The presence of more than one radioactive peptide was expected since the continuous mobility of the enzyme molecule, the flexibility of the arylazido-β-alanyl substituent on the nucleotide, and the finite lifetime of the nitrene could each contribute to the probability of numerous interactions. The possibility that incomplete tryptic digestion had generated several labeled peptides was discounted because of the lengthy digestion period employed, and an alternate explanation involving the presence of more than one specific nucleotide binding site in an enzyme of subunit size ~25,000 was considered unlikely. The additional possibility of bacterial contamination occurring during the 30-hr digestion was negated by the consistency observed in the HPLC profiles of both labeled and unlabeled enzyme samples digested for times varying from 8 to 36 hr. The specificity exhibited by the photoaffinity NADH analog suggests that any of the three labeled peptides may reside in the vicinity of the active site and, therefore, contribute towards the binding affinity of enzyme and nucleotide. Analysis of the composition and sequence of these peptides will allow selection of the most probable contributors to the nucleotide envelope and prediction of possible configurations for its structure.

In summary, therefore, arylazido NADH was examined as a photoaffinity probe for the nucleotide binding site of rat liver dihydropteridine reductase. When a 30-fold excess of the nucleotide analog was irradiated with the reductase, a 26.5% loss of enzymatic activity was observed and 25% uptake of radioactivity occurred if the tritiated derivative was employed. Irradiation in the presence of NADH led to minimal enzymatic activity loss. Inhibition constants of 0.14 and 0.35 μM were determined for the arylazido-β-alanyl NADH derivative relative to NADH and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterine respectively. The results suggested specific interaction of the NADH analog at the reductase nucleotide binding site, and the radioactive arylazido-β-alanyl NADH-labeled enzyme was digested and subjected to HPLC analysis to afford radiolabeled peptides as a prelude to active-site structural characterization.

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Effects of drugs on the activity of histamine-*N*-methyltransferase from guinea pig skin

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The principal metabolic pathway of histamine in cutaneous tissue is mediated by histamine-*N*-methyltransferase (HMT) [1–3]. We have succeeded recently in purifying the enzyme from guinea pig skin and have demonstrated that biogenic amines which contain a CH_2—CH_2—NH_2 moiety next to a hydrophobic group (serotonin, tryptamine, dopamine, tyramine,) have intense inhibitory effects on the enzyme activity [4]. Although numerous kinds of drugs containing such a structure are now widely used clinically, and inhibitory effects of antihistaminics, tranquilizers, local anesthetics and antimalarials on HMT activity have been demonstrated [5–7], the molecular mechanism has not been elucidated. We decided, therefore, to examine the structure–inhibition relationship as a possible explanation for the inhibitory effects of these drugs, and to ascertain whether other drugs containing such a structure, whose effects on the enzyme activity have not yet been reported, also inhibited the activity of HMT purified from guinea pig skin.

Materials and methods

Materials. The following chemicals were used: histamine dihydrochloride (Wako, Tokyo, Japan), *S*-adenosyl-L-methionine (SAM; Boehringer, Mannheim West Germany), and *S*-[*methyl*-^{3}H]adenosyl-L-methionine (15 Ci/mmol, Radiochemical Centre, Amersham, Bucks, UK). DEAE-cellulose was from Whatman, Clifton, NJ, U.S.A. Diphenhydramine, diphenylpyraline, promethazine, pyrilamine, tripelennamine, cimetidine, chloropromazine, haloperidol, imipramine, trifluoperazine, dibucaine, lidocaine, procaine, chloroquine and quinacrine were from Sigma, St. Louis, MO, U.S.A. The generous

supply of the following drugs was greatly appreciated: famotidine (Yamanouchi Pharmaceutical Co., Ltd.), ranitidine (Glaxo Japan), metoclopramide (Fujisawa Pharmaceutical Co.), clomiphene (Shionogi & Co., Ltd.), dimethylaminoethyl reserpilinate (Green Cross Co.) and guanethidine (Ciba-Geigy Japan).

Other chemicals were of analytical grade and were obtained commercially.

Enzyme preparation. Two Dunkin–Hartley guinea pigs weighing 300–350 g were killed, and the cutaneous tissues of the abdominal and dorsal surfaces were stripped off. The tissues were homogenized, and the partially purified HMT was prepared as described elsewhere [4]. The specific activity of the purified enzyme that was obtained by DEAE-cellulose was 760 pmol/min/mg protein (X 24.8-fold purified when compared with the original homogenate, and apparent K_m values for histamine and SAM were 20 and 1.7 μM respectively), since further purified enzyme preparation was unstable [4]. The enzyme preparation was stored at −80° until used.

Enzyme assay. Enzyme activity was determined by the method described by Axelrod [8] with slight modifications reported previously [9]. Partially purified HMT (15 μg) was incubated with ^{3}H-labeled-SAM (2 μM, 0.1 μCi) and histamine (0.2 mM) in 50 mM sodium phosphate buffer (pH 7.8) containing 0.02% bovine serum albumin in a total volume of 0.5 ml at 37° for 30 min.

Others. These compounds were dissolved in distilled water just before use. The incubation was run in triplicate. The amount of protein was determined by the method of Lowry *et al.* [10].

Results

Effects of antihistaminic agents, tranquilizers, local anesthetics and antimalarials. The activity of guinea pig skin histamine-*N*-methyltransferase (HMT) was inhibited by both groups of antihistaminics, H_1 and H_2 antagonists, though the former had more potent effects (Table 1). The tranquilizers, local anesthetics and antimalarials examined in the present study significantly inhibited HMT activity, although haloperidol and lidocaine exhibited only slight effects. Of these drugs, antimalarials had the greatest inhibitory effects, while those caused by chlorpromazine, imipramine, trifluoperazine and dibucaine were as great as those caused by H_1 antagonists and more intense than that of procaine. These effects were clearly dose dependent, and the mode of inhibition of these drugs was competitive with respect to histamine (Fig. 1).

Effects of other drugs. Metoclopramide (antiemetic), clomiphene (Gonad-stimulating principle), dimethylaminoethyl reserpilinate and guanethidine (antihypertensives) inhibited HMT activity (Table 1). These effects were clearly dose dependent, and the mode of inhibition was competitive with respect to histamine.

Table 1. Effects of various drugs on guinea pig skin histamine-*N*-methyltransferase activity

Compounds	IC_{50} (μM)	K_i (μM)		Inhibition mode	
		Histamine	SAM	Histamine	SAM
None	—	—	—	—	—
H_1 antagonists					
Diphenhydramine	100	4.0	Not done	Competitive	Not done
Diphenylpyraline	79	6.0	44	Competitive	Competitive
Promethazine*	10	1.0	3.6	Competitive	Competitive
Pyrilamine	22	1.5	Not done	Competitive	Not done
Tripelennamine	25	2.0	Not done	Competitive	Not done
H_2 antagonists					
Cimetidine	251	36	90	Competitive	Competitive
Famotidine*	200	50	120	Competitive	Competitive
Ranitidine	133	12	42	Competitive	Competitive
Tranquilizers					
Chlorpromazine	14	0.60	4.0	Competitive	Competitive
Haloperidol	—	—	—	—	—
Imipramine	20	1.0	Not done	Competitive	Not done
Trifluoperazine*	15	2.0	8.0	Competitive	Competitive
Local anesthetics					
Dibucaine	9.4	1.2	Not done	Competitive	Not done
Lidocaine	—	—	—	—	—
Procaine*	89	12	56	Competitive	Competitive
Antimalarials					
Chloroquine	0.60	0.08	Not done	Competitive	Not done
Quinacrine	0.16	0.02	Not done	Competitive	Not done
Other drugs					
Metoclopramide	25	3.0	Not done	Competitive	Not done
Clomiphene	2.0	0.25	Not done	Competitive	Not done
Dimethylaminoethyl reserpilinate	0.89	0.09	Not done	Competitive	Not done
Guanethidine	7.1	0.60	Not done	Competitive	Not done

Partially purified enzyme (15 μg) was incubated with *S*-[*methyl*-^{3}H]adenosyl-L-methionine ([^{3}H]SAM) (2 μM, 0.1 μCi), histamine (0.2 mM) and various compounds, as indicated, in 50 mM sodium phosphate buffer (pH 7.8) containing 0.02% bovine serum albumin in a total volume of 0.5 ml at 37° for 30 min. The enzymatically produced ^{3}H-labeled methylhistamine was extracted and counted. The IC_{50} value was calculated graphically from three to six determinations over concentrations of 10^{-8} to 10^{-3} M. The K_i value was determined graphically using Tamiya's plots (apparent K_m values for histamine and SAM were 20 and 1.7 μM respectively).

* Lineweaver–Burk plots of these drugs are shown in Fig. 1.

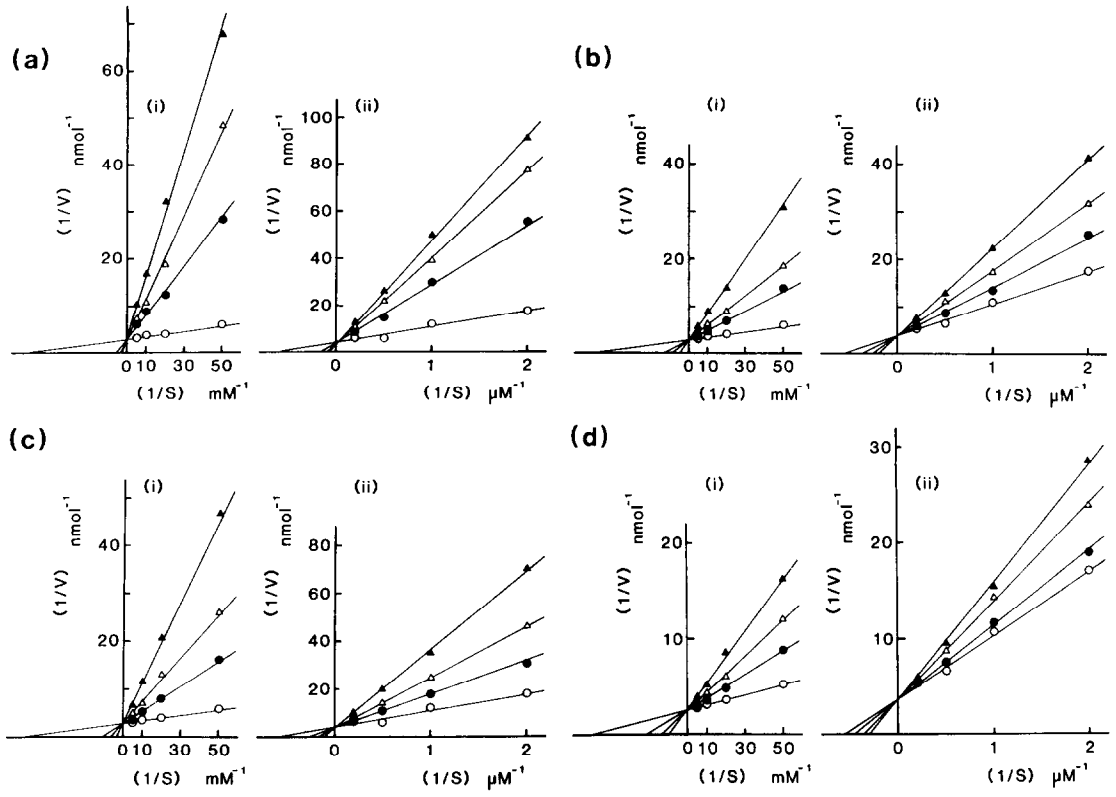

Fig. 1. Effects of representative antihistaminic agents, tranquilizers and local anesthetics on histamine-*N*-methyltransferase activity: Lineweaver–Burk plots. Key: (a) promethazine: no addition (○), 10 μM (●), 30 μM (△) and 50 μM (▲); (b) famotidine: no addition (○), 100 μM (●), 200 μM (△) and 300 μM (▲); (c) trifluoperazine: no addition (○), 10 μm (●), 30 μM (△) and 50 μM (▲); (d) procaine: no addition (○), 10 μM (●), 30 μM (△) and 50 μM (▲). (i) Double-reciprocal plots with respect to histamine. The concentration of *S*-adenosyl-L-methionine (SAM) was fixed at 2 μM, while that of histamine was varied between 0.02 and 0.2 mM. (ii) Double-reciprocal plots with respect to SAM. The concentration of histamine was fixed at 0.2 mM, while that of SAM was varied between 0.5 and 5 μM. Other details were identical to those described in the legend of Table 1.

Discussion

Histamine is metabolized by two main pathways, methylation by HMT and oxidative deamination by diamine oxidase (DAO), in mammalian tissues. DAO is distributed in certain tissues such as placenta, small intestine, kidney and thyroid [11, 12], whereas HMT appears to be ubiquitous [13]. We have already demonstrated that HMT mediates the principal metabolic pathway of the amine in cutaneous tissue of humans [1], guinea pigs [2] and mice [3] and that the enzyme activity is inhibited by certain biogenic amines, in which the CH_2—CH_2—NH_2 structure neighbouring the hydrophobic groups is significant to the inhibition [4].

As shown in the present study, antihistaminic agents (both H_1 and H_2 antagonists) inhibited HMT activity, although the enhancement of the enzyme activity at higher histamine concentrations, as reported by Taylor and Snyder [14], was not observed in our assays. Chemical formulas of antihistaminics are illustrated in Fig. 2a, and it is clear that compounds which inhibited HMT activity possessed CH_2—CH—N structures, with the exception of famotidine. Although the C-portion or N-terminals of ethylamine were replaced by some alkyl groups, and the H_2 antagonists had more complicated replacement structures, these drugs were considered to have essentially the same basic structure as mentioned above. Similarly, tranquilizers, local anesthetics and antimalarials which inhibited HMT activity also had a CH_2—CH—N structure in their chemical formulas, as indicated in Fig. 2b. Lidocaine and haloperidol caused only slight inhibition when compared with other local anesthetics and tranquilizers. The only differences in their chemical formulas were that the C_2 position of lidocaine was oxygenated and the N-terminals of haloperidol were more complicated and/or more hydrophobic than those of others presented in Fig. 2b. Imipramine, which is not illustrated in this figure, also inhibited the enzyme activity. A possible molecular mechanism for the structure–inhibition relationship between the activity of HMT and the potent inhibitors was demonstrated in our previous investigation [4], that HMT does not appear to detect whether an imidazole ring is present or not, and that the binding site of the histamine molecule to the enzyme is the CH_2—CH_2—NH_2 portion and HMT does not appear to react when this portion is deaminated, acetylated, carboxylated, or hydroxylated. Therefore, these differences were considered to be essential to the inhibitory effects of such drugs.

The inhibitory effects of antihistaminics, tranquilizers, local anesthetics and antimalarials on HMT activity were demonstrated previously [5–7], although, to date, the molecular mechanism has not been clarified. Due to this hypothetical molecular mechanism, we examined the effects of other drugs (metoclopramide, clomiphene, dimethylaminoethyl reserpilinate and guanethidine) that contain a similar CH_2—CH—N structure in their chemical

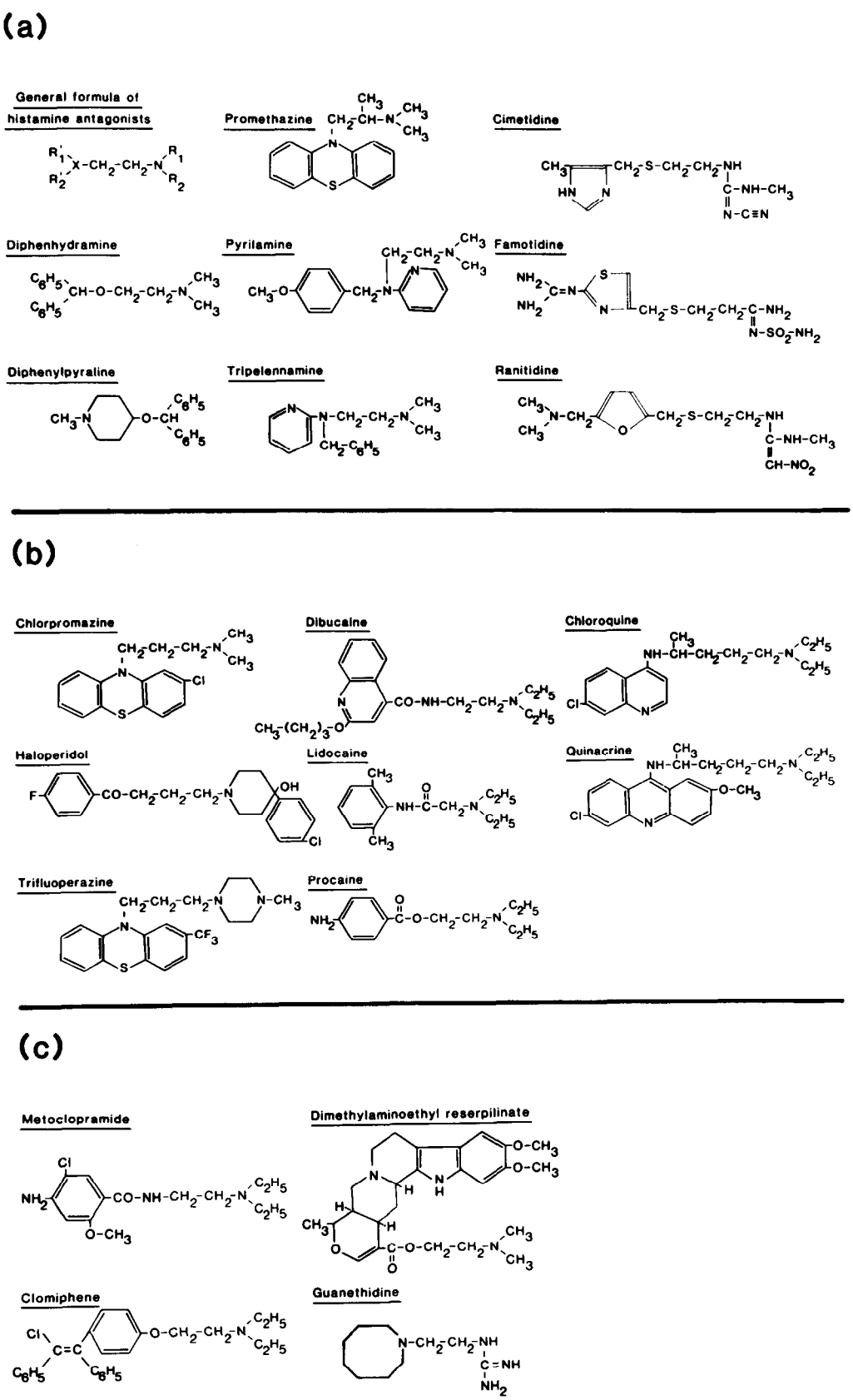

Fig. 2. Structure formulas of antihistaminic agents (a), tranquilizers, local anesthetics and antimalarials (b), and other drugs (c).

formulas (Fig. 2c) and whose effects on HMT activity have not been reported to date. These drugs inhibited the enzyme activity as expected for the structure–inhibition relationship, and the mode of the inhibition was competitive with respect to histamine. Therefore, additional to our previous report [4], it may be concluded that the essential structure of the inhibitors affecting the HMT activity was CH_2—CH—N, except for famotidine. The inhibitory effect of famotidine was evidently competitive with respect to histamine (Fig. 1b–i); therefore, this effect was considered to be due to the inhibition of binding of the amine to the HMT molecule. Thithapandha and Cohn [7] have demonstrated that amodiaquine, chlorguanide and cycloguanil (antimalarials), whose chemical structures are quite different from our hypothetical one, significantly inhibit the activity of HMT derived from guinea pig brain. Taken together, we speculate here that the binding site of the HMT molecule for histamine may not be as definite as demonstrated in the present study; a more detailed molecular mechanism of the inhibitory effect that can explain this discrepancy should be examined.

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Vitamin K reductases in normal and in warfarin-resistant rats

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Vitamin K is required for the formation of gammacarboxyglutamic acid (Gla) residues in proteins. The Gla-residues are formed in a carboxylation reaction in which vitamin K hydroquinone (KH_2) is converted into an epoxide (KO). The conversion of vitamin K quinone (K) into KH_2 may be accomplished either by a dithiol-dependent reductase or by an NADH-dependent enzyme. The dithiol-dependent reductions of KO and K are extremely sensitive to the action of oral anticoagulants such as warfarin [1]. KH_2-dependent carboxylase activity as such can be assessed by reducing vitamin K into KH_2 in a chemical way before it is added to the reaction mixtures. The activity of the two K reductases, on the other hand, is frequently determined in an indirect way, namely by starting the carboxylation reaction with either K + DTT or with K + NADH. A direct measurement of the K reductases by establishing the production of KH_2 is less reliable because the hydroquinone is unstable and rapidly re-oxidized into its quinone form by traces of oxygen.

Materials and methods

Animals. Warfarin-resistant rats of the Scottish resistance strain (HS) were initially obtained from the Agricultural Science Service, Tolworth Laboratory (Tolworth/Surbiton, Surrey, U.K.). Warfarin-susceptible male Wistar rats were obtained from Winkelman (Borchen, F.R.G.). The animals entered the experiments at the age of 16 weeks and from that moment they were housed singly so that their water consumption could be checked. Warfarin treatment was performed by adding 5 mg/l of warfarin and 10 mM sodium phosphate buffer (pH 9.0) to the drinking water. Control animals received the same buffer without warfarin. The buffer was refreshed every day and the daily consumption was 25–30 ml. The intake of warfarin was routinely checked by measuring the serum levels with the method described by Thijssen *et al.* [2]. After 5 days the animals were sacrificed under ether anesthesia and the livers were excised for the preparation of microsomes [3].

Assays. Unless stated otherwise, the microsomal pellets were washed by repeated suspension and centrifugation (1 hr at 105,000 *g*): twice with buffer A (0.1 M NaCl, 50 mM Tris/HCl, pH 7.4) and once with 1 M NaCl in buffer A. The final microsomal pellet was resuspended in buffer A to a protein concentration of 40 mg/ml and stored at −80° until use. KH_2-dependent carboxylase was assayed in 0.25 ml reaction mixtures containing 4 mg of microsomal proteins, 0.5% (w/v) CHAPS, 4 mM FLEEL, 2 mM DTT, 0.1 M NaCl, 1 M $(NH_4)_2SO_4$, 50 mM Tris/HCl, pH 7.4, 0.4 mM KH_2 and 0.01 mCi $NaH^{14}CO_3$. Incubations were performed at 25° for 30 min and the reaction was terminated by adding 1 ml of 5% (w/v) trichloroacetic acid [3]. [K + DTT]-dependent carboxylation was measured under the same conditions but with K instead of KH_2. [K + NADH]-dependent carboxylation was detected by replacing KH_2 by K, and DTT by 2 mM NADH. Endogenous carboxylatable protein precursors were assayed by incubating the reaction mixtures for 1 hr at 25° in the absence of FLEEL and with KH_2 as a coenzyme. Protein concentrations were established according to Sedmak and Grossberg [4].

Results

The *in vitro* sensitivity for warfarin was measured in the hepatic [K + DTT]-dependent carboxylase assay, and the