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The Effect of Thiol and other Group-specific Reagents on Erythrocyte and Plasma Cholinesterases

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That the activity of certain enzymes may depend on the presence in the enzyme molecule of intact sulphhydryl groups is now widely accepted and the cholinesterases have been considered to belong to this class of 'sulphydryl enzymes' (Nachmansohn & Lederer, 1939; Barron & Singer, 1943; Stadie, Riggs & Haugaard, 1945; Thompson, 1948). Many of the relevant observations were, however, made before it was realized that cholinesterases from different sources are not identical in specificity and other properties (Alles & Hawes, 1940; Richter & Croft, 1942; Mendel & Rudney, 1943; Zeller & Bissegger, 1943), and a critical survey of the literature reveals that the evidence for regarding any one cholinesterase as an —SH enzyme rests almost entirely upon inhibition by a few reagents, some by no means specific for —SH groups. The present work was undertaken with the object of investigating the possible sulphhydryl nature of the cholinesterases of the human plasma and erythrocytes and attempting to decide whether sulphhydryl groups can be regarded as having functional significance in relation to the activity of these representative mammalian cholinesterases.

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Table 1 summarizes existing work with —SH inhibitors. The most extensive observations are those of Nachmansohn & Lederer (1939) who found that the cholinesterase of the electric organ of the torpedo was inhibited by such typical thiol reagents as copper, maleic acid, iodoacetate, oxidized glutathione and alloxan. They concluded that torpedo cholinesterase was an —SH enzyme, though with some of these reagents rather large concentrations and prolonged incubation periods were required to produce a significant inhibition. Moreover, the specificity of some of these reagents is doubtful, while other thiol reagents, such as arsenite, were not tested. Mapharside (3-amino-4-hydroxy-phenylarsenoxide) was, however, found by Barron & Singer (1943) to be fairly powerful as an inhibitor of a cholinesterase of unspecified origin (electric-organ cholinesterase) while Thompson (1947) found that pigeon-brain cholinesterase was fairly sensitive to arsenite. Thompson (1948) also reported that pigeon-brain cholinesterase, unlike pigeon-brain 'pyruvate oxidase', could be protected from arsenite by a monothiol, cysteine ester hydrochloride, as well as by the dithiol 2:3-dimercaptopropanol (BAL). He tentatively suggested that cholinesterase is a monothiol enzyme, that is, its activity possibly depends on the presence of one thiol group per active centre in contrast to the 'dithiol enzyme' of pyruvate oxidation which is presumed to form an arsenic complex of stability intermediate between those which arsenic forms with cysteine and with BAL.

Horse serum cholinesterase was found by Massart & Dufait (1939) to be inhibited by arsenite in fairly high concentration. Mackworth (1948) found that this enzyme is not markedly sensitive, as are succinic and triosephosphate dehydrogenases, activated papain and other typical —SH

Table 1. *The effect of thiol reagents on cholinesterases, a résumé of earlier work*

Reagent	Enzyme source	pI*	Inhibition (%)	Author
Arsenite	Horse serum	1.7	87	Massart & Dufait (1939)
	Pigeon brain	4	52	Thompson (1947)
Lewisite	Horse serum	2.4	30	Mackworth (1941)
	Serum†	4	85	Barron <i>et al.</i> (1947)
	Pigeon brain	3	19	Thompson (1947)
Phenyldichloroarsine	Pigeon brain	3	57	Thompson (1947)
3-Amino-4-hydroxyphenylarsenoxide	Electric organ (?)	4.2	57	Barron & Singer (1943)
Copper	Torpedo	3.3	38	Nachmansohn & Lederer (1939)
	Horse serum	2.7	87	Mackworth (1941)
Maleic acid‡	Torpedo	1.5	22	Nachmansohn & Lederer (1939)
Iodoacetate‡	Torpedo	1.7	88	Nachmansohn & Lederer (1939)
Iodoacetamide	Cat serum	?	Nil	Wels & Repke (1947)
Glutathione‡ (oxidized)	Torpedo	2	24	Nachmansohn & Lederer (1939)
Iodine‡	Torpedo	5.75	85	Nachmansohn & Lederer (1939)
Alloxan§	Torpedo	3.4	38	Nachmansohn & Lederer (1939)
o-Iodosobenzene	Human plasma	?	Nil	Stadie <i>et al.</i> (1945)
Bromobenzyl cyanide	Horse serum	1.6	70	Mackworth (1948)
Chloropicrin	Horse serum	1.6	Nil	Mackworth (1948)
Bromoacetophenone	Horse serum	3	84	Mackworth (1948)
Ethyl iodoacetate	Horse serum	2.8	Nil	Mackworth (1948)
Oxygen (high pressure)	Rat brain	—	Nil	Stadie <i>et al.</i> (1945)

* Calculated from authors' data where necessary.

† Species not stated.

‡ Partly reactivated by glutathione.

§ Incubated with enzyme 4–10 hr.

enzymes, to lachrymators which are known (Bacq, 1946; Dixon, 1948) to combine readily with protein —SH groups. Typical —SH inhibitors to which various cholinesterases appear to be rather insensitive are lewisite (Mackworth, 1941; Thompson, 1947; but contrast Barron, Miller, Bartlett, Meyer & Singer, 1947), phenyldichloroarsine (Thompson, 1947), iodoacetamide (Wels & Repke, 1947) and o-iodosobenzene (Stadie *et al.* 1945) which is generally inhibitory to —SH enzymes (Dickens, 1946). Finally, the difference in sensitivity of cholinesterases of different origin to certain inhibitors listed in Table 1 underlines the need for comparative studies.

MATERIALS AND METHODS

Enzyme preparations

Human erythrocyte cholinesterase. A preparation obtained by the method of Mounter & Whittaker (1950) was further purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and differential centrifugation of the dispersed and dialysed precipitate (for details see Mounter, 1952). It gave 5700 $\mu\text{l. acid (or CO}_2\text{)}/\text{mg. N/hr.}$ using 30 mm-acetyl- β -methylcholine as substrate.

Human plasma cholinesterase. This was prepared from reconstituted dried transfusion plasma by the method of Strelitz (1944) (stage 3 of her preparation). Initial activity 150 $\mu\text{l.}/\text{mg. dry wt.}/\text{hr.}$

Reagents and substrates

Acetyl- β -methylcholine chloride (30 mm) and, in a few experiments, acetylcholine perchlorate (6 mm), were used as substrates for erythrocyte cholinesterase; acetylcholine perchlorate (15 mm) was the substrate for plasma cholinesterase. The reagents employed for inhibition studies were the best commercial grade, or laboratory samples available from previous work. Acid and alkaline reagents were

brought to pH 7.4 before use. Lewisite and diphenylchloroarsine were dissolved in a small volume of isopropanol and diluted with 0.2% NaHCO_3 before use. The amount of alcohol thus added to the enzyme was found in control experiments to be without effect. Oxidized glutathione was kindly provided by Dr R. V. Cecil.

Methods

Determination of enzyme activity was made by Ammon's (1933) modification of the Warburg technique, in which CO_2 displaced from a $\text{NaHCO}_3\text{-CO}_2$ buffer at pH 7.4 and 38° by acid produced during hydrolysis is measured manometrically. The solution in each Warburg flask (total vol. 3 ml.) contained 0.2% NaHCO_3 in equilibrium with 5% CO_2 in N_2 as the gas phase. The enzyme and reagent were contained in the main compartment of the flasks and the substrate in the side bulb; in reactivation experiments, the reactivating agent was placed in the second side bulb of double side-bulb flasks. The flasks were attached to the manometers, gassed for 3 min. and placed in the thermostat. After equilibrating for 10 min. the substrate was added from the side bulb and readings commenced 5 min. later. In reactivation experiments the initial incubation was continued for 15 min., the reactivator was then added and the incubation continued for a further 10 min. before addition of the substrate. The inhibition due to reactivator was controlled separately. Rates of hydrolysis and percentage inhibitions were constant during the experimental period. Enzyme and inhibitor were usually in contact 20 min. before addition of substrate and in some experiments with arsenite and mercurials in which the time of contact was varied, this was found to be adequate for the attainment of maximum inhibition.

In a few experiments the photometric method of Hestrin (1949) was used. All experiments included controls without enzymes.

RESULTS

Inhibition experiments with thiol reagents

Results obtained at pH 7.4 are summarized in Table 2. Where possible, the pI_{50} value (pI_x denotes the Briggsian logarithm of the molar concentration of inhibitor required to produce $x\%$ inhibition, with sign reversed) has been obtained by interpolation. When 50% inhibition was not attained, the pI_{50} value is recorded as 'less than' the highest pI value used, and the inhibition corresponding to this pI value is given in an adjoining column.

Table 2. *Inhibition of cholinesterases by thiol reagents*

Reagent	Red-cell cholinesterase		Plasma cholinesterase	
	pI_{50}	Inhibition (%) at highest pI used	pI_{50}	Inhibition (%) at highest pI used
Sodium arsenite	4.2	—	3.4	—
Lewisite	<3	22	<3	12
Phenylarsenoxide	<3	35	<3	25
Diphenylarsine chloride	<3	31	<3	26
Phenylmercuric nitrate	<3	15	4	—
<i>p</i> -Chloromercuribenzoic acid	2.2	—	2.6	—
Mercuric chloride	2.6	—	3.8	—
Copper sulphate	<2	45	3.7	—
Silver nitrate	<3	0	<3	10
Potassium antimonyl tartrate	<2	10	<2	10
Stibacetin	<3	5	<3	0
Sodium maleate	<1	20	<1	12
Sodium iodoacetate	<2	10	<2	5
Glutathione (oxidized)	<2	0	<2	0
Potassium ferricyanide	<2	0	<2	0
Iodine	3.6	—	3.6	—

Arsenicals. Appreciable inhibition was obtained with sodium arsenite, the erythrocyte enzyme being more sensitive than that of the plasma. In experiments with varying times of contact of enzyme and inhibitor before addition of substrate (5–60 min.) inhibition was found to be maximal at 5 min. By contrast, the inhibiting effect of organic arsenicals, normally very powerful inhibitors of sulphydryl enzymes (reviewed by Stocken & Thompson, 1949), is much less than the inorganic arsenite ion, as was noted for pigeon-brain and horse-serum cholinesterase by Thompson (1947) and Mackworth (1941).

As the As—S link is readily hydrolysed in alkaline solution (Cohen, King & Strangeways, 1931) it was thought to be of some importance to determine the effect of pH on the inhibition of blood cholinesterases by arsenite. In the experiments with erythrocyte cholinesterase, acetylcholine (2.2 mM) and the enzyme were incubated at 38° in 0.034M-phosphate buffer of the required pH, in the presence and absence of 0.5 mM-arsenite. Other tubes, containing substrate, but no enzyme or inhibitor, or buffer alone, were run as a non-enzymic control and as a reagent blank respectively. Samples (1 ml.) were withdrawn at 20 min. inter-

vals and the acetylcholine content determined by Hestrin's (1949) method. Experiments with plasma cholinesterase had 5.5 mM-acetylcholine and 1 mM-arsenite, but were otherwise identical. Rates of hydrolysis were linear with time, and from them the percentage inhibition was calculated in the usual way. Fig. 1 shows the inhibition plotted as a function of pH. The values obtained at pH 7.4 with the Warburg technique are inserted for comparison; the agreement is satisfactory considering that both buffer and substrate concentration were different. It will be seen that the inhibition increases with

increasing pH and is maximal at the highest pH used (pH 8). If the arsenic had been combining with a monothiol grouping, one would have expected the

Fig. 1. Effect of pH on inhibition of cholinesterases by arsenite (0.5 mM, erythrocyte enzyme; 1.0 mM, plasma enzyme).

inhibition to be maximal on the acid side of neutrality as an alkaline reaction favours dissociation of thioarsenites. This point is discussed more fully below.

Mercurials. The readiness of thiol groups to combine with mercury compounds is one of their earliest recorded properties. In the present study, mercuric chloride, phenylmercuric nitrate, and *p*-chloromercuribenzoic acid were tried as inhibitors. Of these substances, mercuric chloride was the most

Reversal of heavy-metal inhibition by thiols

The ability of mono- and di-thiols and of a 1:2-diol, catechol, to reverse the inhibitory action of heavy metals was also examined. The results are presented in Table 3. Taking first the results with

Table 3. *Reversal of metal inhibition of cholinesterases by thiols*

Inhibitor (I)	Concn. (mm)	Reactivator (R)	Concn. (mm)	Inhibition (%)			Reactivation (%)
				(I)	(I + R)	(R)	$100 \frac{I - (I + R)}{I - R}$
A. Plasma cholinesterase							
Arsenite	1	BAL	10	56	0	0	100
	1	BAL	3	60	0	4	107
	0.3	2-Mercaptoethanol	10	49	20	4	65
	1	2-Mercaptoethanol	10	63	28	5	60
	0.3	Glutathione	10	48	42	2	13
	1	Glutathione	10	65	58	14	14
	0.3	Cysteine	10	44	44	20	0
	1	Cysteine	10	69	60	22	19
	1	Catechol	10	64	62	0	3
Mercuric chloride	0.5	BAL	5	97	11	5	94
	0.5	2-Mercaptoethanol	10	98	0	0	100
	0.5	Glutathione	10	98	25	6	80
	0.5	Cysteine	10	98	13	16	103
Phenylmercuric nitrate	1	BAL	10	100	18	10	91
	1	2-Mercaptoethanol	10	94	32	0	66
Copper sulphate	0.3	BAL	5	62	12	10	96
	0.3	2-Mercaptoethanol	10	62	4	1	95
	0.5	Glutathione	10	80	20	15	92
	0.3	Cysteine	10	65	30	20	78
B. Erythrocyte cholinesterase							
Arsenite	1	BAL	10	98	35	33	97
	1	BAL	3	95	30	29	99
	1	2-Mercaptoethanol	10	92	82	6	12
	0.5	2-Mercaptoethanol	10	85	59	6	33
	1	Glutathione	10	94	90	3	4
	0.3	Glutathione	10	85	82	4	4
	1	Cysteine	10	98	90	6	9
	0.3	Cysteine	10	83	90	18	None
	1	Catechol	10	91	92	7	None
Lewisite	1	BAL	10	25	33	30	Reactivator more toxic than inhibitor
Mercuric chloride	0.5	BAL	10	11	23	25	
Phenylmercuric nitrate	1	BAL	10	12	27	25	

powerful inhibitor of plasma cholinesterase, phenylmercuric nitrate was also effective, but *p*-chloromercuribenzoate only gave 22% inhibition at 1 mM. Erythrocyte cholinesterase is only slightly inhibited by all these substances. No increase in inhibition was found on prolonged incubation with the mercurials.

Other thiol reagents. Iodoacetate, oxidized glutathione, ferricyanide and maleate had little or no effect on either enzyme; incubation for as long as 4 hr. with maleate produced no more than 10% inhibition. Iodine strongly inhibited both cholinesterases.

plasma cholinesterase, it will be seen that the dithiol BAL is consistently effective in reversing the inhibition due to arsenic, mercury or copper. By contrast, the monothiols show variation in effectiveness. Thus 2-mercaptoethanol, but not glutathione or cysteine, was effective against arsenite, but all were fully effective against mercury and copper. Catechol was uniformly ineffective.

Experiments with the erythrocyte enzyme were limited by the very high concentration of most inhibitors required to produce appreciable inhibition. At the same concentrations relative to the inhibitor as were used with the plasma enzyme, BAL was

actually more toxic to the enzyme than lewisite or mercury; it was, however, an effective antidote for arsenite, while glutathione, cysteine and catechol were not. 2-Mercaptoethanol proved less effective than in the experiments with the plasma enzyme.

Experiments with reagents other than thiol reagents

A few experiments were done with reagents specific for other groups. Table 4 shows that neither enzyme is sensitive to reagents for carbonyl ester groups or iron or to chelating agents. A possible significant degree of inhibition was obtained with 8-hydroxyquinoline.

esterases can inhibit unpurified preparations in extremely low concentrations.

The problem of interpreting conflicting results with thiol reagents has been discussed *inter alia* by Hellerman (1937) and by Barron & Singer (1945). The latter authors stress possible variations in the reactivity of 'essential' —SH groups and conclude that the arsenicals and *p*-chloromercuribenzoic acid are the most reactive —SH reagents. Our results show that neither cholinesterase is markedly sensitive to *p*-chloromercuribenzoic acid, though the plasma enzyme is fairly sensitive to other mercurials, and among the various arsenicals tested, only

Table 4. *Effect of reagents (other than thiol reagents) on blood cholinesterases*

Reagent	Diagnostic for	Concn. (mm)	pI	Inhibition (%)	
				Erythrocyte	Plasma
Hydroxylamine	—CO—	5	2.3	0	0
	—COOR				
Phenylhydrazine	—CO—	5	2.3	15	0
Semicarbazide	—CO—	5	2.3	0	0
Sodium bisulphite	—CO—	10	2	41	25
Sodium cyanide	Fe	1	3	0	0
Sodium azide	Fe	1	3	0	0
Thiourea	Metals	1	3	0	0
8-Hydroxyquinoline	Metals	3	2.5	20	67

DISCUSSION

The results presented above are inconclusive in that the functional groups of the two cholinesterases studied have not been identified. Only two inhibitors having pI_{50} values of 4 or more were encountered: sodium arsenite (red-cell cholinesterase) and phenylmercuric nitrate (plasma cholinesterase). The plasma enzyme was moderately sensitive ($pI_{50} > 3$) to AsO_3^{2-} , Hg^{2+} (as chloride), Cu^{2+} (as sulphate) and both were fairly sensitive to iodine ($pI_{50} = 3.5$). Both enzymes were usually inhibited by metallic —SH reagents in sufficiently high concentration, but clearly differed from typical —SH enzymes in being either much less sensitive or quite insensitive to most —SH reagents tested. The results are thus generally in line with those obtained with other cholinesterases (Table 1), and while certain differences in sensitivity undoubtedly exist, the resemblances between the cholinesterases are more striking than these differences. It is unlikely that the pattern of the results would have been different if more highly purified enzymes had been used as most of the work with typical —SH enzymes has been done with impure preparations, and the amount of the less inhibiting reagents was frequently greatly in excess of the total protein content of the reaction mixture. It seems inconceivable that inert protein could take up many times its weight of reagent before any thiol groups in the enzyme were attacked, especially as the specific anticholin-

sodium arsenite was an effective inhibitor, although this is usually less inhibitory than the organic arsenicals to arsenic-sensitive enzymes. A possibly significant comparison may be made between the cholinesterases and D-amino-acid oxidase, another enzyme whose status as an —SH enzyme is doubtful. Like the cholinesterases, this enzyme is sensitive to trivalent arsenic and mercurials (Barron & Singer, 1943) but not to lachrymators (Mackworth, 1948) or high pressure oxygen (Dickens, 1946). Conceivably, the cholinesterases and D-amino-acid oxidase have the same group in their active centres which may be an —SH group strongly modified by a neighbouring group, a group capable of being converted to an —SH group by some but not all —SH inhibitors, or some quite different group possessing some sulphhydryl properties.

Goldstein & Doherty (1951) have reached the conclusion that the inhibition of plasma cholinesterase by mercuric chloride is not due to reaction with 'essential' thiol groups. Several of their observations are, however, in conflict with ours. They found that mercuric chloride inhibition was progressive, the time course of inhibition being dependent on mercuric chloride concentration, that a 2.5-fold excess of BAL was not able to effect reactivation of the enzyme after inhibition and that the enzyme was uninhibited by 3 mm *p*-chloromercuribenzoic acid. In our experiments, inhibition by mercuric chloride was always complete in less than 30 min., a tenfold excess of BAL was able to

effect almost complete reactivation of the enzyme and 2.5 mM-*p*-chloromercuribenzoic acid gave 50% inhibition. Our preparation also appeared to be more sensitive to mercuric chloride than theirs.

Hydrogen-ion concentration is known to affect the reactivity of —SH groups and the stability of mercaptides. It is conceivable, though unlikely, that the relatively feeble inhibition of these enzymes by arsenicals might be due to dissociation of the

Fig. 2. Correlation between cholinesterase activity and inhibition by arsenite.

putative thioarsenite link of the arsenical-enzyme complex, at the slightly alkaline pH of the experiment. We have found, however, that arsenite inhibition actually decreases with decreasing pH, though this should favour thioarsenite formation. Indeed, there exists a good correlation between enzyme activity and the inhibition produced by a given concentration of arsenite, as is shown in Fig. 2. This suggests an alternative explanation of the inhibitory effect of arsenic. Wilson & Bergmann (1950*a, b*) have explained the effect of pH on the activity of electric-organ cholinesterase and inhibition by certain anticholinesterases by postulating the existence, in the part of the active centre concerned with fission of the ester link of the substrate, of a group, or groups, which (a) act as a base and accept a proton in acid solution, and (b) act as an acid and liberate a proton in alkaline solution. The forms produced by addition of a proton to the basic group (denoted by Y— in structure I) or removal of a proton from the acidic group (XH in I) are supposed to be enzymically inactive. The nucleophilic proton-accepting basic group is supposed to form a link with the electrophilic carbon of the acyl group of a substrate molecule, while the acidic group forms a link (possibly a hydrogen bond) with one or

other of the ester oxygens, leading to the electronic shifts which disrupt the ester link. The co-operation of both these groups is conceived to be necessary for rupture of the link.

It seems certain that a large number of structures other than carboxylic esters would also interact, to varying extents, with such a system and would thereby possess varying degrees of anticholinesterase activity which would vary with the pH in a similar way to the enzyme activity. If this hypothesis can be generalized it may account for the anticholinesterase activity not only of L-amino-acids (Bergmann, Wilson & Nachmansohn, 1950), and of many organic phosphoric esters, but also of inorganic pyrophosphate, citrate, tartrate, atoxyl, and finally arsenite and related organic arsenicals (I) (for literature, see Augustinsson, 1948). The fact

that atoxyl is to some extent inhibitory is significant for the point of view expressed here as pentavalent arsenicals are normally non-inhibitory to —SH enzymes.

The only non-metallic —SH reagent appreciably inhibitory was iodine. Torpedo cholinesterase is also sensitive to this reagent (see Table 1). Iodine is however a highly reactive substance and oxidation of —SH groups is only one of many ways in which it could conceivably inactivate an enzyme.

Both cholinesterases were quite insensitive to group-specific reagents other than —SH reagents with the possible exception of hydroxyquinoline ($pI_{20} = 2.5$, erythrocyte; $pI_{67} = 2.5$, plasma). Since the other chelating agent tested, thiourea, was non-inhibitory, this can hardly be regarded as evidence for the participation of a metal ion in the active centre.

In conclusion, there seems to be little or no evidence for the participation of —SH groups, carbonyl groups, or metal ions in the active centres of these enzymes. No class of group-specific reagents is consistently inhibitory, and we believe that such inhibitions as have been observed with individual members of these classes are probably to be explained by a non-specific reaction of the substance concerned. In our present state of knowledge of the constitution of the active centres of these enzymes, it is hardly possible to decide just what these non-specific reactions are. Further studies of the effect of pH on them might be of value.

SUMMARY

1. The inhibitory effects of a number of thiol reagents and other group-specific reagents on the cholinesterases of human blood have been studied.

2. Although the enzymes were moderately sensitive to arsenite and iodine, and were usually inhibited by other heavy metal reagents in sufficiently high concentration, they differed from typical —SH enzymes in being either much less sensitive or quite insensitive to most —SH reagents

tried. Further, arsenite inhibition was reduced by pH, not increased as would be expected if a thio-arsenite link had been formed.

3. It is concluded that these cholinesterases are in all probability not —SH enzymes and that there is also no evidence for the participation of metal ions, carbonyl groups or carboxylate groups in the active centre.

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The Effect of Dosage on Sexual Differences in the Iron Metabolism of Rats

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Both rats and human beings exhibit sexual differences in the metabolism of iron. Widdowson & McCance (1948) outlined the information available and showed that, given equal opportunities, female rats stored much more iron in their livers than males. They also showed that this was true of some but not of all other species of animals, and that in poultry it applied to the spleen as well as the liver. At that time it had been shown (Brenner, Brookes & Roberts, 1942) that female rats could store vitamin

A in their livers with much greater facility than males, and it was suggested by Widdowson & McCance that the storage of vitamin A might in some way be coupled with that of iron. More recently, Moore & Sharman (1950) and Moore, Sharman & Ward (1951) have pointed out that the level of dosage makes a great difference to the ease with which vitamin A is accumulated in the organs of the rat. At comparatively low intakes (40 i.u./day) the liver of the female contained over twice as