3. Treatment of cephalosporin C with dilute acid also yields the nucleus of cephalosporin C_c. The formation of this compound involves hydrolysis of an acetoxy group and subsequent lactonization, as well as removal of the side chain. The same compound is formed on hydrolysis of cephalosporin C_c.

4. Hydrolysis of cephalosporin C_A (pyridine) in dilute acid yields the nucleus of cephalosporin C_A (pyridine). This nucleus is also formed when 7-aminocephalosporanic acid reacts with aqueous pyridine.

We are indebted to Mrs M. Loveridge, Miss M. Arber and Miss O. Breitenmoser for skilful technical assistance. We wish to thank the Lilly Research Laboratories and Merck Sharp and Dohme Inc. for gifts of 6-aminopenicillanic acid, and Dr M. R. Pollock for purified penicillinase.

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Studies on Vitamin A Esterase

4. THE HYDROLYSIS AND SYNTHESIS OF VITAMIN A ESTERS BY RAT INTESTINAL MUCOSAE*

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(*Received* 24 *August* 1960)

Most of the work on vitamin A esterase has been on the hydrolysis of the esters; studies on the esterification are very few. In a preliminary communication High, Bright & Powell (1956) have reported esterification of vitamin A by small intestine and kidneys of rats. Krinsky (1958) has made a rather detailed investigation of esterification of vitamin A by cattle retina. While the present work was in progress, Pollard & Bieri (1960) reported their experiments on the esterification of vitamin A by acetone-dried pancreas of rat and chick.

Since the work of Gray & Cawley (1942) and of Clausen (1943) it has been generally assumed that vitamin A esters undergo hydrolysis and re-esterification during the process of absorption from the small intestine. More recently we have demonstrated that irrespective of whether vitamin A is given orally to rats as the free alcohol, as its acetate or as its palmitate, the esters found in the intestinal muscles, mesenteric lymph, blood and liver are invariably those of higher fatty acids (Mahadevan, Krishnamurthy & Ganguly, 1959), thus supporting the possibility of hydrolysis and re-esterification in the intestine during absorption.

It is demonstrated in this report that the mucosae of the small intestine of rats show hydrolytic and esterifying activities towards the vitamin. Some of the general properties of these activities are described and it is indicated that the esterification of vitamin A is apparently independent of coenzyme A and adenosine triphosphate requirements.

* Part 3. Krishnamurthy, Seshadri Sastry & Ganguly (1958).

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MATERIALS AND METHODS

Materials. Sodium taurocholate and palmitic acid were obtained from British Drug Houses Ltd. The palmitic acid was recrystallized from ethanol before use. Coenzyme A (CoA), *p*-chloromercuribenzoate and the disodium-trihydrate salt of adenosine 5′-triphosphate (ATP) were from Sigma Chemical Co. Tween 20 (polyoxyethylene sorbitan monolaurate) was from the Atlas Powder Co., Wilmington, Delaware, U.S.A., and diethylamine acetarsol (Acetylarsan) from May and Baker Ltd., Bombay, India. Di*iso*propyl phosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP) and diethyl *p*-nitrophenyl phosphate (E–600) were kindly procured for us by Dr S. Y. Thompson of the

National Institute for Research in Dairying, Reading (U.K.) from different sources in England. Sodium barbitone (veronal) was the product of E. Merck. The rest of the materials used have already been described by Ganguly, Krishnamurthy & Mahadevan (1959).

Preparation of the substrates. In our previous studies (Seshadri Sastry, Krishnamurthy & Ganguly, 1957) and in the preliminary experiments of the present study very low concentrations (0·2%, w/v) of Tween 80 (polyoxyethylene sorbitan mono-oleate) were used for dispersing the vitamin A. As will be seen later on, this detergent shows powerful inhibitory effect on the esterifying, but not on the hydrolytic system. Subsequently we found ethanol to be a suitable dispersing medium and all the experiments described here were carried out with ethanol as the dispersing agent (see also Pollard & Bieri, 1960).

The crystalline vitamin A samples (alcohol, acetate or palmitate) were dissolved in light petroleum and were purified by chromatography through alumina columns (Ganguly, Krinsky, Mehl & Deuel, 1952). The ester fractions were evaporated to dryness and taken up in ethanol so as to obtain a final concentration of 500 μg./ml. The vitamin A alcohol was similarly dissolved in ethanol, but here 1 mg. of palmitic acid/ml. was also added.

Preparation and assay of the enzymes. Initially the experiments were carried out with whole homogenates of the intestinal mucosal cells of normal stock rats of this Institute colony. Later on we noticed that feeding with a high-cholesterol diet for about 5 weeks markedly increased the specific activity of the mucosal enzymes of rats (Murthy, Mahadevan & Ganguly, 1960) and it was possible to obtain soluble enzymes in highly active form from acetone-dried powder prepared from the intestinal mucosae of such rats. No appreciable differences in the general properties of the enzymes prepared from these two sources were found and we have therefore used the soluble enzymes for the present investigation.

Young rats of both sexes were kept for 5 weeks on a diet consisting of starch (54%), casein (20%), sucrose (10%), salt mixture (5%), groundnut oil (10%), cholesterol (1%) and adequate supplements of all vitamins, and then were killed. The composition of the diet and the treatment of these animals are described in more detail in Murthy *et al.* (1960). The separation of the contents, mucosae and muscles of the small intestine was carried out as described earlier (Ganguly *et al.* 1959). The mucosae were ground in a pestle and mortar in 10 vol. of acetone at −15°. The mixture was centrifuged and the precipitate was washed three times with fresh acetone at −15°. Finally the residual acetone was removed under suction. The enzymic activities of the acetone-dried powders, thus prepared, were stable for more than 2 months at 0–5°, but once the enzymes were obtained in water solution they became rather unstable.

For preparation of the soluble enzymes, 1 g. of the acetone-dried powder was kept suspended in 25 ml. of cold water for 1 hr. at 0° and the solid was then removed by centrifuging at 10 000*g* for 15 min. The clear supernatant contained about 30% of the proteins of the original powder and retained about 90% of the activity. It was used as the source of the enzyme for the present study. As far as possible all manipulations were carried out in the cold room at 0–5°.

Unless otherwise stated, the reaction mixtures contained 3 ml. of 0·1 M-veronal buffer (Michaelis, 1931) of the appropriate pH (pH 8·6 for hydrolytic systems and pH 6·6 for esterification), 0·5 ml. of the ethanolic solution of the substrate (prepared as described above) containing 250 μg. of vitamin A alcohol, acetate or the palmitate and 0·5 ml. of the enzyme solution (6 mg. of protein for the hydrolytic reactions and 1·5 mg. of protein for esterification). The hydrolytic system for the palmitate always contained 0·5 ml. of a 10% (w/v) solution of sodium taurocholate, with the exception of the experiments of Tables 1 and 4 and Fig. 6, where the total amounts of the bile salt added are given. The final volume was made up to 5 ml. with water. All incubations were carried out at 37° for 30 min. The reactions were stopped by the addition of 5 ml. of ethanol. The amounts of free and esterified vitamin A formed were then determined according to Seshadri Sastry *et al.* (1957) and were taken as the measures of the enzyme activities.

Proteins were estimated by the biuret method (Robinson & Hogdon, 1940).

RESULTS

Effect of enzyme concentration. As shown in Fig. 1, the hydrolytic activity was proportional to protein concentration up to 10 mg./reaction mixture, and the process of esterification was proportional to protein concentration up to 1·5 mg. The specific activity for the esterification was 24 μg. of vitamin A alcohol esterified/mg. of protein/30 min., whereas for the hydrolysis of the acetate and the palmitate it was 6·7 and 4·0 μg. of alcohol liberated/mg. of protein/30 min. respectively. However, in these experiments bile salt was used with the palmitate, in the absence of which the specific activity

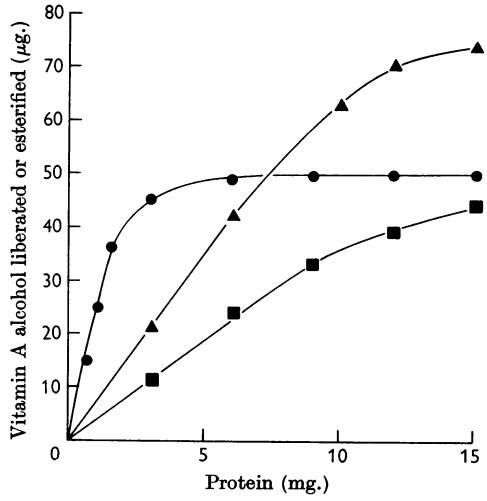

Fig. 1. Effect of enzyme concentration. Conditions employed were the same as those described in the Materials and Methods section. Enzyme extracts containing the given amounts of proteins were added to each reaction mixture. ▲, Hydrolysis of acetate; ■, hydrolysis of palmitate; ●, esterification of alcohol.

for the palmitate hydrolysis was 0·9 μg. of alcohol liberated/mg. of protein/30 min. In subsequent experiments we have used 6 mg. of protein for the hydrolytic studies and 1·5 mg. of protein for the esterification studies.

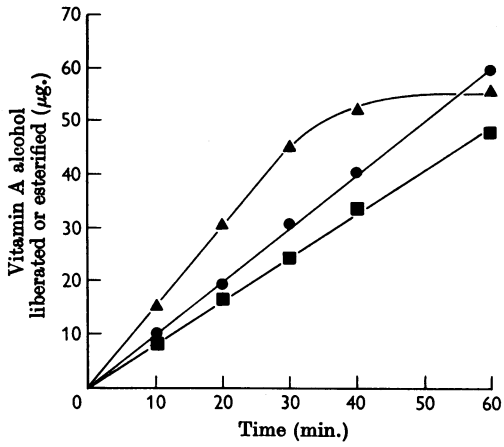

Fig. 2. Time course of the reaction. Conditions were the same as those described in the Materials and Methods section. Samples were withdrawn and analysed at the given time intervals. ▲, Hydrolysis of acetate; ■, hydrolysis of palmitate; ●, esterification of alcohol.

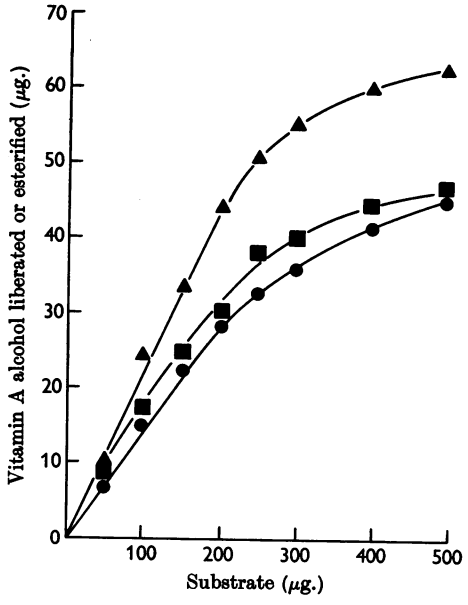

Fig. 3. Effect of substrate concentration. For each reaction a solution of the given amount of the substrate in 0·5 ml. of ethanol was added. Other conditions were the same as those described in the Materials and Methods section. ▲, Hydrolysis of acetate; ■, hydrolysis of palmitate; ●, esterification of alcohol.

Time course of the reactions. Both hydrolysis and synthesis were linear with time up to 30 min. (Fig. 2). The rate of esterification was markedly higher than the rate of hydrolysis of either substrate; the specific activity of the enzyme was almost identical with that found in the experiment described above.

Effect of substrate concentration. The effect of increasing the substrate concentrations on the three activities is shown in Fig. 3.

Effect of pH *and buffers.* The hydrolysis of both the acetate and the palmitate was optimum at pH 8·6, and the esterification had optimum activity at pH 6·6 (Fig. 4). With the rat-liver enzyme (Seshadri Sastry *et al.* 1957) there were two peaks at pH 6·6 and 8·6 for the hydrolysis of the acetate, with optimum activity at pH 8·6. In the present case the smaller peak at pH 6·6 was absent.

Substituting phosphate for veronal buffer did not shift the pH optimum of the esterifying enzyme, although the activity was 30 % more with the phosphate buffer. A similar comparison was not made with the hydrolytic systems, as pH 8·6 is outside the range of phosphate buffer. Since the veronal buffer could cover both the pH ranges of 6·6 and 8·6, we chose this particular buffer.

Effect of fatty acid concentration on the esterification. Vitamin A could not be esterified with acetic acid, butyric acid, *iso*valeric acid or hexanoic acid, but it readily formed esters with lauric

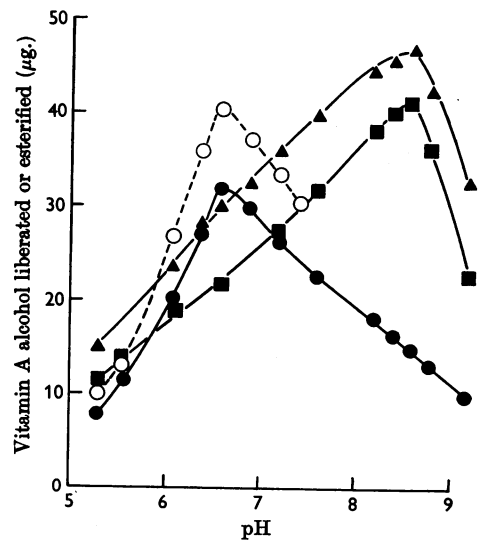

Fig. 4. Effect of pH and buffers. Conditions were the same as those described in the Materials and Methods section. Buffers (0·1 M) of the given pH were used. ▲, Hydrolysis of acetate (in veronal buffer); ■, hydrolysis of palmitate (in veronal buffer); ●, esterification of alcohol (in veronal buffer); ○, esterification of alcohol (in phosphate buffer).

Table 1. *Effect of Tween 20 and sodium taurocholate on hydrolysis and esterification*

Conditions were as described in the Materials and Methods section except that 0·5 ml. of water solutions of Tween or sodium taurocholate containing the corresponding amounts of the detergents were added for each reaction. In the experiments with Tween 20 for palmitate hydrolysis 50 mg. of the bile salt was always present.

	Tween 20 or taurocholate in reaction mixture (mg.)	Vitamin A alcohol (μg.)		
		Liberated		Esterified
		Vitamin A acetate	Vitamin A palmitate	Vitamin A alcohol
Tween 20	0	53·5	26·5	33·0
	2	53·7	27·0	24·5
	5	53·7	26·5	4·3
	10	56·4	11·3	4·3
	25	51·0	8·0	2·8
	50	44·8	8·0	2·8
Taurocholate	0	54·6	5·8	33·0
	10	53·6	15·0	28·5
	25	56·4	18·6	14·3
	50	51·0	26·5	5·7
	100	54·8	29·5	3·6

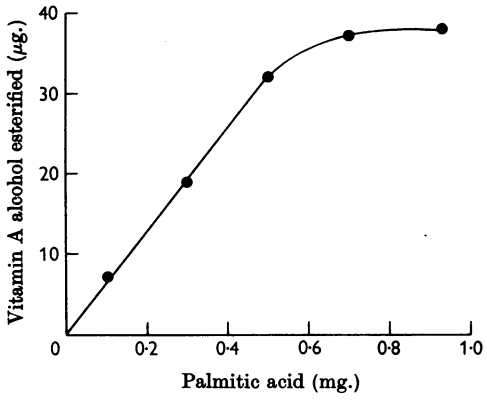

Fig. 5. Effect of fatty acid concentration on esterification. For each reaction 250 μg. of vitamin A alcohol, with the given amount of palmitic acid, in 0·5 ml. of ethanol was added. Other conditions were the same as those described in the Materials and Methods section.

acid, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. The presence or absence of unsaturation in the fatty acid did not appear to make any significant difference in the degree of esterification.

The effect of increasing concentrations of the fatty acid (palmitic acid) on the esterification of vitamin A is shown in Fig. 5. With up to 0·5 mg. of the fatty acid/5 ml. of reaction mixture the esterification was proportional to its concentration and above this concentration there was no further increase in esterification nor was there any inhibition.

Effect of coenzyme A and adenosine triphosphate on esterification. The esterification of vitamin A by the crude soluble enzymes was independent of CoA and ATP. Even with enzyme preparations purified 20- to 30-fold and dialysed for 24 hr. against cold distilled water there was no requirement for CoA or ATP. Similar results were obtained with cattle retina (Krinsky, 1958) and with chick pancreas (Pollard & Bieri, 1960). Recent work on the biosynthesis of triglycerides and phospholipids (Weiss, Kennedy & Kiyasu, 1960) and on acetylation of choline (Berman, Wilson & Nachmansohn, 1953) has shown that in some esterification reactions the fatty acids must be activated to the acyl-CoA derivatives by CoA and ATP; nevertheless, all esterification reactions do not seem to be energy-dependent. Thus not only the esterification of vitamin A, but the esterification of cholesterol by hog pancreas (Hernandez & Chaikoff, 1957) or by rat-intestinal mucosae, is independent of CoA and ATP (S. K. Murthy, S. Mahadevan & J. Ganguly, unpublished work).

Effect of Tween 20 and sodium taurocholate. In previous studies with the hydrolytic enzymes Tween dispersions of vitamin A were consistently used (McGugan & Laughland, 1952; Ganguly & Deuel, 1953; Ganguly, 1954; Seshadri Sastry *et al.* 1957). However, Tween compounds appear to act as inhibitors for the esterification of vitamin A (Krinsky, 1958). The effect of Tween 20 on the systems from the intestinal mucosae was therefore investigated. The detergent had little or no effect on the hydrolysis of the acetate and the palmitate up to a concentration of 5 mg./reaction mixture, but at higher concentrations the palmitate hydrolysis was inhibited to about 70% (Table 1). At the same time it had a pronounced inhibitory action on the esterifying reaction. Thus even at a concentration of 5 mg./reaction mixture the inhibition was about 85%. In separate experiments without the tauro-

cholate, Tween 20 up to a concentration of 5 mg./reaction mixture had no appreciable effect on the hydrolysis of the palmitate. Our preliminary results with Tween 80 (polyoxyethylene sorbitan monooleate) indicated that this compound was an even more potent inhibitor of the esterification. These results thus confirm the observations of Krinsky (1958). Cleland & Kennedy (1960) have reported that Tween 20 inhibits the biosynthesis of psychosine.

In contrast, as further shown in Table 1, the bile salt had a marked activating effect on the hydrolysis of the palmitate, without exerting similar action on the acetate hydrolysis. Thus the acetate was hydrolysed equally well irrespective of whether the bile salt was absent or present even at a concentration of 50 mg., whereas the palmitate hydrolysis had increased 4·4-fold with a change in the concentration of the detergent from 0 to 50 mg. The hydrolysis of the palmitate was only about 5 μg. of vitamin A alcohol liberated/30 min./reaction mixture without the bile salt. This is obviously too low for studies of reasonable accuracy. We have therefore used 50 mg. of bile salt/reaction mixture for the hydrolytic system for the palmitate.

The esterification, however, was progressively inhibited with increasing concentrations of the bile salt. This is quite different from the behaviour of cholesterol, where both hydrolysis and esterification by the same mucosal enzymes require the presence of the bile salt (Swell & Treadwell, 1955; S. K. Murthy, S. Mahadevan & J. Ganguly, unpublished work).

Effect of inhibitors

Metal ions, alkaloids and arsenicals. The effects of the various compounds on the activities are summarized in Table 2. Most of the compounds tested did not have any appreciable effect on the three systems at a final concentration of 0·1 mM. However, at 1 mM concentration some interesting differences in the three systems were evident. Thus calcium chloride had considerable activating effect on the hydrolysis of the palmitate and not of the acetate, but at the same time it had an inhibitory action on the esterification process. It is possible that this may be due to the formation of insoluble calcium salts of the free palmitic acid. With the other compounds, there was variation in the degree of inhibition of the three systems. Three compounds, however, sodium fluoride, sodium arsenite and diethylamine acetarsol, had no effect on esterification but produced considerable inhibition of the hydrolysis of both the acetate and the palmitate. Many of the compounds listed in Table 2 displayed similar effects on the hydrolysis of vitamin A acetate by rat-liver vitamin A esterase (Seshadri Sastry *et al.* 1957).

p-*Chloromercuribenzoate.* This compound had no effect on the three systems. P. Seshadri Sastry & J. Ganguly (unpublished work) have observed that the hydrolysis of vitamin A acetate by rat- and chicken-liver enzymes is inhibited by *p*-chloromercuribenzoate and, according to Krinsky (1958), the esterifying enzyme of cattle retina is also inhibited by this reagent, but Pollard & Bieri (1960) do not consider the chick-pancreas esterifying enzyme to be a sulphydryl one. It is possible that these discrepancies could be due to the fact that not only are they from different tissues, they belong to different species also.

Organophosphorus compounds. Several organophosphorus compounds have been used by other workers for differentiating the esterases of animal tissues (Myers, Schotte & Mendel, 1955). By the procedure described by Aldridge (1954), the effects of three of these compounds, DFP, TEPP and E–600, on the three systems were studied (Table 3).

Table 2. *Effect of metal ions, alkaloids and arsenicals on the hydrolytic and esterifying systems*

Except for the substrate, all other components described in the Materials and Methods section were preincubated with 0·5 ml. of aqueous solution of the given compound for 15 min. at 37°, after which the substrate was added and incubation continued for another 30 min. at the same temperature.

	Activity retained (%)					
	Vitamin A acetate		Vitamin A palmitate		Vitamin A alcohol	
Compound	1 mM	0·1 mM	1 mM	0·1 mM	1 mM	0·1 mM
$HgCl_2$	55·5	88·0	47·5	94·0	65·0	97·8
$MgSO_4,7H_2O$	88·6	97·0	100·0	100·0	85·4	86·4
$CaCl_2$ (anhydrous)	106·0	99·8	162·0	112·0	66·0	84·0
$CuSO_4,5H_2O$	18·4	87·0	65·0	96·4	30·0	92·1
$ZnCl_2$	11·1	69·6	31·0	93·0	18·1	91·2
NaF	79·6	96·0	67·0	95·6	96·3	97·4
NaCN	101·0	99·8	70·1	98·6	100·0	100·0
Na_3AsO_3	55·0	87·0	60·0	96·0	100·0	100·0
Diethylamine acetarsol	46·0	77·7	60·8	95·2	96·6	100·0
Quinine–HCl	60·0	92·8	55·0	98·8	30·0	96·3

Table 3. *Effect of organophosphorus compounds on the hydrolytic and esterifying systems*

Conditions and procedures were the same as described for Table 2.

	Inhibition (%)					
Final concn. of inhibitor (μM) ...	100	10	1	0·1	0·01	0·001
Di*iso*propyl phosphorofluoridate						
Vitamin A acetate	100·0	100·0	100·0	100·0	53·6	5·2
Vitamin A palmitate	74·6	50·0	33·6	2·8	0	0
Vitamin A alcohol	4·8	3·5	0	0	0	0
Diethyl *p*-nitrophenyl phosphate						
Vitamin A acetate	100·0	100·0	100·0	89·0	43·0	14·0
Vitamin A palmitate	72·8	35·0	8·0	0	0	0
Vitamin A alcohol	4·8	4·0	0	0	0	0
Tetraethyl pyrophosphate						
Vitamin A acetate	100·0	100·0	100·0	88·5	26·4	6·6
Vitamin A palmitate	0	0	0	0	0	0
Vitamin A alcohol	0	0	0	0	0	0

Table 4. *Effect of sodium taurocholate on the hydrolytic and esterifying systems in the presence of* 0·1 mM-*di*iso*propyl phosphorofluoridate*

For the hydrolysis of the palmitate the enzyme was preincubated in 0·1 M-veronal buffer, pH 8·6, with 0·1 mM-DFP as described in Table 3. The taurocholate and the substrate were then added and the incubation was continued at 37° for 30 min. The esterification was at pH 6·6 (veronal buffer, 0·1 M) after preincubation of the enzyme with 0·1 mM-DFP as described in Table 3. The rest of the procedure was the same as for hydrolysis.

	Vitamin A alcohol (μg.)	
	Liberated	Esterified
Additions	Vitamin A palmitate	Vitamin A alcohol
Without taurocholate	5·8	35·0
With taurocholate (50 mg.)	35·0	7·0
With taurocholate (50 mg.) + 0·1 mM-DFP	8·7	7·0
With 0·1 mM-DFP	1·2	35·0

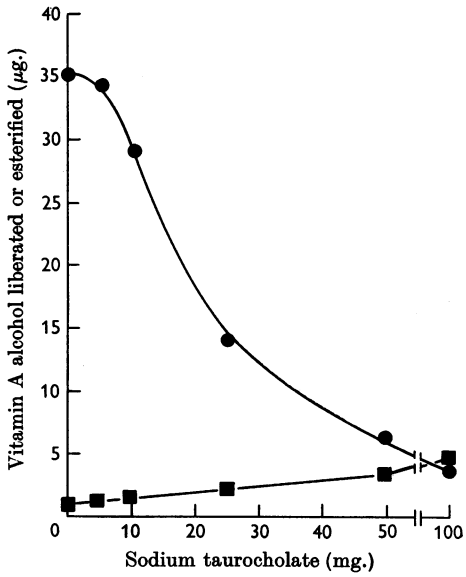

Fig. 6. Effect of sodium taurocholate in the presence of DFP. Reactions were carried out in a total volume of 5 ml. containing a constant amount of DFP (0·1 mM) and the given amounts of the bile salt, as described in the Materials and Methods section. ■, Hydrolysis of palmitate; ●, esterification of alcohol.

With all three of them there was total inhibition of the hydrolysis of the acetate at inhibitor concentrations of 1 μM–0·1 mM. Two of the three inhibitors (DFP and E–600) had considerable effect on the hydrolysis of the palmitate, though it was not as pronounced as with the acetate. The third inhibitor (TEPP) does not appear to have any effect on the hydrolysis of the palmitate.

The esterification reaction, on the other hand, was completely unaffected by all of these compounds. It would thus appear logical to conceive that the esterification of vitamin A by the mucosal enzymes is not a direct reversal of the hydrolytic process and this is further supported by the following experiments with DFP and taurocholate.

*Sodium taurocholate in the presence of di*isopropyl *phosphorofluoridate.* There was little hydrolysis of the palmitate without the bile salt, but the esterification was inhibited by this agent. DFP alone inhibited the hydrolysis, but not the esterification, whereas these two substances added together inhibited both the hydrolytic and esterification reactions (Table 4). In Fig. 6 it is further demonstrated that in the presence of a constant amount of DFP increasing concentrations of the bile salt produced progressive inhibition of the esterification without concomitant increase in hydrolysis.

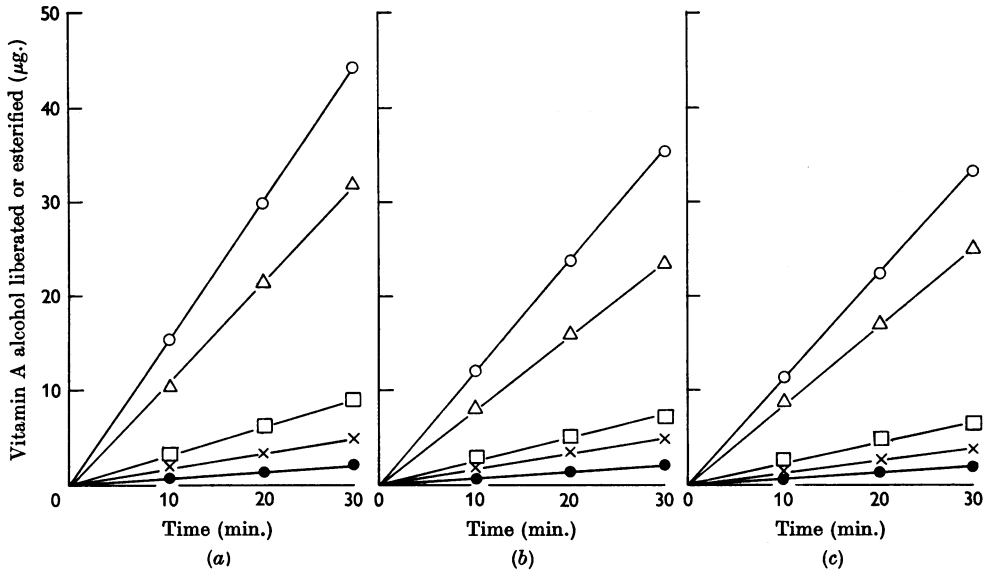

Fig. 7. Intracellular distribution of the hydrolytic and synthetic activities of intestinal mucosae of normal rats. For each reaction, cell fractions equivalent to 100 mg. of the original mucosal tissue were used. Other conditions were similar to those described in the Materials and Methods section. (*a*) Hydrolysis of acetate; (*b*) hydrolysis of palmitate; (*c*) esterification of alcohol. ○, Whole homogenate; ●, nuclear fraction; ×, mitochondrial fraction; △, microsomal fraction; □, supernatant fraction.

Intracellular distribution of the hydrolytic and esterifying activities of the mucosae

The mucosae of the small intestine of normal stock male rats were collected, homogenized in 0·25 M-sucrose and fractionated by differential centrifuging into the nuclear, mitochondrial, microsomal and supernatant fractions as described by Ganguly *et al.* (1959). Fig. 7 shows that these activities are mostly localized in the microsomal fraction of the homogenate. Earlier workers found the hydrolytic and esterifying systems of the cells of other tissues also to be associated with the particulate materials of the cell homogenate. Thus the hydrolytic enzyme is in the microsomal fraction in rat liver (Ganguly & Deuel, 1953) and in the nuclear and microsomal fractions in chicken liver (Krishnamurthy, Seshadri Sastry & Ganguly, 1958); the esterifying enzyme in cattle retina is sedimentable by centrifuging at 35000*g* (Krinsky, 1958).

Other properties of the mucosal enzymes. Prolonged dialysis of the mucosal-cell homogenates for 24 hr. against frequently changed cold distilled water did not alter the activities to any appreciable extent. The homogenate enzymes were stable for at least 30 days when stored at 4°, but the soluble enzymes, as prepared from the acetone-dried mucosae, lost 40 % of their activities at the same temperature in 1 day.

Distribution of the activities in normal rats. The activities were present in measurable amounts in the contents, mucosae and muscles of the small intestine, as well as in the pancreas of the normal stock rats (Table 5). The specific activities of the enzymes of intestinal contents were the highest. The kidneys did not show any appreciable activity

Table 5. *Tissue distribution of the activities in the normal stock rat*

Conditions were the same as in the Materials and Methods section except that 5–20 mg. of protein was used for each reaction.

	Vitamin A alcohol (μg./mg. of protein/30 min.)		
	Liberated		Esterified
	With 50 mg. of bile salt	Without bile salt	Without bile salt
	Vitamin A palmitate		Vitamin A alcohol
Pancreas	13·0	6·4	7·6
Small intestine			
Contents	40·0	27·0	55·5
Mucosae	2·3	Nil	2·6
Muscle	9·2	Nil	5·3
Plasma	Nil	Nil	0·1
Liver	0·13	Nil	Nil
Kidneys	0·12	Nil	0·2
Spleen	0·08	Nil	0·25

with respect to esterification and this seems to agree with the findings of Pollard & Bieri (1960), but not with those of High *et al.* (1956).

DISCUSSION

The work of Gray & Cawley (1942) had implied the presence of both hydrolytic and synthetic activities for vitamin A esters in the small intestine and our studies have demonstrated that these activities are present not only in the lumen, but also in the muscles and mucosae of the small intestine of rats. Rapid hydrolysis of vitamin A esters and esterification of the free vitamin A can be demonstrated with the mucosal-cell homogenates, as well as with the soluble enzymes prepared from the acetone-dried mucosae.

Optimum rates of hydrolysis took place at pH 8·6, whereas the esterification was optimum at pH 6·6. Sodium arsenite, sodium fluoride and diethylamine acetarsol were able to inhibit partially the hydrolysis, but not the esterification. On the other hand, Tween 20 even at 0·1 % concentration exerted a pronounced inhibitory action on the esterification, but at the same time had no effect on the hydrolysis. Finally the organophosphorus compounds were very powerful inhibitors of the hydrolytic process only. It would thus appear probable that though both activities are recovered from the microsomal fraction of the cell homogenate of the mucosae, the hydrolysis and esterification are not brought about by the reversal of one reaction.

Instances, however, are known where hydrolysis and esterification are not strictly reversible, e.g. the enzymic formation of choline esters (Berman *et al.* 1953) or of triglycerides (Weiss *et al.* 1960). But in such cases the fatty acids pass through a state of activation in the form of acyl-CoA derivatives at the expense of ATP. However, no CoA or ATP seems to be necessary for the esterification of vitamin A or of cholesterol. The exact mechanisms of such esterification processes thus remain obscure.

The fatty acid-specificity experiments have indicated that the esterification of vitamin A by the mucosal enzymes takes place with fatty acids containing more than 10 carbon atoms without any preference for chain length or unsaturation of the acid. Gray & Cawley (1942) have suggested that in rat liver vitamin A is probably stored as its palmitate. More recent work has shown that after feeding of vitamin A acetate the vitamin appears in the muscles of the small intestine as its higher ester (Mahadevan *et al.* 1959). This suggests that the vitamin A esters undergo hydrolysis and are then esterified again inside the mucosal cells after absorption. The most common fatty acids in the animal body and in the normal diet, and available for esterification of vitamin A at the site of absorption, are stearic acid, palmitic acid, oleic acid and linoleic acid. This would therefore explain why only the higher fatty acid esters are found in the animal body (Kaiser & Kagan, 1956).

Poor or no absorption of vitamin A (presumably the natural esters) in biliary diseases is a well-recognized fact; oral administration of extra bile along with the vitamin improves its absorption in such cases (Clausen, 1943). We have demonstrated here that the hydrolysis of the higher esters of the vitamin is rather poor without the bile salt and is greatly facilitated by its presence. These results would thus explain the role of the bile in improving the absorption of vitamin A in biliary diseases.

SUMMARY

1. The hydrolytic and synthetic activities for vitamin A esters are present in the contents, mucosae and muscles of the small intestine, as well as in the pancreas of rats, with the luminar enzymes showing highest specific activities.

2. Both the activities are localized in the microsomal fraction of the mucosal-cell homogenate.

3. The activities of the mucosae can readily be brought into solution by extracting the acetone-dried mucosae with water.

4. Hydrolysis of vitamin A acetate by the soluble enzymes did not require sodium taurocholate; that of the palmitate was very poor in the absence of the bile salt and was greatly activated by it.

5. The hydrolytic and esterifying activities were optimum at pH 8·6 and 6·6 respectively. The hydrolytic activity was preferentially inhibited by sodium fluoride, sodium arsenite, diethylamine acetarsol, di*iso*propyl phosphorofluoridate and diethyl *p*-nitrophenyl phosphate. Tween 20, sodium taurocholate and calcium chloride inhibited esterification alone.

6. No coenzyme A or adenosine triphosphate appeared to be necessary for the esterification of vitamin A.

The authors wish to thank Dr S. Y. Thompson of National Institute for Research in Dairying, Reading (U.K.), for kindly procuring the organophosphorus compounds for us, and Voltas Ltd., Bombay, for the gift of synthetic vitamin A (F. Hoffmann–La Roche and Co. Ltd., Basle 2, Switzerland). Financial assistance from the Indian Council of Medical Research, New Delhi, is acknowledged.

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The Occurrence of Free D-Serine in the Earthworm

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(*Received* 8 *November* 1960)

The occurrence of D-serine in lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate) and serine ethanolamine phosphodiester, isolated from the earthworm, has been reported from this laboratory (Beatty, Magrath & Ennor, 1959; Ennor, Rosenberg, Rossiter, Beatty & Gaffney, 1960; Beatty, Ennor, Rosenberg & Magrath, 1961).

This paper describes the isolation of DL-serine from earthworm tissues and provides conclusive proof that D-serine is present in the tissues immediately before extraction and that it could not arise as a result of racemization of L-serine during the isolation procedure.

A preliminary report of this work has appeared (Rosenberg & Ennor, 1960).

MATERIALS AND METHODS

All reagents were A.R. grade. Ethanol used for chromatography and other purposes was purified by refluxing over KOH and aluminium powder and then by fractional distillation. D-, L- and DL-Serine were purchased from California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A. L-[3-^{14}C]serine was obtained from Dr R. L. Blakley of this Department and was prepared from glycine and [^{14}C]formaldehyde with serine transhydroxymethylase (McDougall & Blakley, 1961). The earthworms used in this work were as described by Rosenberg & Ennor (1959).

Isolation of serine. The experimental procedures used for the isolation of serine from the tissue have been described in detail (Ennor *et al.* 1960), and the various steps will be referred to in general terms only. Such modifications as have been introduced will be described in the text.

D-*Amino acid oxidase.* This was prepared according to Negelein & Brömel (1939) and was taken to stage III [$(NH_4)_2SO_4$ precipitation]. The precipitate containing the enzyme was stored at 0° as a suspension in 50% saturated $(NH_4)_2SO_4$. Portions of this suspension were centrifuged as required and the precipitate was resuspended in 0·01 M-pyrophosphate buffer, pH 8·1, and dialysed against 100 vol. of the same buffer, with two changes, for 24 hr. The dialysed solution was cleared by centrifuging and its volume adjusted with water so that each millilitre of the solution corresponded to 1 g. of the acetone-dried kidney powder used in the preparation. The solution was tested for the presence of catalase according to Herbert (1955) and it was found that 0·1 ml. of the solution decomposed 100 μmoles of H_2O_2 in 30 sec. The solution was kept frozen at −10° and retained activity for at least 3 months. Assays with this enzyme were carried out in Warburg manometers at 37° in air. The vessels contained 2·0 ml. of 0·1 M-sodium pyrophosphate buffer, pH 8·3, the amino acid tested and 0·3 ml. of the enzyme solution, in a total volume of 3 ml.

Ascending paper chromatography. This was carried out on Whatman no. 3 papers and the following solvent systems were employed: (I) ethanol–formic acid–water (70:10:20, by vol.); (II) water-saturated phenol; (III) butan-2-one–methylCellosolve (2-methoxyethanol)–acetic acid–water (40:15:6:24). Amino acids and related compounds were detected by dipping the dried papers into 0·2% solution of ninhydrin in acetone, followed by heating for 10 min. at 80°.