Manometric Assay and Properties of Pancreatic Cholesterol Esterase

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It has long been known that enzymically catalysed synthesis and hydrolysis of cholesterol esters occurs in the presence of certain tissues (Kondo, 1910*a, b*; Schultz, 1912; Cytronberg, 1912; Gardner & Lander, 1913; Mueller, 1916*a, b*; Porter, 1916; Nomura, 1924*a, b*; Shope, 1928; Nedswedski, 1935, 1936; Klein, 1939; Sperry, 1935; Sperry & Stoyanoff, 1937, 1938). The most extensive recent studies, initiated with the enzyme from pancreas, are those of Yamamoto, Goldstein & Treadwell (1949) and Swell & Treadwell (1950*a*), who stressed the importance of stable emulsions of cholesterol and cholesterol esters and described their preparation. By use of these mixtures numerous properties of the cholesterol esterase present in serum (Swell & Treadwell, 1950*b*) and in homogenates of various organs were studied (Swell, Byron & Treadwell, 1950; Byron, Wood & Treadwell, 1953; Swell, Dailey, Field & Treadwell, 1955). Hydrolysis of cholesteryl acetate catalysed by an enzyme present in the microsomal fraction of rat liver has been observed by Schotz, Rice & Alfin-Slater (1954). Some properties of purified pancreatic cholesterol esterase have been reported by Hernandez & Chaikoff (1957), who devised a rapid turbidimetric method for estimation of enzymic activity.

The present report describes some characteristics of the cholesterol esterase, which is found in extracts of acetone-dried pancreas, and defines the conditions required to demonstrate high catalytic activity. The data were obtained by use of a manometric procedure for estimation of synthesis or hydrolysis of cholesterol esters. Brief reports of some of the data have been made (Korzenovsky, Rust & Diller, 1955; Korzenovsky, Vesely & Diller, 1956).

METHODS

In this paper the expression colloidal substrate is used to describe a properly prepared colloidal system consisting of sterol or sterol ester with albumin, fatty acid and bile acid.

Preparation of colloidal substrate and composition of standard test system for synthesis of sterol esters. The substrate for esterification consisted of sterol, fatty acid (used as the lithium salt unless otherwise noted) and bile acid. Appropriate amounts of these compounds were placed in a flask containing approximately the requisite amount of water. To this was added an amount of ethyl ether equal to

one-half of the volume of water. The flask was shaken until all components dissolved and only one phase was present. The ether was expelled by gentle stirring while warming on the water bath. The temperature was not allowed to rise above 50°. The substrate was adjusted to pH 6.5–6.6 with 0.1M-H₂SO₄ and made up to volume. Ether was again added and the mixture was re-emulsified, by shaking, until it became single-phased. The ether was removed as above. Although the colloidal substrate for esterification may be prepared as described at virtually any concentration, so long as the ingredients are maintained in the proper molar ratio, the following concentrations have been found convenient to use in the standard substrate for synthesis of cholesteryl oleate: 0.04M-cholesterol, 0.12M-lithium oleate and 0.02M-cholic acid; 0.8 ml. of this was used in every assay. The final concentrations in a volume of 2.2 ml. were: 0.014M-NaHCO₃, 0.09M-NaCl or 0.045M-(NH₄)₂SO₄, 0.0145M-cholesterol, 0.044M-lithium oleate and 7.3 mM-cholic acid. To ensure rapid gas equilibration the total fluid volume did not exceed 2.2 ml. The complete reaction mixture after gassing with CO₂ is at pH 6.1–6.2.

Preparation of colloidal substrate and composition of standard test system for hydrolysis of sterol esters. The components of the substrate for hydrolysis, consisting of sterol ester, bile acid and lithium oleate, were placed in an appropriate amount of an aqueous 0.67% solution of crystalline bovine albumin. The mixture was shaken with a volume of ether equal to one-half of the amount of water until only one phase was present. After the ether was removed by gentle heat, the substrate was adjusted to pH 6.9–7.0 and made up to volume with the albumin solution. The remainder of the procedure is as outlined in the previous section. The standard substrate for hydrolysis consists of 0.07M-cholesteryl oleate, 0.014M-cholic acid and 0.035M-lithium oleate emulsified in 0.67% albumin solution as described. The ratio of the first two compounds on a molar basis is 5:1. The concentrations of lithium oleate and albumin are not critical but appear to be minimal as given. The standard test system consists of the following ingredients, which must be placed in the reaction vessel in the order listed to preserve the dispersed character of the substrate: 0.8 ml. of the substrate for hydrolysis, water (in sufficient amount so that the final fluid volume is 2.2 ml.), NaHCO₃, (NH₄)₂SO₄ at pH 7.0, and enzyme. The final concentrations were 0.18M-NaHCO₃, 0.72M-(NH₄)₂SO₄, 0.025M-cholesteryl oleate, 5.1mM-cholic acid, 0.0127M-lithium oleate and 0.24% albumin. After flushing with CO₂ the complete reaction mixture is at pH 6.9–7.0.

Although the substrates prepared as described do not deposit a sediment or change in any discernible way, even after several days of storage, only quantities sufficient for daily use were made at any one time. The mixtures should not be frozen.

Manometry. The reactions were conducted at 37° in single-side-arm Warburg flasks of approx. 16 ml. capacity. The enzyme solution was put in the side arm and all other ingredients in the main compartment unless otherwise indicated. After gassing with 100% CO₂ and temperature equilibration, the reaction was begun by tipping the enzyme into the main compartment. The first reading was made after 3–6 min., and the reaction was allowed to proceed for 30 min. more. Results are reported as μ l. of CO₂. The absorption of gas is designated by a minus sign and is indicative of ester synthesis. Release of CO₂ occurs during ester hydrolysis and is indicated by a plus sign. Bicarbonate solutions were gassed with CO₂ for at least 30 min. before use.

Enzyme solution. Ox pancreas, obtained at slaughter, was chilled in ice immediately after removal from the carcass. Fat and connective tissue were trimmed away and the rest of the gland was cut into small pieces, but not minced. The pieces of pancreas were rapidly stirred with 10 times their volume of acetone at –15° and were left overnight at this temperature. The acetone was decanted and the solid material was passed through an ordinary meat grinder into a volume of acetone equal to the first used. The grinding operation and subsequent filtrations were done at –10° to –15°. The suspension was filtered on a Büchner filter without permitting the cake to dry. The moist cake was washed on the filter with fresh acetone, then with acetone-ether (1:1, v/v) and finally with ether in amounts equal to twice the volume of the filter cake. The residue was sucked dry and was spread on a clean surface to eliminate the ether. The dry powder has been kept at –15° for periods longer than 1 year without appreciable loss of activity. A solution of the enzyme was prepared by suspending 1 g. of powder in 10 ml. of 0.01 M-NaCl or water at 4°. The suspension was stirred at 4° for 30–60 min. and then was filtered through a hard-surface paper. The clear amber-coloured solution contained 15–20 mg. of protein/ml. as determined according to Weichselbaum (1946). It may be dialysed for 48 hr. against 0.01 M-NaCl or for 4–6 hr. against water without loss of activity. Almost all of the enzymically active protein present in the extract was precipitated upon increasing the concentration of (NH₄)₂SO₄ from 25 to 50% of saturation. The precipitate was dissolved in water and was dialysed for 4–6 hr. against water or for

24 hr. against 0.01 M-NaCl. The solution contained 5–7 mg. of protein/ml., indicating an approximately threefold concentration of the enzyme. About 0.3 ml. of either enzyme preparation was used in each Warburg flask. The crude extract and the fraction obtained by (NH₄)₂SO₄ precipitation were found equally satisfactory in the manometric procedure.

Chemicals. Sterols were obtained from commercial sources; they were recrystallized until m.p. values and X-ray-diffraction patterns agreed with published data. β -Sitosterol was of cotton seed origin. β -Norcholesterol was kindly supplied by Dr W. G. Dauben (University of California, Berkeley). Oleic acid was purified according to Hilditch (1947). The lithium salt was obtained by neutralizing the purified acid with LiOH followed by precipitation with acetone. The crystalline bile acids were a generous gift of Professor S. Bergström (Lund, Norway), to whom a debt of gratitude is expressed.

RESULTS

Estimation of cholesterol-esterase activity. The manometric estimation of cholesterol-esterase activity is based on the assumption that for each mole of ester which is synthesized or hydrolysed an equivalent of CO₂ gas is exchanged. The estimation of ester hydrolysis, by measurement of CO₂ gas released from the aqueous phase of a sodium bicarbonate–CO₂ buffer system, is a well-established analytical procedure. The reverse process, i.e. the measurement of the amount of CO₂ gas absorbed into the aqueous phase of a sodium bicarbonate–CO₂ buffer system to estimate ester synthesis, is not a method in common use. It is evident from Table 1 that, under the conditions specified, the manometric determination of synthesis or hydrolysis of cholesteryl oleate is in fair agreement with results obtained by the colorimetric procedure of Sperry & Webb (1950). Table 2 shows that the CO₂ gas absorbed into the aqueous phase appears as bicarbonate or a closely related compound. The

Table 1. Comparison of manometric and colorimetric estimation of cholesterol-esterase activity

The standard test systems for synthesis and hydrolysis of cholesteryl oleate were used except that the reactions were allowed to proceed for the times indicated. Cholesterol was determined by the method of Sperry & Webb (1950) on a sample of the reaction mixture used for manometric estimation. Synthesis of cholesteryl oleate is indicated by a decrease (–) of cholesterol or CO₂ and hydrolysis by an increase (+) of these compounds.

Method of estimation	...	Colorimetric (A)	Manometric (B)	
Reaction	Time of incubation (min.)	Cholesterol (μ moles)	CO ₂ (μ moles)	B:A
Synthesis	30	–3.0	–3.3	1.10
	75	–7.3	–8.1	1.11
	165	–15.6	–13.9	0.89
Averages		–8.6	–8.4	1.03
Hydrolysis	30	+6.8	+6.0	0.88
	60	+8.5	+9.6	1.13
	110	+11.0	+12.1	1.11
Averages		+8.8	+9.2	1.04

amount of CO₂ gas absorbed during esterification is very nearly equal to the amount released upon addition of mineral acid. Table 3 shows that 42 % of the radioactivity initially present as [4-¹⁴C]-cholesterol is found in a compound that reacts with digitonin only after hydrolysis. Nearly the same percentage of the initial radioactivity is associated with a compound that follows the elution pattern of an authentic sample of cholesteryl oleate during isolation by silicic acid chromatography. The enzyme has been found useful for preparation of labelled sterol esters.

Table 2. *Conversion of carbon dioxide into bicarbonate during synthesis of cholesteryl oleate*

To accommodate the large change in gas volume a differential manometer of the Summerson type was used. The volumes of each side of the assembled manometer were nearly identical as judged by the volume of CO₂ released from a NaHCO₃ solution. The reaction was conducted in double-side-arm flasks. The main compartments of each flask contained an identical amount of the standard reaction mixture for synthesis of cholesteryl oleate. The enzyme was placed in one side arm and 0.2 ml. of N-H₂SO₄ in the other. The reaction was begun in flask 3 by tipping the enzyme into the main compartment. After 2 hr. of activity, acid was added from the second side arm and the contents of both side arms and the main compartment of flask 6 were mixed. The data are given as millimetres of change in manometer fluid.

	Reaction flask 3 (mm.)	Control flask 6 (mm.)	Total change (mm.)
After 2 hr. of activity	- 132	+ 68	- 200
After acidification	+ 232	+ 26	+ 206

Properties of cholesterol esterase

Influence of substrate composition and concentration upon rate of esterification. Dependence of the rate of cholesteryl oleate synthesis upon the concentration of cholesterol, oleate and cholic acid is illustrated in Figs. 1-3. These data were obtained with substrates in which the concentration of only one component at a time was varied from zero to the levels indicated on the graphs. However, it was not possible to prepare a stable emulsion of cholesterol and cholic acid in the complete absence of oleate. It is evident from a consideration of the data that the three compounds must be combined in a certain ratio for maximum activity. On a molar basis the optimum proportions of cholesterol, oleate and cholic acid are approximately 2:6:1, or 3:6:1 if maximum activity is taken to occur over the range indicated by the horizontal portion of each graph. Somewhat higher activities are attained with the former ratio. Under these conditions maximum activity occurs when 32 μ -moles of cholesterol, 96 μ -moles of oleate and 16 μ -moles of cholic acid are present in a total volume of 2.2 ml. Swell, Field & Treadwell (1953) reported maximum activity when cholesterol, oleic acid and sodium taurocholate were present in a molar ratio of 1:2:1. To obtain the clear emulsion required by their turbidimetric-assay procedure Hernandez & Chaikoff (1957) combined the same three compounds so that the ratio was 1:2:4.5.

Influence of substrate composition and concentration upon rate of hydrolysis. The effect of cholic acid concentration upon rate of hydrolysis of cholesteryl oleate is given in Fig. 4. The optimum

Table 3. *Enzymic synthesis of [4-¹⁴C]cholesterol ester*

Standard test system for synthesis of cholesteryl oleate was used except that the substrate was prepared to contain about 0.033 μ C of [4-¹⁴C]cholesterol/ml. The reaction was allowed to proceed for about 2 hr. Part (2 ml.) of the reaction mixture (total volume 2.2 ml.) was extracted in 50 ml. of acetone-ethanol (1:1, v/v). The extract was centrifuged and a portion of the supernatant was used to prepare cholesterol digitonide before and after hydrolysis according to Sperry & Webb (1950). The digitonide was dissolved in acetic acid and was transferred into scintillating fluid for counting. A second portion of the supernatant was dried, and the residue was dissolved in light petroleum and placed on the silicic acid column of Fillerup & Mead (1953) for isolation of free and ester cholesterol. The recovery was about 87 %. The eluates were dried and the residue was dissolved in scintillation fluid. All counting was done with a Packard Tri-Carb liquid-scintillation counter. The counting efficiency was standardized by the addition of internal standard to each sample.

	Counts/min./2.2 ml. of reaction mixture			Ester (%)
	Total	Free	Ester	
Isolated as cholesterol digitonide				
Control	40 150	39 600	550*	1.3
Test	40 150	23 320	16 830*	42.0
Isolated as free and esterified cholesterol by chromatography				
Test	34 643*	20 350	14 293	41.5

* Calculated values.

molar ratio of cholesteryl oleate to cholic acid is 5:1. In order to prepare a satisfactory emulsion of this as well as other sterol esters it has been found advantageous to add lithium oleate and albumin, as already indicated. With the complete substrate, maximum activity occurs when 56 μ moles of cholesteryl oleate, 9.6 μ moles of cholic acid, 28 μ moles of lithium oleate and albumin are present in a total volume of 2.2 ml.

Effect of various bile acids upon rate of synthesis and hydrolysis. In the preceding sections it has been tacitly assumed that the stimulatory properties of cholic acid are unique. That this is so for both synthesis and hydrolysis of cholesteryl oleate is seen from the data presented in Table 4. None of the common bile acids, either free or conjugated, possesses the effectiveness of cholic acid. All other

Fig. 1. Effect of cholesterol concentration on rate of cholesteryl oleate synthesis. The standard test system was used except that the substrates were prepared to give the range of cholesterol concentrations shown.

Fig. 2. Effect of oleic acid concentration on rate of cholesteryl oleate synthesis. The standard test system was used except that the substrates were prepared to give the indicated range of oleic acid concentrations.

Fig. 3. Rate of esterification as a function of bile acid concentration. The standard test system was used except that substrates were prepared to give the concentrations of cholic acid (O), taurodeoxycholic acid (Δ) and taurocholic acid ($\square$) shown.

Fig. 4. Effect of cholic acid concentration on rate of cholesteryl oleate hydrolysis. The standard test system was used except that the substrates were prepared to give the required range of cholic acid concentrations.

bile acids tested in the esterifying system are only slightly stimulatory and all to about the same degree. The virtual lack of effect over a considerable range of concentrations is shown for two bile acids in Fig. 3. In the hydrolytic system, conjugates of cholic acid are somewhat more effective than are conjugates of deoxycholic acid. Neither system is stimulated significantly by 3:7:12-trioxocholanic acid. Similar results have been obtained with β -sitosterol.

Effect of ionic environment upon the rate of synthesis and hydrolysis. The activity of an extract of acetone-dried pancreas is considerably increased upon the addition of appropriate amounts of salt to the reaction mixture. Dialysis of enzyme solutions against water invariably results in extensive inactivation. But activity is restored if salt is incorporated into the reaction mixture.

Table 4. *Influence of various bile acids on the rate of synthesis and hydrolysis of cholesteryl oleate*

Standard test systems were used. The bile acids listed were substituted for cholic acid in the standard substrates. The reaction period was 30 min.

Bile acid	Synthesis (- μ l. of CO_2)	Hydrolysis (+ μ l. of CO_2)
None	11	4
Cholic	65	124
Deoxycholic	14	13
Lithocholic	14	0
Taurocholic	16	42
Taurodeoxycholic	17	21
Glycocholic	17	49
Glycodeoxycholic	20	20
Glycolithocholic	15	2
3:7:12-Trioxocholanic	17	12

Fig. 5. Effect of ionic environment on rate of esterification. The standard test system was used except that $(\text{NH}_4)_2\text{SO}_4$ (O), NaCl ($\square$) or dextrose ($\triangle$) was used in the reaction mixture to give the concentrations shown.

The magnitude of the effect due to salt upon the rate of cholesteryl oleate synthesis is shown in Fig. 5. A suitable ionic environment is established with a variety of electrolytes; the effect is not due to a particular ion. However, un-ionized compounds, as dextrose, are totally ineffective (Fig. 5). According to the data of Fig. 5, 200 μ moles of sodium chloride is equivalent to 100 μ moles of ammonium sulphate. These are optimum concentrations under the conditions imposed; greater amounts cause formation of a precipitate. Effectiveness in this reaction is governed by the total number of cationic and anionic charges. Thus Na^+ ion and NH_4^+ ion are equivalent and 2 Cl^- ions are replaceable by SO_4^{2-} ion. On this basis sodium chloride and ammonium sulphate are equally effective if the former is present at a concentration twice that of the latter. The activating effect of various univalent ions upon synthesis of cholesteryl oleate has also been reported by Hernandez & Chaikoff (1957).

The response of the hydrolytic system to various amounts of sodium chloride or ammonium sulphate indicates that these electrolytes are equally effective if the comparison is made on the basis of ionic strength. Thus 400 μ moles of ammonium sulphate are as effective as 1200 μ moles of sodium chloride. These are optimum concentrations in the standard test system.

Similar results for both systems have been obtained with other electrolytes, although the choice is limited to ions which will not form insoluble compounds with any of the ingredients present in the reaction mixture.

Because it is not possible to prepare a reaction mixture which is devoid of ionizable compounds, considerable activity exists even in the absence of added electrolyte (Fig. 5).

The relationship between substrate concentration and concentration of ammonium sulphate required for maximum activity is not the same for both systems. The amount of ammonium sulphate needed for optimum activity increases as the concentration of substrate for esterification decreases. In the standard test system 0.8 ml. of substrate requires about 100 μ moles of ammonium sulphate. When the volume of substrate is reduced to 0.2 ml., 250 μ moles of ammonium sulphate are needed. At all substrate concentrations tested when the amount of ammonium sulphate exceeds the optimum, a precipitate forms and the activity decreases markedly. In the hydrolytic system the optimum concentration of ammonium sulphate is independent of the substrate concentration (Fig. 6).

The concentration of electrolyte required for maximum esterifying activity is influenced by the nature of the sterol. The amount of either sodium chloride or ammonium sulphate that will permit

maximum activity with β -sitosterol is approximately one-half of that needed with cholesterol.

Effect of pH upon activity. The pH optimum for synthesis of cholesteryl oleate is pH 6.1 and for hydrolysis it is pH 7.0. Swell & Treadwell (1950a, 1955) reported pH 6.2, as did Hernandez & Chaikoff (1957) for synthesis, and pH 6.6 for hydrolysis of cholesteryl oleate (Yamamoto *et al.* 1949).

Effect of enzyme concentration upon activity. Under the conditions specified, the rate of synthesis and hydrolysis of cholesteryl oleate is proportional to the enzyme concentration within rather narrow limits (Fig. 7).

Relation between sterol structure and rate of esterification with oleate. The use of optimum conditions empirically established for synthesis of cholesteryl oleate to determine the rate of synthesis of other sterol esters may lead to ambiguous conclusions. β -Sitosterol is esterified 37% faster than is cholesterol if the comparison is made with the optimum electrolyte concentration for each, i.e. 23 mM-ammonium sulphate for β -sitosterol and 45 mM-ammonium sulphate for cholesterol, all other conditions being as in the standard test system. If, however, the optimum electrolyte concentration for cholesterol (45 mM-ammonium

sulphate) is used, then the rate with β -sitosterol is 43% slower than with cholesterol. This rate, or even a lesser one, has been the usual one reported (Swell, Field & Treadwell, 1954; Hernandez & Chaikoff, 1957). Further, in order to obtain maximum activity with β -sitosterol, the molar ratio of sterol:oleate:cholate must be altered from 2:6:1, as is optimum for cholesterol, to 6:6:1. When this is done, β -sitosterol is esterified about 300% faster than is cholesterol.

The data of Table 5 indicate that under optimum conditions for synthesis of cholesteryl oleate, cholesterol-esterase activity is confined to those sterols in which the steric orientation of the hydroxyl group at C-3 is β , as *epicholesterol* and *epicholestanol* are not esterified. Both cholesterol and β -sitosterol are esterified at rates somewhat greater than their respective C-5 unsaturated analogues. Hence, saturation of the double bond at C-5 is permitted but only if rings A and B are joined in the *trans*-decalin manner; no reaction occurs with coprostanol. Hernandez & Chaikoff (1957) have reported similar results. The importance of an intact phenanthrene nucleus and the presence of a hydroxyl group at C-3 is emphasized by the lack of activity with β -norcholesterol and cholesteryl chloride.

Fig. 6. Effect of substrate concentration on optimum electrolyte concentration in the hydrolytic system. The standard test system was used except that the substrate was used in amounts of 0.9 ml. (○) and 0.3 ml. (●) and the concentration of $(\text{NH}_4)_2\text{SO}_4$ was varied over the range shown.

Fig. 7. Rate of synthesis (○) and hydrolysis (●) of cholesteryl oleate as a function of enzyme concentration. The standard test system was used.

Table 5. *Effect of sterol structure on the rate of esterification with oleate*

The standard substrate for esterification was prepared as described but concentration of sterol was reduced to 0.025M. The standard test system was used except that the amount of substrate per flask was reduced to 0.5 ml.

	- μ l. of CO ₂ /30 min.
Cholesterol	103
<i>epi</i> Cholesterol	5
Cholestanol	139
<i>epi</i> Cholestanol	5
Coprostanol	7
β -Sitosterol	71
β -Sitostanol	95
β -Norcholesterol	0
Cholesteryl chloride	0

DISCUSSION

The catalytic activity described in this paper has been attributed to the enzyme known as cholesterol esterase, even though some of the properties obtained by use of the manometric method of estimation differ from those reported by previous workers. The data demonstrate that with the proper ionic environment, pH, bile acid and substrate concentration and composition, the rate of both the synthetic and hydrolytic reactions is so high that it is sufficient to make observations over relatively short periods, during which time linear rates prevail. Furthermore, activity is proportional to enzyme concentration, albeit the range is limited. Conclusions, depending upon differences in rate, have been made on the basis of data obtained under these conditions. Data obtained under conditions requiring long periods of incubation, especially with homogenates, may not be directly comparable.

Although the enzyme is readily obtainable in soluble form and appears susceptible to purification by methods currently in use (Hernandez & Chaikoff, 1957), no firm evidence is yet available which distinguishes cholesterol esterase from other esterases, especially lipase. The definitive answer to this important question must await fractionation. A comparison of some catalytic properties has led Swell *et al.* (1955) to suggest that cholesterol esterase and lipase are unique enzymes.

The beneficial effects of electrolytes, in colloidal systems serving as substrate for enzymic attack, have recently been noted by Mattson & Beck (1955) for lipase and by Hernandez & Chaikoff (1957) for cholesterol esterase. It has been shown in the present report that the concentration of a given electrolyte required for optimum activity is governed by the type of reaction being catalysed, i.e. synthesis or hydrolysis of cholesteryl oleate, and by the nature of the sterol. The latter case deserves particular emphasis inasmuch as it

illustrates that differences in sterol structure, however minor, may exert a profound effect upon the physical character of the colloidal substrate of which the sterol is a part. For instance, the electrolyte concentration required to reach maximum rate of synthesis of cholesteryl oleate is about twice that needed for synthesis of β -sitosteryl oleate. Furthermore, the relationship between substrate concentration and the optimum electrolyte concentration differs. In the system for synthesis of cholesteryl oleate, substrate concentration is inversely related to electrolyte concentration, whereas in the hydrolytic system the electrolyte concentration is independent of substrate concentration. These data indicate that the obvious action of the electrolyte is on the substrate, although effects on the enzyme protein are not ruled out. The difference between the responses of the two systems toward electrolyte may indicate dissimilar function, at least in part. It may be that the ionic environment changes the electrical properties of the colloidal substrate in such a manner as to make it more readily accessible to the active centres of the enzyme, as well as permitting the arrangement of the components of the colloidal substrate in a definite way suitable for enzymic attack. In addition, the rate is favourably affected because the optimum concentration of electrolyte for the two systems is so different that the reverse reaction is inhibited, especially at pH values which are not advantageous to either reaction. In the hydrolytic system this occurs because the products of the reaction are removed from colloidal solution, and thus from the sphere of effective enzymic action, whereas the electrolyte concentration in the system for synthesis is too low for rapid hydrolysis. Mattson & Beck (1955) suggest that the stimulation of lipase by high electrolyte concentration is brought about by alteration of the enzyme protein into a more reactive form.

Although the stimulatory effect of bile acids upon the activity of cholesterol esterase has been known since the observations of Mueller (1916b), there is no clear evidence in the literature to implicate a particular bile acid as being responsible for the phenomenon. According to the data of Swell *et al.* (1953) both taurocholate and cholate stimulate the synthesis of cholesteryl oleate more effectively than do any of the other common bile acids. Of these two, the former is the more active. In the hydrolytic system taurocholate and cholate are reported as approximately equally effective, although the published data would indicate that cholate is the more stimulatory (Swell *et al.* 1953).

The unique effect of cholic acid, as described, was observed by comparison only with pure bile acids and under conditions such that the bile acid in question was in colloidal solution with the other

components of the substrate. The high specificity of the bile acid requirement in both systems strongly suggests a substrate-enzyme type of relationship. Two properties of bile acids in general are pertinent to consider in the present situation: surface activity and ability to form molecular compounds, even though of low stability. It is tempting to speculate that these two properties not only need to be combined in one chemical substance, but that in addition certain structural features must be present in the molecule to permit interaction with the enzyme protein in proximity to the catalytic site. Interaction with other parts of the enzyme, i.e. non-specific interactions, are not excluded but do not lead to enhanced catalysis. The dual actions of surface activity and molecular-compound formation of the bile acid, occurring near the catalytic site, permit the colloidal substrate to form a complex with the enzyme much more readily than it could in the absence of bile acid or in the presence of those bile acids not fully satisfying the structural requirements for specificity.

SUMMARY

1. The synthesis and hydrolysis of cholesteryl oleate catalysed by pancreatic cholesterol esterase has been followed manometrically in a sodium bicarbonate-carbon dioxide buffer system.

2. The optimum composition, as well as the concentrations of the components, of the colloidal substrate for synthesis and for hydrolysis of cholesteryl oleate has been determined empirically.

3. Data which define the colloidal substrate and other conditions for optimum activity with a particular sterol may not be applicable to a different sterol.

4. The stimulatory capacity of cholic acid, not shared by other bile acids, suggests a relationship with the enzyme of a highly specific nature. Its function is postulated to be that of a specific detergent and former of molecular compounds, acting, by virtue of its unique structure, in the vicinity of the reactive sites.

5. The electrolyte requirement, satisfied by various ions, is governed by the substrate but may also affect the enzyme protein.

6. Cholesterol-esterase activity is confined to sterols with a β -oriented hydroxyl group at C-3, with rings A and B arranged as in *trans*-decalin and with the phenanthrene nucleus intact.

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