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ACTION OF *n*-ALKANES ON DRUG-METABOLIZING ENZYMES FROM GUINEA-PIG LIVER

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Abstract—Treatment of guinea-pig liver microsomal preparations with *n*-pentane, *n*-hexane and *n*-heptane enhanced both *p*-nitrophenol and *o*-aminophenol glucuronidation. The apparent K_m values of UDPglucuronate, for both *p*-nitrophenol and *o*-aminophenol UDPglucuronyltransferase, and of *o*-aminophenol were considerably increased, whereas the apparent K_m value of *p*-nitrophenol was the same after *n*-pentane treatment. No solubilization of UDPglucuronyltransferase or other microsomal proteins was observed after treatment with the *n*-alkanes. However, a substantial release of phospholipids from microsomal membranes occurred. The relative capacity of the different alkanes to release phospholipid was the same as the relative activation of UDPglucuronyltransferase. Aniline *p*-hydroxylation was considerably increased by the alkanes at concentrations of about 15–30 times lower than those necessary for optimal activation of UDPglucuronyltransferase. At these concentrations no activation of the aminopyrine *N*-demethylation was observed, whereas at higher concentrations a gradual decrease in both *N*-demethylating and *p*-hydroxylating activities occurred. *n*-Heptane, at a concentration which optimally activated aniline *p*-hydroxylation, markedly increased the rate of NADPH-cytochrome P-450 reduction. This suggests that the increase of the *p*-hydroxylating rate by *n*-heptane is related to a reversal of the cytochrome P-450 reduction by aniline.

Most of the enzymes which are involved in the metabolism of compounds foreign to the organism are located in the endoplasmic reticulum of the liver cells, firmly bound with this network of membranes. Studies with membrane perturbing agents have indicated that the activity of these enzymes is dependent upon phospholipids and membrane structure. Detergents [1–3], phospholipases [4, 5], ageing [6, 7], and sonication [8] enhance the *in vitro* activity of UDPglucuronyltransferase (EC 2.4.1.17). The mechanism of activation has not been fully elucidated. It has been suggested that the glucuronidating enzymes are covered by other membrane components [9, 22]. Treatment of liver microsomal preparations with surfactants may alter the membrane structure, resulting in an enhanced membrane permeability [2]. On the other hand, it has been postulated that, on treatment, UDPglucuronyltransferase exhibits different conformational states with different kinetic properties [5].

The activities of oxidative drug-metabolizing enzymes of the liver, are inhibited by surfactants [2, 21], chaotropic agents [9], and phospholipase C [16], whereas acetone [16, 26] and magnesium [27] exhibit stimulatory effects.

In our previous work [12] we observed that addition of *n*-hexane to liver microsomal preparations considerably increased UDPglucuronyltransferase activity. No alterations in the *p*-hydroxylation and *N*-demethylation enzyme activities were found with the *n*-hexane concentrations used. In order to understand this, the *in vitro* effects of *n*-pentane, *n*-hexane, and *n*-heptane on drug-metabolizing enzymes are described in this report.

METHODS

Microsomal preparations. Adult male guinea-pigs (325–350 g) were maintained on a Standard Cavy Diet L.C. 23-B (Hope Farms). The animals were killed by decapitation. The livers were immediately removed and cooled in ice. Portions of liver were weighed and finely minced. Homogenates (20%, w/v) were prepared in either ice-cold 0·25 M sucrose solution, containing 50 mM Tris–HCl buffer (pH 7·4), or in ice-cold 0·15 M KCl, using a Teflon–glass Potter-Elvehjem type of homogenizer. The homogenate was centrifuged at 9,000 *g* for 20 min. Microsomes were obtained by centrifuging the 9000 *g* supernatants at 105,000 *g* for 1 hr. The microsomal pellets from the latter step were gently resuspended in the homogenization medium. One ml of suspension contained microsomes derived from 0·2 g of liver.

The microsomal preparations were treated with various amounts of *n*-pentane, *n*-hexane or *n*-heptane. Microsomal suspensions (10 ml) were shaken on ice for 20 min prior to the enzyme assays, with volumes of the *n*-alkanes ranging from 5–700 μl. It was observed that the supernatant hydrocarbon layer fully disappeared after shaking for 20 min.

It is known that alkanes do not mix with water, however, they can absorb or dissolve in protein solutions [28–30]. Therefore, as a measure of the amount of *n*-alkane absorbed or bound by the microsomal particles, the term "concentration" (%, v/v) is used in this paper, indicating the amount of alkane present in the microsomal suspension.

Enzyme assays. Microsomal *N*-demethylation of aminopyrine and *p*-hydroxylation of aniline in the microsomal suspensions were measured according to the method of Henderson and Kersten [13].

UDPglucuronyltransferase activity was measured with the acceptor substrate *p*-nitrophenol or *o*-aminophenol. The *p*-nitrophenol incubation system consisted of 0·10 ml microsomal suspension, 0·05 ml $MgCl_2$ (3·3 mM final concn), 0·15 ml UDPglucuronate (4 mM final concn), 0·1 ml saccharo-1,4-lactone (3 mM final concn), 0·2 ml *p*-nitrophenol (0·4 mM final concn) in Tris–HCl buffer (50 mM, pH

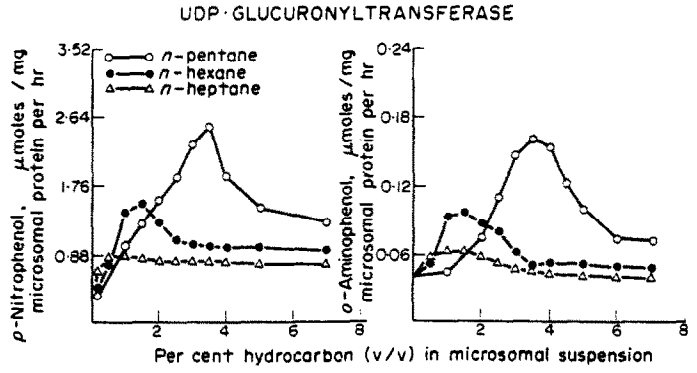

Fig. 1. Effect of *n*-alkanes on the glucuronidation of *p*-nitrophenol and *o*-aminophenol in guinea-pig liver microsomes. Each point indicates the mean of 3–6 experiments.

7·4). The further procedure, except sonication, was accomplished according to the method of Henderson [14]. The *o*-aminophenol incubation system consisted of 0·20 ml microsomal suspension, 0·05 ml $MgCl_2$ (3·3 mM final concn), 0·15 ml UDP-glucuronate (4 mM final concn.), 0·3 ml *o*-aminophenol (0·5 mM final concn, in 0·2% ascorbic acid), 0·1 ml saccharo-1,4-lactone (3 mM final concn) in glycylglycine buffer (50 mM pH 7·7). *o*-Aminophenyl glucuronides were assayed according to the method described by Mills and Smith [15].

NADPH-cytochrome P-450 reductase activity was assayed as described by Gigon *et al.* [23] and subsequently modified by Vainio and Hänninen [16]. The assay mixture (3·0 ml) contained 2–3 mg microsomal protein, 2 mM glucose, and 10 i.u. glucose oxidase (Boehringer, GFR) in KH_2PO_4–Na_2HPO_4 buffer (0·1 M final concn, pH 7·5). The reaction was started by addition of NADPH to a final concn of 0·4 mM. The formation of reduced CO–cytochrome P-450 complex was measured at 450 nm with a Cary 118C spectrophotometer at 20°. The change in absorbance was recorded for approximately 2 min. Due to the spectrophotometer used, the first rapid phase of reduction could not be measured.

Analytical methods. Protein was determined by the method of Lowry *et al.* [18], with bovine serum albumin as standard.

Phospholipids were estimated by their content of total phosphorus, assuming that 1 g of phosphorus is derived from 25 g phospholipid. After treatment of the microsomal suspensions with the *n*-alkanes, the microsomes were recentrifuged at 105,000 *g* for 1 hr. Total lipid phosphorus was extracted from the supernatant and determined by the method of Chen *et al.* [19], modified by Ames and Dubin [20].

RESULTS

Activation of UDPglucuronyltransferase. Treatment of the microsomal preparations with *n*-pentane, *n*-hexane, and *n*-heptane resulted in a substantial activation of both *p*-nitrophenol and *o*-aminophenol glucuronidation (Fig. 1). The concentration at which the *n*-alkanes display their maximum effect, increases with a decrease in the carbon chain length. On the other hand, it is apparent that the extent of activation decreases with the chain length of the *n*-alkanes, viz. the longer the carbon chain, the smaller the effect.

Treatment with *n*-pentane, *n*-hexane, and *n*-heptane, respectively, led to an approximately 9-, 5- and 3-fold increase in the rate of *p*-nitrophenol glucuronidation, and to a 4-, 3- and 2-fold increase in *o*-aminophenol glucuronidation (Table 1). It is also evident in this table that the extent of the activation by *n*-pentane is comparable with the maximum activation due to Triton X-100. No further increase in the activity of *p*-nitrophenol UDPglucuronyltransferase occurred after successive treatment of microsomes with Triton X-100 and *n*-alkanes. In fact a decrease in *o*-aminophenol glucuronidation was observed.

A comparison of the apparent K_m values of the respective susbstrates and UDPglucuronate for the glucuronidating enzymes in untreated and *n*-pentane treated microsomes is given in Table 2. The apparent K_m of *p*-nitrophenol was not appreciably changed by *n*-pentane, in contrast to the K_m value of *o*-aminophenol. The K_m values of UDPglucuronate considerably increased both for *p*-nitrophenol UDPglucuronyltransferase and *o*-aminophenol UDPglucuronyltransferase.

Release of microsomal components. In order to determine whether the activation is due to solubilization of the glucuronidating enzymes, the microsomal suspension was recentrifuged at 105,000 *g*. The 105,000 *g* pellet and supernatant were tested for glucuronidating activity and protein content. The results are presented in Table 3. It can be seen that, at concentrations which lead to maximum activation,

Table 1. Effect of some *n*-alkanes on UDPglucuronyltransferase in native and in Triton X-100 activated microsomes from guinea-pig liver

Treatment†	*p*-Nitrophenol glucuronidation*		*o*-Aminophenol glucuronidation*	
Triton X-100	+	−	+	−
Control	2·34	0·27	0·171	0·038
n-Pentane	2·41	2·56	0·085	0·165
n-Hexane	2·30	1·46	0·056	0·091
n-Heptane	2·29	0·80	0·044	0·059

* Expressed as μmoles substrate conjugated per hr per mg microsomal protein.

† Triton X-100, *n*-pentane, *n*-hexane and *n*-heptane were added to the microsomal suspensions in concns of 0·25, 3·5, 1·5, and 1·0% (v/v) respectively.

Table 2. Effect of *n*-pentane on the apparent K_m-values of *p*-nitrophenol, *o*-aminophenol, and UDPglucuronate for UDPglucuronyltransferase in guinea-pig liver microsomes

Substrate	Apparent K_m (mM)	
	Untreated	Treated with *n*-pentane (3·5% v/v)
p-Nitrophenol	0·12	0·10
UDPglucuronate	0·13	0·43
o-Aminophenol	0·04	0·19
UDPglucuronate	0·01	0·04

K_m-values of *p*-nitrophenol and *o*-aminophenol were determined with concentrations ranging from 0·02 to 0·2 mM and from 0·06 to 0·30 mM, respectively, and a fixed UDPglucuronate concentration of 3·0 mM.

The K_m of UDPglucuronate for *p*-nitrophenol UDPglucuronyltransferase was measured with UDP-glucuronate concentrations ranging from 0·1 to 1·0 mM and a fixed *p*-nitrophenol concentration of 0·3 mM.

The K_m of UDPglucuronate for *o*-aminophenol glucuronidation was determined with concentrations ranging from 0·01 to 0·10 mM and a constant concentration of *o*-aminophenol of 0·3 mM.

the *n*-alkanes did not solubilize UDPglucuronyltransferase or other microsomal proteins. It appeared however that treatment of the microsomes with the *n*-alkanes at these concentrations caused a substantial release of phospholipids from the membranes. The relative capacity of the different alkanes to release phospholipid (Table 4) is the same as the relative activation of UDPglucuronyltransferase (Table 1).

Effect on the oxidative enzyme system. The *n*-alkanes, at low concentrations, had no influence on the *in vitro* rate of aminopyrine *N*-demethylation. However, after treatment of the microsomes with *n*-alkanes at concentrations above 0·5 per cent (v/v) a gradual decrease in *N*-demethylating activity was observed (Fig. 2).

Table 4. Release of phospholipid from guinea-pig liver microsomal preparations after treatment with *n*-alkanes

Treatment*	mg phospholipid / mg protein	Per cent release†
Untreated	0·72	1·4
n-Pentane	0·50	31·5
n-Hexane	0·55	24·6
n-Heptane	0·62	15·1

* 10 ml microsomal preparations were shaken on ice with 0·35 ml *n*-pentane, 0·15 ml *n*-hexane, and 0·1 ml *n*-heptane, respectively, for 20 min.

† Native, control microsomes contained 7·3 mg phospholipid per mg protein.

After treatment with *n*-pentane, *n*-hexane or *n*-heptane the *in vitro* *p*-hydroxylation of aniline increased to 140, 170 and 270 per cent, respectively (Fig. 3). It should be noted that this stimulation occurred at hydrocarbon concentrations of about 15–30 times lower than those necessary for optimal activation of

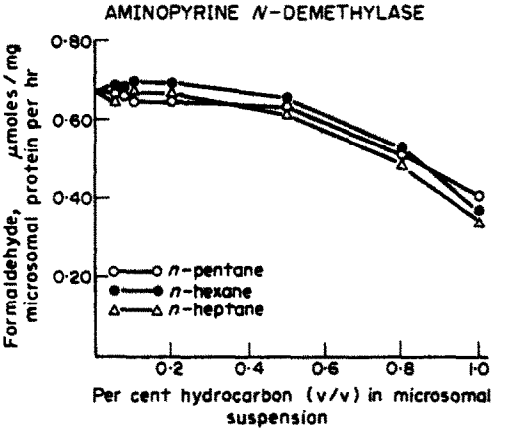

Fig. 2. Effect of *n*-alkanes on the *N*-demethylation of aminopyrine in guinea-pig liver microsomes. Each point indicates the mean of 3–6 experiments.

Table 3. Distribution of UDPglucuronyltransferase activity and protein between the 105,000 *g* pellet and supernatant after treatment of guinea-pig liver microsomes with *n*-alkanes

Treatment*	UDPglucuronyltransferase activity†			
	In pellet		In supernatant	
	p-Nitrophenol	*o*-Aminophenol	*p*-Nitrophenol	*o*-Aminophenol
Control	7·15	1·26	0·23	0·02
n-Pentane	71·20	6·40	0·22	0·08
n-Hexane	42·83	3·14	0·25	0·02
n-Heptane	23·21	2·29	0·24	0·01

	Protein concentration‡	
	In pellet	In supernatant
Control	28·6	2·16
n-Pentane	27·8	2·24
n-Hexane	29·4	2·26
n-Heptane	29·0	2·10

* Microsomal suspensions were treated with *n*-pentane (3·5%), *n*-hexane (1·5%), and *n*-heptane (1·0%), for 20 min, and then re-centrifuged at 105,000 *g* for 1 hr.

† Expressed as μmoles conjugated per hr per g fresh liver.

‡ In mg per g fresh liver.

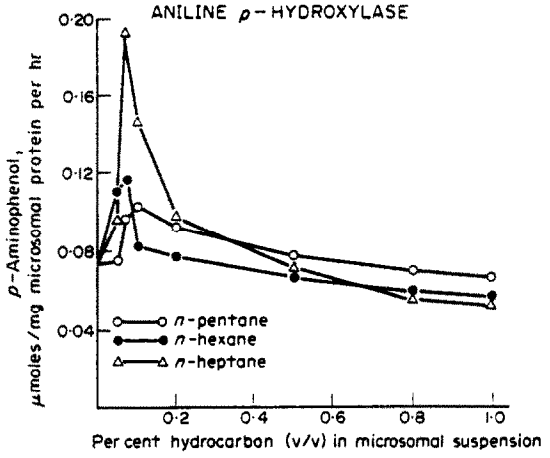

Fig. 3. Effect of *n*-alkanes on the *p*-hydroxylation of aniline in guinea-pig liver microsomes. Each point indicates the mean of 3–6 experiments.

UDPglucuronyltransferase. Contrary to UDPglucuronyltransferase activation, *n*-heptane was the most active stimulant of *p*-hydroxylase activity.

Effect on the rate of cytochrome P-450 reduction. In order to understand the mechanism of the accelerating action on the aniline hydroxylation, the influence of *n*-heptane on the rate of NADPH-linked cytochrome P-450 reduction was studied. As can be seen in Table 5, *n*-heptane at a concentration of 0·07 per cent, which is optimal for activating aniline hydroxylation, was found to increase markedly the rate of NADPH-cytochrome P-450 reduction both in the absence and in the presence of aniline. Aniline inhibited significantly the rate of cytochrome P-450 reduction. Addition of *n*-heptane to the hepatic microsomes in a concentration of 1·0 per cent, which did not exert an effect on the *p*-hydroxylating activity, had no significant influence on cytochrome P-450 reduction.

DISCUSSION

The results demonstrate that *n*-pentane, *n*-hexane, and *n*-heptane are able to activate *p*-nitrophenol and *o*-aminophenol UDPglucuronyltransferase to a degree comparable with the action of Triton X-100. Recently, a slight activating effect of *n*-hexane on the *in vitro* glucuronidation of *p*-nitrophenol in rat liver microsomes has been reported by Vainio [22]. However, the concentrations of *n*-hexane used were much higher than those found to be optimal for activation of guinea-pig UDPglucuronyltransferase in the present study.

Table 5. Effect of *n*-heptane on the NADPH-cytochrome P-450 reductase activity in hepatic microsomes from the guinea-pig

Addition	Rate of cytochrome P-450 reduction* Aniline (2·5 mM)	No aniline
None (control)	0·136 ± 0·009 (5)	0·186 ± 0·009 (5)
n-Heptane (0·07%)	0·196 ± 0·005† (4)	0·259 ± 0·018‡ (5)
n-Heptane (1·0%)	0·150 ± 0·005 (4)	0·168 ± 0·005 (4)

* Mean values, expressed as nmoles converted per mg microsomal protein per min, are given ± 2 S.E.; the number of experiments is given in parentheses.

† Significantly different from control with aniline at $0·001 < P < 0·01$ (Student's *t*-test).

‡ Significantly different from control without aniline at $0·001 < P < 0·01$ (Student's *t*-test).

It is generally accepted that the activity of UDPglucuronyltransferase is strongly dependent upon the structure of the microsomal membrane. Disruption of the microsomal vesicles with several surfactants caused an activation of UDPglucuronyltransferase concomitant with a release of proteins from the microsomes [2]. On treatment of guinea-pig liver microsomes with *n*-alkanes, solubilization of glucuronidating enzymes or releases of other proteins was not observed, whereas a liberation of phospholipids from the microsomal membranes occurred.

The dependency of UDPglucuronyltransferase activity on membrane phospholipids has been demonstrated previously by Graham and Wood [24]. They reported that treatment of microsomal preparations with phospholipase A or C, which attack specifically the membrane phospholipids, led to an inactivation of UDPglucuronyltransferase. Further studies done by Hänninen and Puukka [4] and Vessey and Zakim [5] revealed a severalfold increase in the rate of glucuronidation after a much milder treatment with phospholipase A and C. These authors concluded that the structure of the phospholipid environment in the membrane limits the maximal activity of UDPglucuronyltransferase [11]. The correspondence of the UDPglucuronyltransferase activation with the release of phospholipids from the microsomes, which we found after treatment with *n*-alkanes (see Table 4), is in good agreement with this concept. *n*-Pentane, which is the most active of the tested alkanes in accelerating the glucuronidation rate, also caused the greatest release of phospholipids.

The treatment of the microsomal membranes with *n*-alkanes increased the apparent K_m values of UDPglucuronate. The same effect was observed for the phospholipase treatment [4, 5], and after treatment with fatty acids [25]. It was suggested that such alterations in K_m values must be ascribed to conformational changes in the microsomal proteins. Our findings are consistent with this suggestion and, therefore, further support the idea that membrane phospholipids have an important role in regulating UDPglucuronyltransferase activity in the endoplasmic reticulum.

With reference to the mixed-function oxidase system, it appeared that the *n*-alkanes exhibited a stimulatory effect on the rate of microsomal aniline *p*-hydroxylation (Fig. 3), whereas for the same concentrations the *N*-demethylation of aminopyrine was not influenced. This strongly resembles the enhancement of aniline *p*-hydroxylation by acetone in rat liver microsomes [16, 26]. Acetone only activates the oxidation of compounds producing type II difference spectra. Vainio and Hänninen [16] concluded that the stimulatory effect of acetone may be explained in terms of a reversal of the inhibition of the cytochrome P-450 reduction by aniline. Our finding that *n*-heptane, at a concentration which maximally stimulates aniline *p*-hydroxylation, enhanced the NADPH-cytochrome P-450 reductase activity (Table 5) is in good agreement with their concept. It should be emphasized that, in contrast to acetone,

n-heptane (which appeared to produce a type I difference spectrum with guinea-pig liver microsomes) as well as other type I substrates [17] accelerated the reduction of cytochrome P-450.

The present results as well as the data reported by Vainio and Hänninen [16] are difficult to reconcile with the conclusions of Schenkman [10] and of Takeshige and Minakami [21], that the reduction of cytochrome P-450 cannot be the rate-limiting step in the oxidation of aniline. Schenkman [10] suggested that aniline slows its own metabolism by interfering with the oxygen activation by cytochrome P-450. Further investigations are needed in order to determine whether the n-alkanes can counteract such an interference of the oxygen activation by aniline, or whether the enhancement of the cytochrome P-450 reduction by the presence of n-alkanes accounts for the acceleration of the aniline oxidation.

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REFERENCES

1. G. J. Mulder, *Biochem. J.* **117**, 319 (1970).
2. H. Vainio and O. Hänninen, *Suomen Kemistilehti* B **45**, 56 (1972).
3. R. D. Howland, A. Burkhalter, A. J. Trevor, S. Hegeman and D. Y. Shirachi, *Biochem. J.* **125**, 991 (1971).
4. O. Hänninen and R. Puukka, *Chem. Biol. Interact.* **3**, 282 (1971).
5. D. A. Vessey and D. Zakim, *J. biol. Chem.* **246**, 4649 (1971).
6. G. W. Lucier, O. S. McDaniel and H. B. Matthews, *Archs Biochem. Biophys.* **145**, 520 (1971).
7. A. Winsnes, *Biochim. biophys. Acta* **191**, 279 (1969).
8. P. Th. Henderson, *Life Sci.* **9**, 511 (1970).
9. H. Vainio, *Biochem. biophys. Acta* **307**, 152 (1973).
10. J. B. Schenkman, *Molec. Pharmac.* **8**, 178 (1972).
11. D. Zakim, J. Goldenberg and D. A. Vessey, *Eur. J. Biochem.* **38**, 59 (1973).
12. W. R. F. Notten and P. Th. Henderson, *Biochem. Pharmac.* **24**, 127 (1975).
13. P. Th. Henderson and K. J. Kersten, *Biochem. Pharmac.* **19**, 2343 (1971).
14. P. Th. Henderson, *Life Sci.* **10**, 1 (1971).
15. G. T. Mills and E. E. B. Smith, in *Methods of Enzymatic Analysis*. (Ed. H. H. Bergmeyer). Academic Press, New York (1965).
16. H. Vainio and O. Hänninen, *Xenobiotica* **2**, 259 (1972).
17. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5**, 109 (1969).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. P. S. Chen, T. Y. Toribara and H. Warner, *Anal. Chem.* **28**, 1756 (1956).
20. B. N. Ames and D. T. Dubin, *J. biol. Chem.* **235**, 769 (1960).
21. K. Takeshige, A. Ito and S. Minakami, *J. Biochem.* **72**, 1361 (1972).
22. H. Vainio, *Acta Pharmac. Toxicol.* **34**, 152 (1974).
23. P. L. Gigon, T. E. Gram and J. R. Gillette, *Biochem. Biophys. Res. Commun.* **31**, 558 (1968).
24. A. B. Graham and G. C. Wood, *Biochem. Biophys. Res. Commun.* **37**, 567 (1969).
25. R. Puukka and O. Hänninen, *Int. J. Biochem.* **3**, 357 (1972).
26. H. Clark and G. Powis, *Biochem. Pharmac.* **23**, 1015 (1974).
27. M. A. Peters and J. R. Fouts, *J. Pharmac. exp. Ther.* **173**, 233 (1970).
28. A. Mohammadzadeh-k, R. E. Feeney, and R. B. Samuels, *Biochem. biophys. Acta* **147**, 583 (1967).
29. A. Mohammadzadeh-k, R. E. Feeney, and L. M. Smith, *Biochim. biophys. Acta* **194**, 246 (1969).
30. A. Mohammadzadeh-k, L. M. Smith, and R. E. Feeney, *Biochim. biophys. Acta* **194**, 256 (1969).