was obtained by phenelzine at 0·11 mM and 100 per cent inhibition by phenelzine at 0·7 mM. The incubation mixture contained a suspension of microsomes from rabbit liver, pethidine (3·33 mM) and an NADPH generating system plus the other additions described in the Materials and Methods section.

Inhibition of morphine N-*demethylating system of rat liver.* Morphine *N*-demethylase of microsomes plus supernatant (preparation M + S) from rat liver was sensitive to phenelzine, 50 per cent inhibition being given by 0·05 mM and 100 per cent inhibition by 0·65 mM phenelzine. The K_m for morphine was 0·29 ± 0·04 mM, S.D. of 4 experiments. Preliminary results indicate that morphine acts as a competitive inhibitor

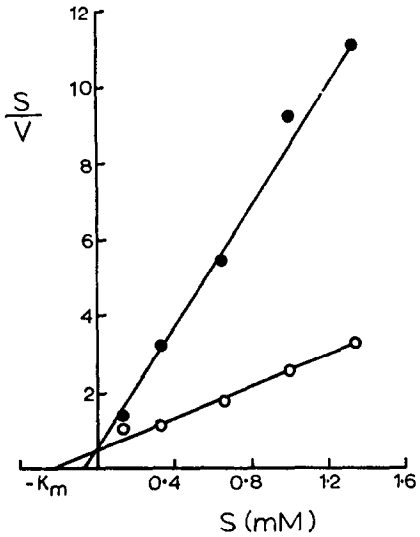

FIG. 6. The inhibition of pethidine *N*-demethylating activity by phenelzine. S/V Against S plots for increasing amounts of NADPH (○) and NADPH plus phenelzine (●). The phenelzine concentration was 0·133 mM. S is the concentration of NADPH added to incubation mixtures containing a constant amount of preparation M and pethidine (3·33 mM). Other details are given in the Materials and Methods section.

in the pethidine *N*-demethylating system. It is therefore possible that morphine is *N*-demethylated by the same enzyme as that which acts upon pethidine.

Inhibition of imipramine N-*demethylating system of rat liver.* The *N*-demethylation of imipramine (K_m for imipramine was 0·08 mM, average of three determinations) was also inhibited by phenelzine (Fig. 7).

The inhibition appeared to be competitive (Fig. 7) and assuming competitive inhibition the K_i for phenelzine was 0·04 mM.

Imipramine also inhibited the pethidine *N*-demethylase system of preparation M + S from rat liver. The inhibition appeared to be competitive and the approximate K_i for imipramine was 0·03 mM. The kinetics were complicated by the fact that imipramine itself was being *N*-demethylated to some extent under these conditions and the K_i value can only be an approximate one.

The effect of phenelzine and SKF 525A on rat microsomal cytochrome c reductase activity

Table 1 shows that addition of either phenelzine (0·1–0·5 mM) or SKF 525A (0·1 mM) to the assay system caused only a small inhibition of this activity.

NADPH *oxidation*

The K_m for NADPH in the microsomal 'NADPH oxidase' system of rat liver was $4{\cdot}6 \times 10^{-6}$ M (average of three determinations, $5{\cdot}4 \times 10^{-6}$, $4{\cdot}0 \times 10^{-6}$, and $4{\cdot}4 \times 10^{-6}$ M). The apparent energy of activation of microsomal 'NADPH oxidase' of rat liver over the range 20–30° was 6700 cal/mole (average of two determinations, 6200 and 7200 cal/mole).

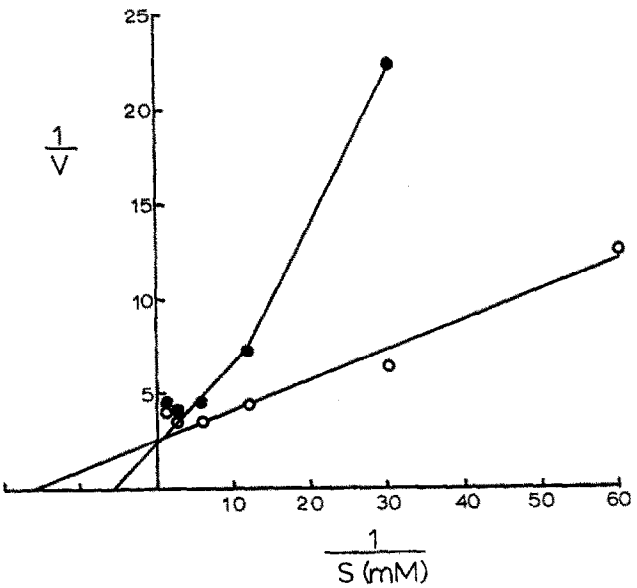

FIG. 7. Reciprocal plot of imipramine *N*-demethylating activity in the presence (●) and absence of phenelzine (○). S is the imipramine concentration mM. Phenelzine was added at a final concentration of 0·05 mM. Preparation M + S of rat liver was used. Experimental details are given in the Materials and Methods section.

TABLE 1. MICROSOMAL CYTOCHROME C REDUCTASE

The assay was carried out as described in the Materials and Methods section. Drugs were added as shown in the table.

Expt.	Addition	$\Delta E_{550m\mu}$/3 min	Per cent inhibition
1	none	0·260	0
	Phenelzine (0·1 mM)	0·260	0
2	none	0·239	0
	Phenelzine (0·5 mM)	0·211	12
	SKF 525A (0·1 mM)	0·225	6

The K_m for NADPH in the microsomal 'NADPH oxidase' system of rabbit liver was $2{\cdot}0 \times 10^{-6}$ M.

It was possible to demonstrate a consistent stimulation of the rate of NADPH oxidation by the addition of pethidine to incubation mixtures containing microsomal preparations from rabbit liver (see Fig. 8). Microsomal preparations from rat liver

were stimulated less consistently by pethidine. The reason for this species difference is not clear. Fig. 9 shows the stimulation of the rate of NADPH oxidation by increasing pethidine concentration. In the experiment of Fig. 9, a microsomal preparation from rabbit liver was used. The K_m for pethidine of the pethidine-stimulated activity was approximately 0·1 mM

Table 2 shows the effect on the rate of NADPH oxidation of adding various amounts of phenelzine, pethidinic acid or pethidine. Experiment 1 of Table 2 shows that

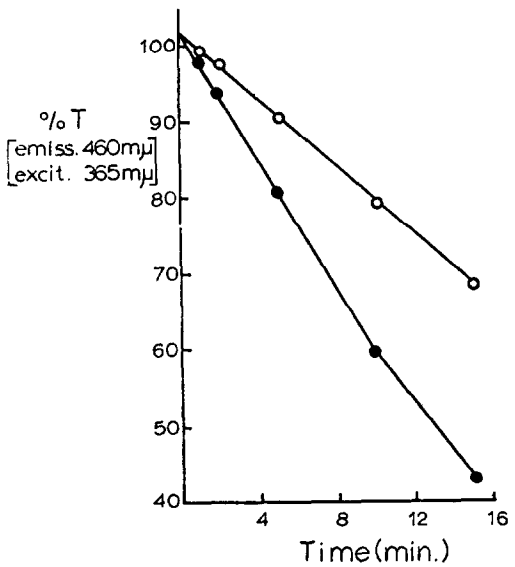

FIG. 8. Stimulation of oxidation of NADPH by rabbit liver microsomes. NADPH was tested alone (○) and in the presence of 2·5 mM pethidine (●). The assay system is described in the Materials and Methods section.

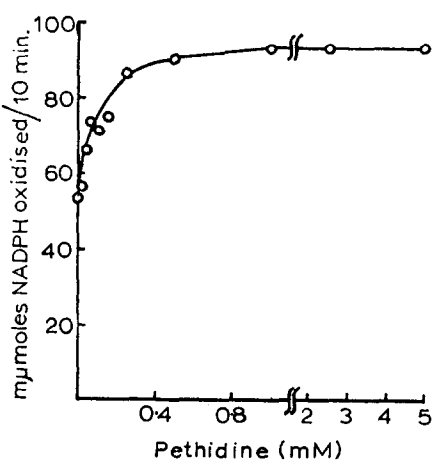

FIG. 9. Stimulation of NADPH oxidation by pethidine. The assay system is described in the Materials and Methods section. A microsomal suspension from rabbit liver was used.

phenelzine (2·5 mM) inhibited endogenous NADPH oxidation by a significant amount and also completely abolished the activity due to stimulation by pethidine (2·5 mM). The activity in the presence of pethidine and phenelzine was the same as that obtained with phenelzine alone. Pethidinic acid stimulated by 8 per cent. Experiment 2 in Table 2 and Fig. 10 show that the activity due to the addition of pethidine (after

TABLE 2. EFFECT OF PETHIDINE, PETHIDINIC ACID AND PHENELZINE ON NADPH OXIDATION

The assay system is described in the Materials and Methods section. A microsomal suspension from rabbit liver was used.

Expt.	Additions (mM)	Rate of NADPH oxidation mμmole/10 min	Percentage stimulation (+) or inhibition (−)
1	None	80	0
	Pethidine (2·5)	116	+45
	Phenelzine (2·5)	60	−25
	Pethidine (2·5) + phenelzine (2·5)	60	−25
	Pethidinic acid (2·5)	87	+ 8
2	None	102	0
	Phenelzine (0·05)	90	−12
	Phenelzine (0·1)	80	−22
	Phenelzine (0·15)	80	−22
	Phenelzine (0·25)	79	−23
	Phenelzine (0·5)	75	−27
	Pethidine (2·5)	161	+58
	Pethidine (2·5) + phenelzine (0·05)	128	+25
	Pethidine (2·5) + phenelzine (0·1)	118	+15
	Pethidine (2·5) + phenelzine (0·15)	111	+ 9
	Pethidine (2·5) + phenelzine (0·25)	102	0
	Pethidine (2·5) + phenelzine (0·5)	95	− 7

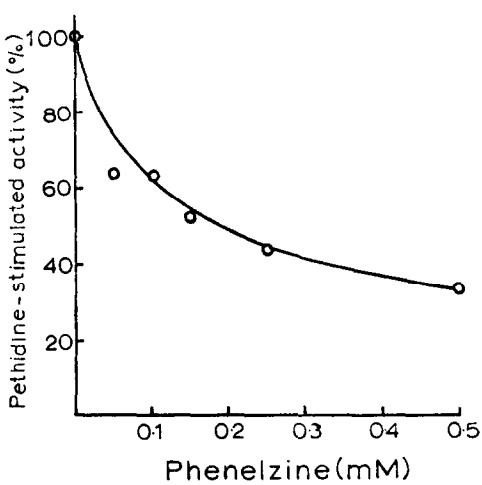

FIG. 10. Inhibition of pethidine-stimulated NADPH oxidation by phenelzine. The data from which this graph is calculated are shown in Table 3 (expt. 2). The percentage activity refers to the difference in rates of NADPH oxidation in the presence or absence of pethidine with increasing phenelzine concentration.

correcting for any effect which phenelzine had on the endogenous NADPH oxidation) rapidly declined with increasing phenelzine concentration. 50 per cent inhibition of the pethidine-stimulated activity was given by 0·17 mM phenelzine. The inhibition of the pethidine-stimulated activity by phenelzine could be completely reversed by increasing the pethidine concentration (Table 3) indicating competitive inhibition. In the experiment of Table 3 a low phenelzine concentration was used (0·05 mM).

Other effects of phenelzine

Phenelzine (0·33–3·33 mM) did not inhibit the microsomal non-specific esterase of rat liver. The non-specific alkaline phosphatase of rat liver microsomes was not inhibited by either 5 or 10 mM phenelzine and the glucose 6-phosphatase of rat liver microsomes was only slightly inhibited by 20 mM phenelzine (8%).

Phenelzine (5 mM) had no effect on the rate of microsomal swelling. The spectra of phenelzine alone, pethidine alone, and phenelzine with pethidine, all at pH 7·3, showed no evidence of a complex between the two drugs.

TABLE 3. EFFECT OF INCREASING PETHIDINE CONCENTRATION ON THE INHIBITION OF PETHIDINE STIMULATED NADPH OXIDATION BY PHENELZINE

The assay system is described in the Materials and Methods section. A rabbit liver microsomal suspension was used.

Pethidine concentration (mM)	Percentage inhibition of the pethidine stimulated NADPH oxidation by phenelzine (0·05 mM)
0·1	53
0·25	47
0·50	39
1·0	0
2·5	0

Under the conditions of assay for pethidine *N*-demethylase, phenelzine did not disappear and so could not have been metabolised to any appreciable extent. Thus it is unlikely that the inhibitory effects were caused by a metabolite of phenelzine.

DISCUSSION

Inhibition of pethidine metabolism by phenelzine. Papp and Benaim,[21] London and Milne[22] and Brownlee and Williams[18, 19] suggested that phenelzine causes the potentiation of pethidine and other narcotic analgesics in man and in animals by interfering with the metabolism of these drugs in liver. In rat and human liver, pethidine is either hydrolysed to pethidinic acid and ethanol,[10, 12, 28] or *N*-demethylated to norpethidine and formaldehyde.[10–12] Norpethidine may then be hydrolysed to norpethidinic acid and ethanol.[12] Pethidinic acid does not seem to be *N*-demethylated.[10] The experiments described here show that phenelzine has little effect on the hydrolysis reaction but is a potent inhibitor of the *N*-demethylation systems of rat and rabbit liver. Whether inhibition of the *N*-demethylation of pethidine can account for the potentiation of this drug by phenelzine is under further investigation. Loveless and Maxwell[20] questioned whether interference with the metabolism of pethidine could account for the observations they made of the effects of combining phenelzine and the analgesic in rabbits.

Pethidine N-*demethylating system*

The *N*-demethylation system of liver microsomes is closely associated with the electron transporting complex in these particles and the main features are probably as shown in Fig. 11, (see Omura, Cooper, Rosenthal and Estabrook[32]).

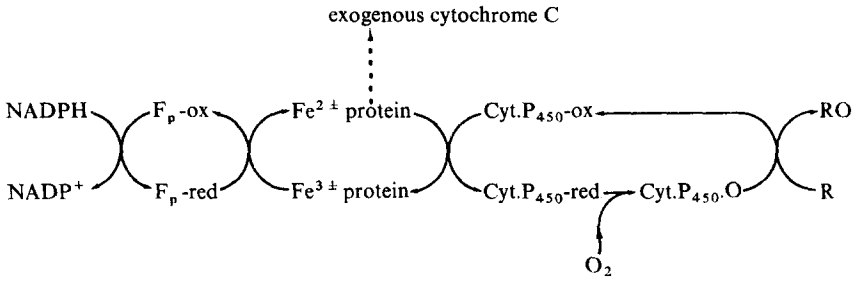

FIG. 11.

The terminal stage of the pathway is the reaction of the 'active-oxygen donor' (Cyt. P_{450}·0) with drugs undergoing oxidative dealkylation, deamination, sulphoxidation, hydroxylation etc; cytochrome P_{450}·0 may be the hypothetical hydroxyl donor suggested by Brodie.[33, 34] These terminal reactions are probably catalysed by *N*-demethylases, hydroxylases, etc. Endogenous NADPH oxidation by isolated microsomal preparations may be due to the reaction of the 'active-oxygen donor' with water (perhaps with the formation of hydrogen peroxide[35]) or other endogenous substrates. It is probable that in intact cells NADPH oxidation is coupled to oxidative dealkylation etc.

The *N*-demethylation of pethidine can be represented as follows:—

$$RNCH_3 + \text{Cyt. } P_{450}\cdot 0 \xrightarrow{(a)} [RNCH_2OH] \xrightarrow{(b)} RNH + HCHO$$

Brodie[33] has suggested that the intermediate $RNCH_2OH$ is unstable and breaks down (reaction b) to the secondary amine and formaldehyde. Whether both reactions (a) and (b) are spontaneous or involve one or more enzymes is not known.

That the *N*-demethylase system is associated with the endoplasmic reticulum is strongly suggested by the distribution of the enzyme activity among the fractions (Fig. 1). The microsomal fraction contained 77 per cent of the total activity and had a specific activity almost 14 times higher than the next most active fraction. The activity associated with other fractions was probably due to contamination by microsomes since the fractions were not washed. This distribution agrees with previous work by Clouet[1] and Axelrod.[6] The K_m value for the pethidine *N*-demethylase system of rat liver microsomes for pethidine (0·35 ± 0·14 mM) is of the same order as the K_m values for other drugs undergoing oxidative metabolism; e.g. aminopyrine, 0·8 mM (Ernster and Orrenius[36]); ethylmorphine, 0·25–0·93 mM (Rubin *et al.*[5]); hexobarbitone, 0·8–1·0 mM (Rubin *et al.*[5]) and chlorpromazine, 0·18–0·70 mM (Rubin *et al.*[5]). How close the K_m value obtained for pethidine is to the true equilibrium dissociation

constant K_S is not known. The actual concentration of pethidine in the microsomal membranes may be different from that in the aqueous phase.

Differences between the N-demethylating system and the NADPH-*oxidase system*

An interesting difference is seen between the K_m for NADPH in the pethidine *N*-demethylating system (0·4 mM) and the K_m for NADPH in the NADPH-oxidase system (2×10^{-6} M). This may be due to the rapid destruction of NADPH by NADPH-oxidase in the *N*-demethylation assay system. In one experiment 20 per cent of the NADPH originally added (1 μmole) remained after 60 min incubation. For this reason an NADPH generating system was preferred.

The apparent energy of activation for the *N*-demethylating system is 17,000 cal/mole whilst that for the NADPH-oxidase system is 6700 cal/mole. This difference may be due to the involvement of additional enzymes in the *N*-demethylating system.

Structure action relationships. The inhibition by phenelzine of the pethidine *N*-demethylating system is very likely due to the hydrazine moiety but the phenethyl group is probably required to confer enough lipid solubility for the inhibitor to diffuse into microsomal membranes (Clark and Thompson[26]). An uptake of inhibitor into the microsomes is suggested by the apparent increase in K_i with the amount of microsomal protein added.

This view is strengthened by the observation that the inhibition by phenelzine is almost completely reversed by adding inactive microsomal protein to the system (Fig. 5). Other hydrazine derivatives are known to be taken up by tissue homogenates.[37]

The main effect of phenelzine is probably at the terminal end of the pathway shown in Fig. 11 by a direct inhibition of the *N*-demethylating enzyme. Evidence for this is as follows:

(i) The inhibition of pethidine metabolism by phenelzine can be reversed substantially by pethidine, showing that this inhibition is principally competitive. It is known that pethidine reacts at the terminal end of the pathway and so it is likely that phenelzine acts there also. The fact that the inhibition by phenelzine cannot be reversed by NADPH, which is known to act at the beginning of the pathway, is also consistent with a terminal site of action of phenelzine.

(ii) The activities of the first two enzymes in the pathway, namely the flavoprotein and the iron-containing protein were estimated by adding cytochrome c to the assay system. NADPH reduced added cytochrome c by a linked reaction involving these two enzymes (microsomal NADPH-cytochrome c reductase) (see Fig. 11) and the rate of reduction was followed. Phenelzine in amounts which significantly inhibited the *N*-demethylation of pethidine caused only a small reduction of this activity (Table 1) indicating that the main effect of phenelzine is at a point on the pathway more distal to the iron-containing protein (see Fig. 11).

(iii) One would expect that drugs undergoing oxidative *N*-demethylation would stimulate the rate of NADPH oxidation by hepatic microsomes. Such a stimulation was found consistently with pethidine using microsomes from rabbit liver although a less consistent effect was seen in microsomes from rat liver. Pethidinic acid gave only a small stimulation. The pethidine-stimulated activity was much more sensitive to phenelzine than endogenous NADPH oxidation (Table 2). The additional activity caused by pethidine probably represented the reaction of pethidine with the 'active-oxygen donor' catalysed by the *N*-demethylase and is a measure of the rate of

N-demethylation. The value of K_m (0·1 mM) and the fact that the inhibition by phenelzine was reversed by high concentration of pethidine (Table 3) strengthens this view. Therefore the sensitivity of pethidine-stimulated oxidation to phenelzine indicates that *N*-demethylase is most strongly inhibited.

(iv) Preliminary results using an oxygen electrode showed that oxygen uptake of microsomes from rabbit liver in the presence of NADPH was significantly stimulated by pethidine. This pethidine-stimulated uptake was abolished by phenelzine in concentrations which caused only a small reduction in oxygen uptake in the absence of pethidine which is also consistent with a terminal site of action for phenelzine.

(v) The K_i value for imipramine when this acts as an inhibitor of the *N*-demethylation of pethidine was of the same order as its K_m when it acts as a substrate for its own *N*-demethylation. Thus imipramine *N*-demethylase and pethidine *N*-demethylase may be the same enzyme. Since phenelzine inhibited both systems with a similar K_i value for each, this provides strong evidence for the suggested action of phenelzine upon the *N*-demethylase enzyme. Similarly, preliminary evidence indicates that morphine *N*-demethylase and pethidine *N*-demethylase may also be the same enzyme since phenelzine inhibited the *N*-demethylation of both substrates.

In conclusion, the inhibition of the microsomal *N*-demethylation of pethidine by phenelzine is most likely to be due to a direct effect upon the terminal reaction of the pathway. It is unlikely to be due to a non-specific effect upon microsomal membranes or to the formation of a complex between inhibitor and substrate.

Acknowledgements—I wish to thank Professor J. W. Thompson for suggesting the project, for his help and encouragement during the work and for reading the manuscript. Dr. H. S. A. Sherratt also made helpful comments on the manuscript. I am indebted to Mrs. G. Widdrington for her skilled technical assistance throughout the work, to Mr. J. L. Haynes for technical assistance, especially in relation to the construction of apparatus; and to the Medical Research Council for generous financial support. I also wish to express my thanks to Miss M. Markey for her secretarial assistance.

REFERENCES

1. D. H. CLOUET, *J. Pharmac.* **144** 354 (1964).
2. L. LEADBETTER and D. R. DAVIES, *Biochem. Pharmac.* **13**, 1569 (1964).
3. R. E. MCMAHON and N. R. EASTON, *J. Pharmac.* **135**, 128 (1962).
4. J. R. FOUTS and B. B. BRODIE, *J. Pharmac.* **116**, 480 (1956).
5. A. RUBIN, T. R. TEPHLY and G. J. MANNERING, *Biochem. Pharmac.* **13**, 1007 (1964).
6. J. AXELROD, *J. Pharmac.* **117**, 322 (1956).
7. J. AXELROD, *J. Pharmac.* **114**, 430 (1955).
8. J. B. ROTH and J. BUKOVSKY, *J. Pharmac.* **131**, 275 (1961).
9. B. N. LA DU, L. GAUDETTE, N. TROUSOF and B. B. BRODIE, *J. biol. Chem.* **214**, 741 (1955).
10. J. J. BURNS, B. L. BERGER, P. A. LIEF, A. WOLLACK, E. M. PAPPER and B. B. BRODIE, *J. Pharmac.* **117**, 289 (1956).
11. N. P. PLOTNIKOFF, H. W. ELLIOTT and E. L. WAY, *J. Pharmac.* **104**, 377 (1952).
12. N. P. PLOTNIKOFF, E. L. WAY and H. W. ELLIOTT, *J. Pharmac.* **117**, 414 (1956).
13. H. PALMER, *Br. Med. J.* **2**, 944 (1960).
14. D. D. H. CRAIG, *Lancet* **ii**, 559 (1962).
15. D. C. TAYLOR, *Lancet* **ii**, 401 (1962).
16. J. J. BRADLEY and J. G. FRANCIS, *Lancet*, **i**, 386 (1963).
17. D. PELLS COCKS and A. PASSMORE-ROWE, *Br. Med. J.* **2**, 1545 (1962).
18. G. BROWNLEE and G. W. WILLIAMS, *Lancet* **i**, 669 (1963).
19. G. BROWNLEE and G. W. WILLIAMS, *Lancet* **i**, 1323 (1963).
20. A. H. LOVELESS and D. R. MAXWELL, *Br. J. Pharmac.* **25**, 158 (1965).
21. C. PAPP and S. BENAIM, *Br. Med. J.* **2**, 1070 (1958).

22. D. R. LONDON and M. D. MILNE, *Br. Med. J.* **2**, 1752 (1962).
23. M. J. LAROCHE and B. B. BRODIE, *J. Pharmac.* **130**, 134 (1960).
24. M. W. ANDERS and G. T. MANNERING, *Molec. Pharmac.* **2**, 319 (1966).
25. R. KATO, E. CHIESARA and P. VASSANELLI, *Biochem. Pharmac.* **13**, 69 (1964).
26. B. CLARK and J. W. THOMPSON, *Fedn Eur. Biochem. Soc. 3rd Meet. Warsaw* Abstract F247 (1966).
27. G. L. MILLER, *Analyt. Chem.* **31**, 964 (1959).
28. F. BERNHEIM and M. L. C. BERNHEIM, *J. Pharmac.* **85**, 75 (1945).
29. T. NASH, *Biochem. J.* **55**, 416 (1953).
30. J. C. ARCOS and M. ARCOS, *Biochim. Biophys. Acta* **28**, 10 (1958).
31. J. W. PORTEOUS and B. CLARK, *Biochem. J.* **96**, 159 (1965).
32. T. OMURA, R. SATO, D. Y. COOPER, O. ROSENTHAL and R. W. ESTABROOK, *Fedn Proc.* **24**, 1181 (1965).
33. B. B. BRODIE, in *Ciba Symposium on Enzymes and Drug Action*, p. 317. Little, Brown, Boston (1962).
35. J. R. GILLETTE, B. B. BRODIE and B. N. LA DU, *J. Pharmac.* **119**, 532 (1957).
36. L. ERNSTER and S. ORRENIUS, *Fedn Proc.* **24**, 1190 (1965).
37. A. HORITA, *J. Pharmac.* **142**, 141 (1964).