Acetyladenylic acid, which may be an intermediate in the activation of acetate (Berg, 1956), on the other hand is a labile substance, which is rapidly hydrolysed at pH 2. Nothing seems, however, to be known about the possible presence of acetyladenylic acid in biological material.

The method described has been used by us extensively for determination of acetate in liver suspensions (Lundquist, Fugmann, Kläning & Rasmussen, 1959) and in blood during alcohol metabolism in man, where the concentration of acetate reaches values of about 50 $\mu\text{g./ml.}$ (Fugmann *et al.* 1958; Lundquist, 1960). At concentrations of this magnitude the diffusion step may be omitted, when the concentration of acetoacetate can be shown to be small and constant.

SUMMARY

1. A specific enzymic method for the quantitative determination of acetate in the range 1–15 $\mu\text{g.}$ in a volume not exceeding 3 ml. is described.

2. The procedure involves diffusion in Conway microdiffusion units and reaction with sulphanilamide by means of a pigeon-liver enzyme preparation, which is stable at -20° for at least 1 year.

3. The method is highly specific for acetate. Interference from a number of substances examined is negligible.

4. The accuracy is about 0.3 $\mu\text{g.}$ (s.d.) independent of the concentration in the range covered.

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Studies on Vitamin A Esterase

5. A COMPARATIVE STUDY OF VITAMIN A ESTERASE AND CHOLESTEROL ESTERASE OF RAT AND CHICKEN LIVER*

By P. SESHADRI SASTRY AND J. GANGULY

Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

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Although esterases have received a great deal of attention for a long time, the existence of vitamin A esterase and cholesterol esterase was recognized only relatively recently; however, information about the identity and specificity of these two esterases does not seem to be available. Seshadri Sastry, Krishnamurthy & Ganguly (1957) reported the general properties of vitamin A esterase of rat liver and demonstrated by differential-inactivation studies that the common esterase (the enzyme hydrolysing short-chain esters of simple

alcohols, e.g. ethyl butyrate) and vitamin A esterase of the same tissue are not identical. All attempts at solubilization of vitamin A-esterase activity from rat liver were unsuccessful.

During the course of these investigations it was noticed that the common esterase, vitamin A-esterase and cholesterol-esterase activities of chicken-liver homogenate are distributed between the nuclear and microsomal fractions, whereas in rat-liver homogenates they are present only in the microsomal fraction (Krishnamurthy, Seshadri Sastry & Ganguly, 1958). Cases of bimodal distribution of enzymes, e.g. aryl sulphatases in the rat-

* Part 4: Mahadevan, Murthy, Krishnamurthy & Ganguly (1961).

liver cell, are known, however, but the enzymes belonging to separate cell fractions were found to have different properties (Dodgson, Spencer & Thomas, 1955). It was therefore of interest to make a comparative study of the esterase activities of the microsomal fraction, and of the nuclear and microsomal fractions from homogenates of rat and chicken liver respectively.

This paper describes a comparison of the properties of vitamin A esterase and cholesterol esterase of rat liver and of the nuclear and microsomal fractions of chicken-liver homogenate. It is also shown that a soluble preparation containing the three esterase activities can be obtained from the nuclear fraction of chicken liver. It was possible to obtain partial separation of the three activities by subjecting the soluble preparation to electrophoresis and ammonium sulphate fractionation. From the results it would appear that the three activities are due to separate enzymes.

MATERIALS AND METHODS

Crystalline synthetic vitamin A acetate, obtained from Hoffmann-La Roche, was chromatographed on alumina before use (Ganguly, Krinsky, Mehl & Deuel, 1952). Crystalline cholesterol acetate, ethyl butyrate and digitonin were from British Drug Houses Ltd.; the ethyl butyrate was redistilled before use. Tween 80 (polyoxyethylene sorbitan mono-oleate) was from Atlas Powder Co., Wilmington, Delaware, U.S.A. and *p*-chloromercuribenzoate from Sigma Chemical Co. The organophosphorus compounds were kindly procured for us by Dr S. Y. Thompson of the National Institute for Research in Dairying, Reading. Lissapol-N, an alkyl aryl polyethoxy alcohol, was from the Imperial Chemical Industries Ltd.

Treatment of animals, preparation of the enzymes and dispersion of vitamin A acetate were as described by Seshadri Sastry *et al.* (1957) and Krishnamurthy *et al.* (1958). Cholesterol acetate was dispersed according to the method of Meier, Siperstein & Chaikoff (1952) with Tween 80. Unless otherwise stated, 1 ml. of the substrate (containing 2 mg. of vitamin A acetate or cholesterol acetate) and 8 ml. of 0.1 M-HCl-veronal buffer (pH 8.6 for vitamin A acetate and pH 7.4 for cholesterol acetate) were equilibrated for 10 min. at 37°, after which 1 ml. of the enzyme preparation (equivalent to 200 mg. of rat liver or 50 mg. of chicken liver) was added and the reaction was carried out for the given length of time at the same temperature. The total volume of the reaction mixture was always adjusted to 10 ml. with the buffer and the reaction was stopped by the addition of an equal volume of ethanol. It was then extracted twice with light petroleum (b.p. 40–60°) in a separating funnel. The free cholesterol was determined by digitonin-precipitation according to Sperry & Webb (1950). In all the experiments controls to account for the cholesterol originating from the enzyme samples were employed. The estimation of vitamin A and assay of the common esterase were as described before (Seshadri Sastry *et al.* 1957).

Proteins were determined by the biuret method (Robinson & Hogden, 1940).

RESULTS

Experiments with rat liver

Relative rates of hydrolysis. At any given time up to 45 min. the average rate of hydrolysis of vitamin A acetate was roughly 12 times as fast as that of cholesterol acetate (Fig. 1), and the specific activities for the hydrolysis of the two substrates were 103.6 μ m-moles/30 min./mg. of protein and 8.36 μ m-moles/30 min./mg. of protein respectively. This is in agreement with the experiences of earlier workers (McGugan & Laughland, 1952; Byron, Wood & Treadwell, 1953).

Effect of pH and buffer. Vitamin A esterase showed two pH optima (6.6 and 8.6) with the maximum activity at pH 8.6. In contrast, cholesterol esterase had only one well-defined pH optimum at pH 7.4 (Fig. 2) [contrast Byron *et al.* (1953)]. These workers, however, used larger amounts of cholesterol butyrate as the substrate and their dispersing media and enzyme preparations were quite different. Our results agree more closely with those of Schotz, Rice & Alfin-Slater (1954). Substitution of phosphate for veronal buffer did not have any effect on the pH optima or on the rates of hydrolysis of the two substrates.

Bile salts have been reported to change the pH optima for the hydrolysis of triglycerides by lipase (Borgstrom, 1954). Sodium taurocholate (1%, w/v) activated the hydrolysis of both vitamin A acetate and cholesterol acetate by about 30%, but did not shift the pH optima.

Effect of substrate concentration. Lineweaver & Burk (1934) plots for the hydrolysis of vitamin A acetate and cholesterol acetate are given in Fig. 3. The K_m values were 0.22 and 0.4 mM respectively.

Energy of activation. Preliminary experiments carried out for testing the thermal stability of the

Fig. 1. Relative rates of hydrolysis of vitamin A acetate and cholesterol acetate by rat liver. Conditions were as described in the Materials and Methods section. X, Vitamin A acetate; O, cholesterol acetate.

Fig. 2. Effect of pH on (a) vitamin A esterase and (b) cholesterol esterase of rat liver. Buffers used: sodium phosphate (0.1M) up to pH 8.0 and sodium veronal buffer (0.1M) above pH 8.0 (---); Michaelis veronal buffer for the entire pH range (—). Incubations were for 1 and 12 hr. for vitamin A esterase and cholesterol esterase respectively. Other conditions were as described in the Materials and Methods section.

Fig. 3. Lineweaver & Burk (1934) plots for vitamin A esterase and cholesterol esterase of rat liver. Incubation was for 20 min. and 2 hr. for vitamin A esterase and cholesterol esterase respectively. Other conditions were as described in the Materials and Methods section. $\times$, Vitamin A esterase; $\circ$, cholesterol esterase.

two activities showed that they remain unchanged when incubated for 30 min. at 40°. The velocities of hydrolysis were therefore determined in triplicate at four different points below 40°, as given in Fig. 4, where the Arrhenius (1889) plots for the hydrolysis of vitamin A acetate and cholesterol acetate are given. The energies of activation, as calculated from the plots, were 11 540 cal./mole and 15 680 cal./mole respectively.

Fig. 4. Arrhenius (1889) plots for vitamin A esterase and cholesterol esterase of rat liver. Incubations were for 10 and 60 min. for vitamin A esterase and cholesterol esterase respectively. Other conditions were as described in the Materials and Methods section. Each point represents an average of triplicates. $\times$, Vitamin A esterase; $\circ$, cholesterol esterase.

Effect of aging and heat. Seshadri Sastry *et al.* (1957) demonstrated that vitamin A esterase is relatively more labile towards aging and heat than is the common esterase. With similar techniques the stability of cholesterol esterase was compared with that of vitamin A esterase. Here again, the latter activity was lost more rapidly by both treatments (Fig. 5). The stability characteristics of the cholesterol esterase thus seem to be more like those of the common esterase.

Inhibitor studies

Metal ions, alkaloids and arsenicals. It was possible to differentiate between vitamin A esterase and common esterase with the aid of these compounds as inhibitors, because all of them preferentially inhibit the former activity (Seshadri Sastry *et al.* 1957). In Table 1 the effects of these compounds on cholesterol-esterase activity of rat liver are summarized; the general pattern of inhibition was similar to that of vitamin A esterase.

Inhibition by compounds which combine with sulphhydryl groups. *p*-Chloromercuribenzoate at 1 mM concentration caused pronounced inhibition of vitamin A esterase and cholesterol esterase, whereas iodoacetate had relatively less effect on both activities. Addition of cysteine or glutathione did not reverse the inhibition. The common

esterase remained totally unaffected by both the reagents (Table 2). The common esterase of rat liver has been reported by other investigators to be a non-thiol enzyme (Ludewig & Chanutin, 1950).

Organophosphorus compounds. Organophosphorus compounds are useful for distinguishing various esterases of animal tissues. Most of this work has been confined to the esterases of tissues such as serum, pancreas and small intestine (Aldridge, 1953, 1954; Myers, Schotte, Boer, Borsje-Bakker, 1955), and the liver esterases have received less attention. It is shown in Fig. 6 that four such compounds, namely tetraethyl pyrophosphate, diethyl *p*-nitrophenylphosphate, diisopropyl nitrophenyl phosphate and diisopropyl phosphorofluoridate are highly inhibitory towards all the three esterase activities of rat liver, and it is further shown in Table 3 that there was no significant difference in their relative sensitivity.

Fig. 5. Effect of (a) aging and (b) heat on vitamin A esterase and cholesterol esterase of rat liver. Incubations were for 1 hr. with vitamin A esterase and for 12 hr. with cholesterol esterase. Other conditions were as described in the Materials and Methods section. Zero points in both sets of experiments represent the enzyme activities immediately after preparation. ×, Vitamin A esterase; ○, cholesterol esterase.

Table 1. Effect of metal ions, alkaloids and arsenicals on cholesterol esterase of rat liver

Enzyme (1 ml.) was mixed with 1 ml. of the inhibitor solution and kept for 30 min. at room temperature, after which 7 ml. of the buffer and 1 ml. of the substrate were added and incubation was continued for 12 hr. The rest of the procedure was as described in the Materials and Methods section.

	Percentage inhibition	
	5 mm	0.5 mm
CuSO ₄	98.0	97.0
HgCl ₂	98.0	98.0
ZnCl ₂	78.0	38.0
CdSO ₄	75.2	56.0
MgSO ₄	27.5	14.2
MnSO ₄	40.9	14.2
CoSO ₄	62.5	10.1
NaF	5.0	0.0
CaCl ₂	24.1	0.0
KCN	13.0	4.6
Atoxyl	13.0	0.0
Carbarsone	58.9	13.8
Physostigmine-HCl	97.9	93.1
Quinine-HCl	74.4	13.0

Table 2. Effect of *p*-chloromercuribenzoate and iodoacetic acid on vitamin A esterase, cholesterol esterase and common esterase of rat liver

Enzyme (1 ml.) was incubated with the inhibitor solution for 30 min. at room temperature; 1 ml. of cysteine, reduced glutathione or water, 6 ml. of the buffer and 1 ml. of the substrate were then added. Incubation was continued for 40 min. with vitamin A esterase and common esterase and for 12 hr. with cholesterol esterase.

	Percentage inhibition		
	Vitamin A esterase	Cholesterol esterase	Esterase
<i>p</i> -Chloromercuribenzoate (1 mm)	93.0	79.0	0.0
<i>p</i> -Chloromercuribenzoate (1 mm) + cysteine (10 mm)	95.0	60.0	0.0
<i>p</i> -Chloromercuribenzoate (1 mm) + glutathione (10 mm)	82.0	60.0	0.0
Iodoacetic acid (1 mm)	29.0	21.0	0.0
Iodoacetic acid (1 mm) + cysteine (10 mm)	32.7	37.0	0.0
Iodoacetic acid (1 mm) + glutathione (10 mm)	18.0	21.0	0.0

Fig. 6. Inhibition by organophosphorus compounds of hydrolysis of vitamin A acetate, cholesterol acetate and ethyl butyrate by rat liver. Conditions were as described in Table 1 except that incubation was for 40 min. for vitamin A esterase and common esterase and for 12 hr. with cholesterol esterase. $\times$, Vitamin A esterase; O , cholesterol esterase; $\bullet$, common esterase. Inhibitors: (a) tetraethyl pyrophosphate; (b) diethyl *p*-nitrophenyl phosphate; (c) diisopropyl *p*-nitrophenyl phosphate; (d) diisopropyl phosphorofluoridate.

Table 3. Sensitivity of vitamin A esterase, cholesterol esterase and common esterase of rat liver to organophosphorus compounds

pI_{50} , Negative logarithm of the molar concentration of the compound required to produce 50% inhibition of the activity.

Compound	pI_{50}		
	Vitamin A esterase	Cholesterol esterase	Esterase
Tetraethyl pyrophosphate	6.25	5.75	6.40
Diethyl <i>p</i> -nitrophenyl phosphate	6.60	6.50	6.35
Diisopropyl <i>p</i> -nitrophenyl phosphate	6.75	6.60	6.25
Diisopropyl phosphorofluoridate	6.40	7.00	6.15

According to Myers *et al.* (1955), the pancreatic cholesterol esterase of rats is similarly sensitive to organophosphorus compounds.

Effect of age and sex of the animals. Rats of both sexes were maintained on the normal stock diet after weaning and at the given time intervals two rats of each sex were killed for examination of the enzyme activities of the liver. It is seen from Fig. 7

that vitamin A-esterase activity progressively increased with age up to the third month in the males and up to the fourth month in the females, after which there was an appreciable fall. On the other hand, although the cholesterol esterase also increased with age it started to decline even after the third month. These marked differential changes in the two activities are further

Fig. 7. Effect of age and sex on vitamin A esterase and cholesterol esterase of rat liver. (a) Males; (b) females. Conditions were as described in the Materials and Methods section. Incubations were for 30 min. for vitamin A esterase and for 3 hr. for cholesterol esterase. Each point represents the average of two from two animals. $\times$, Vitamin A esterase; O, cholesterol esterase; Δ , relative ratios.

Fig. 8. pH-activity relationship of (a) vitamin A esterase and (b) cholesterol esterase of the nuclear and microsomal fractions of chicken-liver homogenate. Incubations were for 20 min. for vitamin A esterase and for 2 hr. with cholesterol esterase. Other conditions were as described in the Materials and Methods section. O, Nuclear fraction; $\times$, microsomal fraction.

Fig. 9. Lineweaver-Burk (1934) plots for (a) vitamin A esterase and (b) cholesterol esterase of the nuclear and microsomal fractions of chicken-liver homogenate. Conditions were as described under Fig. 8, except that the pH was 8.6 and 8.2 for vitamin A esterase and cholesterol esterase respectively. O, Nuclear fraction; $\times$, microsomal fraction.

demonstrated in the ratios of the two activities plotted against age in Fig. 7.

Attempts at solubilization. In previous experiments it was not possible to obtain the vitamin A esterase of rat liver in solution by acetone-drying, since this treatment led to a total loss of activity.

However, the common esterase was retained and could be solubilized after treatment with acetone. In the present study similar attempts at solubilization after acetone-drying led to a total loss of the cholesterol-esterase activity.

Table 4. *Effect of inhibitors on vitamin A esterase and cholesterol esterase of chicken-liver nuclei and microsomes*

Conditions were as described under Table 1. Incubation was for 20 min. and 2 hr. for vitamin A esterase and cholesterol esterase respectively.

Inhibitor	Percentage inhibition							
	Vitamin A esterase				Cholesterol esterase			
	Nuclear enzyme		Microsomal enzyme		Nuclear enzyme		Microsomal enzyme	
	6 mM	0.6 mM	6 mM	0.6 mM	5 mM	0.5 mM	5 mM	0.5 mM
HgCl ₂	100.0	100.0	81.0	81.0	96.0	96.0	100.0	100.0
MgCl ₂	26.8	20.0	19.0	17.6	76.0	31.0	100.0	83.0
CaCl ₂	34.2	14.0	17.6	5.0	86.0	4.0	100.0	87.5
ZnCl ₂	100.0	57.2	86.5	56.7	95.0	94.0	10.0	100.0
NaF	11.4	0.0	10.8	0.0	49.0	0.0	95.8	91.7
NaCN	0.0	2.8	0.0	0.0	10.2	10.2	0.0	25.0
CuSO ₄	90.0	90.0	95.0	80.0	100.0	100.0	100.0	100.0
Quinine-HCl	60.8	26.0	79.0	32.0	79.0	31.0	100.0	100.0
Carbarsone	28.3	23.3	52.0	19.0	92.4	31.0	95.8	58.0
Atoxyl	6.0	3.0	8.0	0.0	34.1	10.1	78.5	41.8
Na ₃ AsO ₃	85.8	40.8	52.0	52.0	52.2	24.4	98.5	45.8
Physostigmine-HCl	96.6	90.0	100.0	100.0	100.0	100.0	100.0	100.0

Experiments with chicken liver

pH optima. The curves for the pH optima of hydrolysis of vitamin A acetate and cholesterol acetate by the nuclear and microsomal enzymes of chicken liver are given in Fig. 8. There were no differences in the pH optima for the nuclear and microsomal enzymes with either substrate. The pH optima for vitamin A esterase were similar to those obtained with rat liver, although the peak at pH 6.6 was less pronounced in the avian species. The cholesterol esterase, on the other hand, showed optimum activity at pH 8.2 and 7.4 with chicken and rat livers respectively. This might be due to species difference.

Effect of substrate concentration. The enzymes from the two sources showed similar behaviour towards the respective substrates (Fig. 9). K_m values for vitamin A esterase of the nuclear and microsomal preparations were 0.19 and 0.3 mM; those for the cholesterol esterase of the same sources were 0.28 and 0.15 mM respectively.

Effect of inhibitors. The effects of a number of compounds on the nuclear and microsomal enzymes are summarized in Table 4. The heavy-metal ions and alkaloids proved to be potent inhibitors for the nuclear and microsomal vitamin A esterase with no major difference between the inhibition of the enzymes of the two sources. Most of these test substances exerted more powerful inhibitory action on the cholesterol esterase of both fractions. However, some differences in the behaviour of the nuclear and microsomal cholesterol esterase towards a few of these compounds can be noticed. Thus calcium chloride, sodium fluoride and quinine hydrochloride at 0.5 mM concentrations

caused relatively more inhibition of the microsomal enzyme. Comparison of the relative inhibition of the two esterases revealed that, although the effect due to most of these substances was similar, calcium chloride and sodium fluoride preferentially inhibited the cholesterol esterase. Similar patterns of inhibition were obtained with vitamin A esterase of rat liver (Seshadri Sastry *et al.* 1957).

Solubilization. Unlike rat liver, chicken liver retained some of its vitamin A-esterase and cholesterol-esterase activities on acetone-drying at -10° . However, the enzymes could not be obtained in solution from the acetone-dried powder; the powder itself lost its activities within 48 hr. at $0-5^\circ$.

Lissapol-N has been used successfully for solubilization of aryl sulphatase C of rat liver (Dodgson, Rose & Spencer, 1957). In our hands the same reagent gave a soluble preparation of the three esterases from the nuclear, but not from the microsomal, fraction of chicken liver. The solubilization of the nuclear activities by this detergent was almost instantaneous, whereas the microsomal enzymes remained insoluble even after prolonged incubation with the detergent at 37° . Usually the nuclei from 1 g. of liver prepared according to Krishnamurthy *et al.* (1958) were allowed to stand at 0° for 15 min. in 5 ml. of veronal buffer (pH 8.6, 0.1M) containing 100 mg. of Lissapol-N. The resulting solution was then centrifuged at 18 000g for 2 hr. at $0-5^\circ$, when a negligible amount of sediment was formed. The clear supernatant contained both activities in solution.

The Lissapol treatment led to a marked activation of the cholesterol esterase, but not of the

vitamin A esterase (Table 5). Similar activating effects of detergents on other enzymes, such as aryl sulphatase C (Dodgson *et al.* 1957) and rabbit-muscle phosphorylase (Cowgill, 1955), are known.

Separation of vitamin A esterase, cholesterol esterase and common esterase

Ammonium sulphate fractionation. The Lissapol extracts of chicken-liver nuclei were dialysed against frequently changed cold water at 0° for 12 hr. Solid ammonium sulphate was then added so as to obtain the desired saturation, as given in Table 6, and the mixture was allowed to stand for 15 min. at 0°. The precipitate was centrifuged at 0°, redissolved in cold water and dialysed against cold water for 12 hr. The fraction obtained between 28.5% and 37.5% saturation of ammonium sulphate contained 66.6% of the original vitamin A-esterase activity, whereas nearly 60% of the common esterase was recovered from the fraction above 50% saturation. The cholesterol-esterase activity, on the other hand, was not in any particular fraction, but 72% of it was recovered on recom-

binning all the fractions (Table 6). However, combination of any two fractions did not yield any significant activity. According to Nieft & Deuel (1949), two factors are involved in the hydrolysis of cholesterol esters by intestinal enzymes of rats. It seems probable that more than one factor might be required for the liver enzyme as well.

Electrophoresis. The Lissapol solution, concentrated to half its volume, was subjected to electrophoresis on agar gels according to Das & Giri (1959)

Table 5. *Effect of Lissapol-N on vitamin A esterase and cholesterol esterase of chicken-liver nuclei*

Nuclear suspension (1 ml., representing 50 mg. of the original tissue on wet-weight basis) was incubated with 1 ml. of veronal buffer (0.1M, pH 8.6) containing the given amounts of Lissapol-N for 30 min. at room temperature. Buffer (7 ml.; pH 8.6 for vitamin A esterase and 8.2 for cholesterol esterase) and 1 ml. of the substrate were then added and incubated for 20 min. with vitamin A acetate and for 1 hr. with cholesterol acetate.

Lissapol-N added (mg.)	Percentage activation (+) or inhibition (-)	
	Vitamin A esterase	Cholesterol esterase
5.0	+4.3	+25.0
10.0	0.0	+29.2
15.0	-4.3	+33.3
20.0	0.0	+33.3
25.0	-13.0	+41.7

Fig. 10. Electrophoretic separation of vitamin A esterase and cholesterol esterase of Lissapol extract of chicken-liver nuclei. Conditions were as described in the Materials and Methods section, except that veronal buffer (pH 8.2, 0.1M) was used for cholesterol esterase. Incubations were for 30 min. with vitamin A esterase and for 3 hr. with cholesterol esterase. [Hatched bars], Vitamin A esterase; [White bars], cholesterol esterase.

Table 6. *Ammonium sulphate fractionation of Lissapol extract of chicken-liver nuclei*

Conditions of assay were as described in the Materials and Methods section except that incubations were for 20 min. for vitamin A esterase, 2 hr. for cholesterol esterase and 40 min. for common esterase.

Fraction no.	Saturation (%)	Protein (mg./ml.)	Percentage of original activity recovered from precipitate		
			Vitamin A esterase	Cholesterol esterase	Common esterase
1	0.0	6.0	100.0	100.0	100.0
2	25.0	1.8	2.8	14.7	0.0
3	28.5	0.5	16.6	1.9	0.0
4	37.5	0.8	66.6	3.9	13.3
5	50.0	0.8	11.1	1.9	19.9
6	Above 50.0	2.0	3.7	0.9	59.9
Combined 2-6	—	—	—	72.0	—

for 24 hr. at 0–5° in sodium veronal buffer (pH 8·6, I 0·1) with a voltage gradient of 8·4v/cm. Maximum separation was obtained during this time and longer periods resulted in inactivation of the enzymes. After the separation the gel was cut into 1 cm. segments, which were then separately extracted with 5 ml. of veronal buffer (pH 8·6, 0·1M) by freezing and thawing, followed by centrifuging in the cold. Equal portions of the clear extracts were tested for vitamin A-esterase and cholesterol-esterase activities; the common-esterase activity could not be determined because of the low sensitivity of the method of assay. Fig. 10 shows that it is possible to separate the two esterases and that the vitamin A esterase consistently moved more towards the cathode than did the cholesterol esterase.

DISCUSSION

Much confusion exists in the literature about the identity and specificity of various esterases. Major difficulties have been solubilization and purification of the enzymes, and devising suitable methods of preparation of appropriate substrates and assay conditions. Some inhibitors, such as physostigmine, have proved useful for the identification of choline esterase (Richter & Croft, 1942). More recently organophosphorus compounds have been used for the classification of esterases in general. Aldridge (1953) classified the esterases into A and B types, based upon their response to the organophosphorus compounds. Techniques of differential inactivation have been employed by other workers for identification of some of the esterases, such as choline esterase (Richter & Croft, 1942), cholesterol esterase (Fodor, 1950; Swell, Dailey, Field & Treadwell, 1955) and vitamin A esterase (Seshadri Sastry *et al.* 1957).

The earlier work on the specificity of esterases was largely based on selective-inhibition and differential-inactivation studies, and attempts at actual separation were very few. Probably the most noteworthy work was by Augustinsson (1958), who successfully separated the A-, B- and C-type esterases of plasma by electrophoresis.

The present work strongly suggests that the three esterase activities are not due to the same enzyme. Thus the studies with rat liver reveal the following important points of difference between the three activities. The relative rates of hydrolysis of vitamin A acetate and cholesterol acetate were significantly different, the former substrate being hydrolysed at about twelve times the rate of the latter by the same enzyme preparation. The pH optima for the hydrolysis of vitamin A acetate were at pH 6·6 and 8·6, as against only one at pH 7·4 for the sterol ester. Aging and heat-inactivation experiments have shown that the vitamin A

esterase is more labile than are the others; acetone-treatment, on the other hand, abolished both vitamin A esterase and cholesterol esterase but not the common esterase. The vitamin A esterase increases its activity with age in both sexes, whereas the cholesterol esterase did not show a proportional increase. The strikingly different energies of activation of vitamin A esterase and cholesterol esterase would provide additional support for their non-identity. Finally, various inhibitors were able to differentiate clearly the common esterase from the other two, because most of them preferentially inhibited the activities of the last-named, whereas the common esterase was resistant. It is significant that the hepatic vitamin A esterase and cholesterol esterase are sulphhydryl enzymes, whereas the common esterase is not, as shown by the behaviour towards *p*-chloromercuribenzoate and iodoacetic acid.

These conclusions have been further substantiated by observations on chicken liver. Thus Lissapol markedly activated the cholesterol esterase of the nuclear fraction, but not the vitamin A esterase. On ammonium sulphate fractionation of soluble preparations from the nuclei the vitamin A esterase and common esterase were obtained in two widely separated fractions; the cholesterol esterase, on the other hand, was not in any single fraction (although it could be largely recovered by recombining the fractions). Further, by subjecting a similar soluble preparation to electrophoresis, vitamin A esterase and cholesterol esterase could be separated. Although any single criterion presented here may not be enough to establish the specificity of the three esterases, when all the points are considered together, the evidence seems to support the conclusion that the three esterases are distinct enzymes.

SUMMARY

1. Vitamin A esterase, cholesterol esterase and common esterase of rat and chicken liver were compared with vitamin A acetate, cholesterol acetate and ethyl butyrate as substrates.
2. Vitamin A acetate was hydrolysed by rat liver twelve times as fast as cholesterol acetate; the pH optima for the former were at pH 6·6 and 8·6, with the maximal activity at pH 8·6, and for the latter the optimum pH was 7·4. K_m values were 0·22 and 0·4 mM and the energies of activation were 11 540 cal./mole and 15 680 cal./mole for the respective substrates.
3. Several metal ions, alkaloids and arsenicals have shown comparable inhibitory effects on vitamin A esterase and cholesterol esterase of rat liver. Organophosphorus compounds inhibited all three esterases; *p*-chloromercuribenzoate and

iodoacetic acid inhibited the vitamin A esterase and cholesterol esterase, but not the common esterase.

4. The vitamin A esterase of rat liver was more labile towards aging and heat, and the other two esterases were more stable. Acetone-drying at 0–5° preferentially destroyed vitamin A esterase and cholesterol esterase, but not the common esterase.

5. The vitamin A esterase of nuclear and microsomal fractions of chicken liver had similar pH optima at pH 6.6 and 8.6, with maximal activity at pH 8.6, and their K_m values were 0.19 and 0.3 mM respectively. The cholesterol esterase of both sources had the same pH optimum at pH 8.2, and their K_m values were 0.28 and 0.15 mM respectively.

6. The three esterase activities of the nuclear, but not of the microsomal, preparation from chicken liver were solubilized by Lissapol-N; the detergent activated the nuclear cholesterol esterase, but not the vitamin A esterase.

7. Ammonium sulphate fractionation of the Lissapol extract gave 66.6% of the original vitamin A-esterase activity in the fraction between 28.5 and 37.5% saturation, whereas 60% of the common esterase was recovered from the fraction above 50% saturation. No single fraction gave the cholesterol-esterase activity, but on recombination of all fractions 72% of the original activity was recovered.

8. On electrophoresis of a Lissapol extract of chicken-liver nuclei on agar gel vitamin A esterase and cholesterol esterase could be separated.

9. It is suggested that the three activities are due to distinct enzymes.

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The Effects of X-Irradiation in Solution on the Kinetic, Sedimentation and Fluorescent Properties of Glutamic Acid Dehydrogenase

By S. J. ADELSTEIN* AND LORNA K. MEE
Department of Radiotherapeutics, University of Cambridge

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Investigators of the effects of ionizing radiation on enzyme solutions generally assume that the result of the interaction is an all-or-none loss of

* Moseley Travelling Fellow, Harvard University, 1958–59. Present address: Department of Anatomy, Harvard Medical School, Boston, Massachusetts, U.S.A.

enzymic activity. This tacit assumption fails to recognize that a change in the measured velocity of an enzymic reaction can also reflect a partial loss in functional activity. Studies of the kinetics of X-irradiated chymotrypsin show changes in the Michaelis constant and the maximum activity,