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Fractionation of the Products of the Direct Sulphation of Monosaccharides on Anion-Exchange Resin

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Previous methods for the preparation of monosaccharide sulphate esters have involved either the use of definitive synthetic routes (Percival & Soutar, 1940; Lloyd, 1959*a*; Peat, Turvey, Clancy & Williams, 1960; Guiseley & Ruoff, 1961) or the fractionation of the products of the direct sulphation of monosaccharides by chromatography on cellulose columns (Lloyd, 1959*a*; Turvey & Clancy, 1959; Peat *et al.* 1960), by electrophoresis on cellulose columns (Lloyd, 1960) or by repeated recrystallization of the brucine salt (Guiseley & Ruoff, 1961). The present report describes the use of anion-exchange chromatography as an alternative general method for the isolation of the monosaccharide sulphate products of direct esterification procedures.

MATERIALS AND METHODS

Monosaccharide 6-O-*sulphate esters.* Authentic samples of the potassium salts of the 6-*O*-sulphate esters of D-glucose, D-galactose and *N*-acetyl-D-glucosamine were prepared by the definitive routes described previously (Lloyd, 1959*a*; 1960). Preparations of the potassium salts of D-glucose 6-*O*-sulphate, D-galactose 6-*O*-sulphate, *N*-acetyl-D-glucosamine 6-*O*-sulphate and *N*-acetyl-D-galactosamine 6-*O*-sulphate also were obtained from the products of direct esterification of the appropriate monosaccharides by cellulose-column chromatography (Lloyd, 1959*a*) or cellulose-column electrophoresis (Lloyd, 1960). Similar methods were employed for the isolation of the corresponding disulphate esters,

Sulphation procedures. Preparations of crude monosaccharide sulphate esters for the present fractionation studies were obtained in two ways. In procedure I the monosaccharide (0·01 mole) was dissolved in the minimum amount of dry pyridine in the presence of anhydrous $CaSO_4$ (200 mg.) before cooling to 5° and the dropwise addition of 0·01 mole of chlorosulphonic acid in 0·9 ml. of dry $CHCl_3$. The temperature of the reaction mixture was maintained at 5° during the course of the addition. The mixture was stirred at 5° for 30 min., at 25° for 2 hr. and then allowed to stand at 25° for 2 hr., when the crude sulphate ester separated as an oily lower layer. The upper pyridine layer was removed by decantation and the oil dissolved in the minimum amount of water with vigorous stirring. The remainder of the procedure for the isolation of the potassium salts, including recrystallization via the brucine salt, was according to Lloyd (1960).

For comparative purposes preparations were also obtained from D-glucose and D-galactose by procedure II, namely that of Peat *et al.* (1960), in which the monosaccharide (0·01 mole) dissolved in the minimum amount of dry pyridine was treated with an excess (0·03 mole) of the

pyridine–sulphur trioxide reagent (Baumgarten, 1926) for 6 hr. at 65–70°. The crude ester sulphates were isolated exactly as described by these authors without intermediate brucine recrystallization.

Anion-exchange chromatography. An aqueous slurry of Dowex 1 (OH^- form X 4; 100–200 mesh) was freed from gases under reduced pressure at 10°, and the resin was then packed under gravity flow into a glass tube fitted at the lower end with a sintered-glass plate. For analytical purposes, suitable for the fractionation of 200 mg. of crude monosaccharide sulphate, the column dimensions were 10 cm. × 0·75 cm., and on a preparative scale a column 25 cm. × 1 cm. was used.

During the fractionation columns were kept at 10°. An aqueous solution of the potassium salt of the sulphate ester preparation (200 mg./0·1 ml.) was added to the tube and allowed to drain into the resin under gravity flow. The bed was then washed with water until the eluate was no longer alkaline.

On an analytical scale columns were eluted with a continuous gradient produced by running 0·15 N-H_2SO_4 into a chamber containing 200 ml. of 0·01 N-H_2SO_4 and fitted with a magnetic stirrer. Suitable rates of elution were achieved by fixing the mixing chamber 30 cm. above the level of the resin bed. Estimation of carbohydrate in fractions of the eluate by determination of reducing substance (Dodgson, Lloyd & Spencer, 1957) was started immediately after the sample was applied to the column and continued during the initial washing with water and subsequent elution with H_2SO_4 solutions. In preparative work columns were eluted in a stepwise manner with appropriate concentrations of H_2SO_4 indicated by the gradient-elution experiments (see below).

Carbohydrate material was recovered from the initial alkaline eluate after washing the columns with water and from the H_2SO_4 eluates. In the former case the pooled washings were passed through a Dowex 50 (H^+ form; 50–100 mesh) column and the eluate was then evaporated to dryness by rotary evaporation at 30°, followed by freeze-drying. For the isolation of the esters from the H_2SO_4 eluates fractions corresponding to homogeneous elution peaks were pooled and adjusted at 2° to pH 8 with a saturated aq. $Ba(OH)_2$ solution previously cooled to 2°. Precipitated $BaSO_4$ was removed by centrifuging at 2° and excess of Ba^{2+} ions in the clear supernatant were precipitated as $BaCO_3$ by gassing with CO_2 at 2°; the mixture was then clarified by centrifuging. The clear supernatant was passed through a Dowex 50 (H^+ form; 50–100 mesh) column, the acid eluate and washings were combined and, after the mixture had been adjusted to pH 7·4 with 0·05 N-KOH, it was concentrated to small volume by rotary evaporation at 30° under reduced pressure. The concentrate was treated with 8 vol. of absolute ethanol and the precipitated material allowed to flocculate at 2°. After centrifuging, the precipitate was washed by suspension in ethanol (four times) and ether (four times), with intermediate centrifuging, before drying *in vacuo* over $CaCl_2$.

Paper chromatography and paper electrophoresis. The homogeneity of products was examined by descending chromatography on Whatman no. 1 filter paper for 48 hr. at 20° with butan-1-ol–acetic acid–water (50:12:25, by vol.) as solvent. In comparisons of the products obtained by procedures I and II the chromatographic method of Rees (1960) was also employed. Reducing components were detected with the $AgNO_3$ (Trevelyan, Procter & Harrison, 1950) or *p*-anisidine (Hough, Jones & Wadman, 1950) spray reagents, hexosamines and derivatives according to Partridge (1948) and sulphate esters by the method of Lloyd (1960).

Zone electrophoresis on Whatman no. 1 paper was performed as described previously (Lloyd, 1960).

Cellulose-column chromatography. Comparison of the homogeneity of the monosulphate fractions from D-glucose and D-galactose were also made by chromatography on columns (30 cm. × 1 cm.) of Munktell cellulose powder (L.K.B. Produkter) with isobutyric acid–aq. 0·5 N-NH_3 soln. (5:3, v/v) as developing solvent. Sulphate esters were converted into the free acids by passage through Dowex 50 (H^+ form; 50–100 mesh) and then concentrated by rotary evaporation at 30° before application to the cellulose bed (cf. Lloyd, 1959*a*). Fractions of the eluates corresponding to bands of reducing substance were pooled and concentrated to dryness. The residue was redissolved in the minimum amount of water, passed through a Dowex 50 (H^+ form; 50–100 mesh) column and the acid eluate and washings were adjusted to pH 7·2 with 0·05 N-KOH. The potassium salts of the esters were then isolated as described above.

Analysis. For the determination of ester sulphate, samples were hydrolysed in sealed tubes with 4 N-formic acid for 6 hr. at 110°. Hydrolysates were concentrated to dryness *in vacuo* over KOH at 50°, the residue was dissolved in water and inorganic sulphate determined by the method of Lloyd (1959*b*). Nitrogen was determined with the micro-Kjeldahl apparatus of Markham (1942) and potassium by flame photometer.

Infrared spectroscopy. Infrared spectra were measured with the Perkin–Elmer Infracord spectrophotometer with a rock-salt prism. Compounds were examined as mulls in liquid paraffin (Nujol).

EXPERIMENTAL AND RESULTS

The results of a typical analytical-scale fractionation of the crude products of the direct sulphation of D-glucose according to procedure I are reproduced in Fig. 1. The elution patterns obtained during the fractionation on a similar scale of the products of direct sulphation according to procedure I of D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-fucose and L-fucose were substantially the same. It was found by

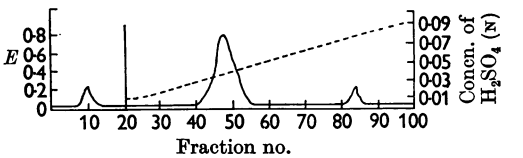

Fig. 1. Analytical-scale fractionation of crude D-glucose sulphate prepared by procedure I. —, *E* of fractions in the Somogyi method (Dodgson *et al.* 1957); - - - -, concentration of H_2SO_4 entering the column. Experimental points have been omitted for clarity.

paper electrophoresis that the components designated 1, 2 and 3 according to the order of elution from the columns corresponded in each instance to unchanged parent compound, monosulphate and disulphate esters respectively (cf. Lloyd, 1960).

When the products of direct sulphation by procedure I were fractionated on a preparative scale the parent compound (component 1) was recovered from the water washings, and concentrations of H_2SO_4 of 0·036N and 0·08N were selected as the most suitable for the general stepwise elution of monosulphate (component 2) and disulphate (component 3) fractions respectively. Results of typical separations of 2 g. of the potassium salts of the crude products obtained by procedure I are given in Table 1. In such separations care was taken that reducing material was absent from the column eluates before a new eluent was applied; the homogeneity of fractions was determined by paper electrophoresis before pooling.

Zone electrophoresis on Whatman no. 1 filter paper (see Lloyd, 1960) of the products of sulphation of D-glucose and D-galactose by procedure II revealed the presence of one immobile and three mobile components. The former corresponded to unchanged parent compound and two of the mobile components corresponded to monosulphate and disulphate esters respectively. The fourth component had an electrophoretic mobility greater than the disulphate and probably corresponded to the trisulphate fraction reported by Peat *et al.* (1960).

The results of the fractionation of the D-glucose sulphate mixture from procedure II on an analytical scale are reproduced in Fig. 2. Fractionation of the corresponding D-galactose sulphate preparation gave substantially similar results. Comparison of the computed areas under the curves in these elution diagrams revealed the presence of appreciably lower amounts of component 2 and higher amounts of components 1 and 3 than in corresponding preparations obtained by procedure I. Attempts to elute material corresponding to the third electrophoretically mobile component from the anion-exchange resin were unsuccessful even when the concentration was increased to 0·5N-H_2SO_4. Fractionation on a preparative scale yielded for each sugar three fractions, analysed as parent compound, monosulphate and disulphate. However, in contrast with material obtained after procedure I (see Table 1), on paper chromatography with the butan-1-ol–acetic acid–water solvent these monosulphate fractions from D-glucose and D-galactose were found to be inhomogeneous. The D-glucose monosulphate fraction contained a major component R_{Glc} (R_F

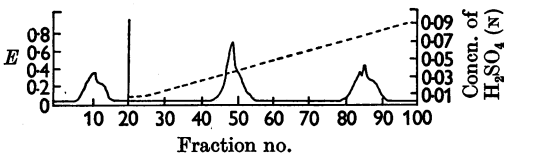

Fig. 2. Analytical-scale fractionation of crude D-glucose sulphate prepared by procedure II. For explanation of curves see Fig. 1.

Table 1. *Products of the Dowex* 1 *fractionation of crude monosaccharide sulphates*

Yields are from 2 g. of the crude potassium salts. R_{Glc} is the chromatographic mobility relative to that of glucose.

	Component 1 (monosaccharide)		Component 2 (monosulphate)							
					Found (%)			Calc. (%)		
Ester	Yield (g.)	R_{Glc}	Yield (g.)	R_{Glc}	N	SO_4	K	N	SO_4	K
D-Glucose	0·09	1·0	1·15	0·66	—	32·9	12·8	—	32·2	13·1
D-Galactose	0·04	0·92	1·4	0·58	—	32·5	13·1	—	32·2	13·1
N-Acetyl-D-glucosamine	0·06	1·24	1·7	0·76	4·4	28·4	11·9	4·1	28·3	11·5
N-Acetyl-D-galactosamine	0·03	1·13	1·1	0·72	4·2	28·2	11·2	4·1	28·3	11·5
D-Fucose	0·02	1·5	1·8	0·92	—	34·2	13·6	—	34·04	13·8
L-Fucose	0·02	1·47	1·7	0·98	—	33·8	13·8	—	34·04	13·8

	Component 3 (disulphate)							
			Found (%)			Calc. (%)		
Ester	Yield (g.)	R_{Glc}	N	SO_4	K	N	SO_4	K
D-Glucose	0·32	0·42	—	46·2	18·9	—	46·1	18·7
D-Galactose	0·21	0·43	—	45·9	18·7	—	46·1	18·7
N-Acetyl-D-glucosamine	0·09	0·54	3·7	41·6	16·7	3·1	42·2	17·2
N-Acetyl-D-galactosamine	0·08	0·54	3·4	41·9	16·5	3·1	42·2	17·2
D-Fucose	0·01	0·59	—	47·6	19·6	—	47·9	19·5
L-Fucose	0·013	0·59	—	47·3	19·2	—	47·9	19·5

relative to glucose) 0·66, identical in mobility with that of authentic D-glucose 6-*O*-sulphate and two minor components R_{Glc} 0·71 and R_{Glc} 0·78, whereas the D-galactose monosulphate contained a major component R_{Glc} 0·58 corresponding to authentic D-galactose 6-*O*-sulphate and two minor components R_{Glc} 0·68 and R_{Glc} 0·70. These results were substantiated by the chromatographic method of Rees (1960) (Table 2).

The monosulphates of D-glucose and D-galactose from procedure II were further fractionated on cellulose-powder columns. Carbohydrate material was eluted in each instance as two distinct bands (see Fig. 3). The material constituting the major band was identical in each instance with the corresponding authentic 6-*O*-sulphate ester on paper chromatography, whereas that associated with the minor band was composed of contaminants observed earlier by paper chromatography. Cellulose-column chromatography of the monosulphate fractions obtained after procedure I (Fig. 3) gave only material corresponding to the authentic 6-*O*-sulphate esters.

Table 2. *Behaviour of* D-*glucose monosulphates and* D-*galactose monosulphates on chromatography by the method of Rees* (1960)

Chromatograms were run on Whatman no. 1 paper for 20 hr. with butan-1-ol–ethanol–water (3:1:1, by vol.), containing 3% (w/v) of cetylpyridinium chloride, as developing solvent.

	R_F components
Authentic esters	
D-Glucose 6-*O*-sulphate	0·44
D-Galactose 6-*O*-sulphate	0·40
Esters from procedure I	
D-Glucose monosulphate	0·44
D-Galactose monosulphate	0·41
Esters from procedure II	
D-Glucose monosulphate	0·44, 0·52, 0·64
D-Galactose monosulphate	0·40, 0·49, 0·63

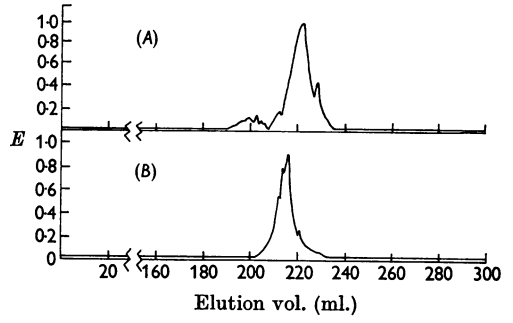

Fig. 3. Cellulose-column chromatography of monosulphate components. Curves represent *E* of fractions in the Somogyi method. (*A*) D-Glucose monosulphate, procedure II; (*B*) D-glucose monosulphate, procedure I.

Infrared spectroscopy. The infrared spectra of the monosulphate fractions isolated after the direct sulphation of D-glucose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-fucose and L-fucose by procedure I and of D-glucose and D-galactose by procedure II all exhibited strong absorption at 1240 cm.$^{-1}$ previously ascribed to vibrations involving the S–O link in chemically synthesized sulphate esters (Lloyd & Dodgson, 1959, 1961; Lloyd, Tudball & Dodgson, 1961). Further, the infrared spectra of the monosulphate esters of D-glucose D-galactose and *N*-acetyl-D-glucosamine isolated after Dowex 1 fractionation of the products of procedure I were identical with those reported previously for the corresponding authentic 6-*O*-sulphate esters (Lloyd & Dodgson, 1959, 1961). This was also true for *N*-acetyl-D-galactosamine monosulphate obtained by ion-exchange chromatography and *N*-acetyl-D-galactosamine 6-*O*-sulphate obtained by cellulose-column chromatography (Lloyd, 1959*a*). The infrared spectra of the disulphate esters all exhibited bands of strong intensity at 1240 cm.$^{-1}$ (S–O) and between 790 and 850 cm.$^{-1}$ (C–O–S) as described by Lloyd & Dodgson (1961).

The infrared spectra of the monosulphate fractions isolated by ion-exchange chromatography after the direct sulphation by procedure II of D-glucose and D-galactose were different from those described above, exhibiting new bands at 849–855 cm.$^{-1}$ and 833–835 cm.$^{-1}$ in addition to the band at 820 cm.$^{-1}$ previously ascribed to vibrations involving the C–O–S linkage (cf. Lloyd & Dodgson, 1959, 1961; Lloyd, Tudball & Dodgson, 1961). However, after refractionation of these monosulphate components by cellulose-column chromatography, material isolated from the major zones (see Fig. 3) gave spectra identical in each instance with the corresponding authentic 6-*O*-sulphate ester.

DISCUSSION

It has now been established that the principal monosulphate products of the direct sulphation of D-glucose, D-galactose and *N*-acetyl-D-glucosamine with chlorosulphonic acid or pyridine–sulphur trioxide are identical in each instance with the corresponding 6-*O*-sulphate esters prepared by definitive synthesis (Lloyd, 1959*a*, 1960; Lloyd & Dodgson, 1959, 1961; Turvey & Clancy, 1959; Peat *et al.* 1960; Guiseley & Ruoff, 1961; Saito, Noguchi & Komatsu, 1961*a*, *b*). Further, after the preliminary allocation of the sulphate group to position 6 of the hexosamine ring in the monosulphate product of the direct sulphation of *N*-acetyl-D-galactosamine (Lloyd, 1959*a*), it has been

confirmed that this material is also a 6-*O*-sulphate (Lloyd, 1960; Lloyd & Dodgson, 1959, 1961; Suzuki & Strominger, 1960*a*, *b*). Chemical sulphation, particularly with chlorosulphonic acid at low temperature, thus closely resembles toluene-*p*-sulphonylation, methane sulphonylation and triphenylmethylation (see Helferich, 1948; Tipson, 1953), in that occupation of the primary alcohol group of monosaccharides is favoured.

The 6-*O*-sulphate esters of monosaccharides are known to have a wide distribution in Nature. It is now accepted that D-galactose 6-*O*-sulphate forms part of the structure of bovine cerebron sulphatide A (Thannhauser, Fellig & Schmidt, 1955) and *N*-acetyl-D-glucosamine 6-*O*-sulphate and *N*-acetyl-D-galactosamine 6-*O*-sulphate residues exist in the polymer chains of keratosulphate and chondroitin sulphate C fractions of mammalian connective tissues respectively (Meyer, 1960; Hirano, Hoffman & Meyer, 1960). Further support for these assignments has been obtained from comparisons of the infrared spectra of monosaccharide 6-*O*-sulphates of known configuration and of those naturally occurring compounds in which such residues are said to exist (Lloyd & Dodgson, 1959, 1961; Lloyd Dodgson, Price & Rose, 1961). Observations on the metabolism of hexose and *N*-acetylhexosamine 6-*O*[^{35}S]-sulphates *in vivo* have been reported (Lloyd, 1961*a*, *b*; Lloyd, 1962; Lloyd, James & Dodgson, 1962), and the use of the 6-*O*-sulphate esters of hexoses and *N*-acetylhexosamines as assay substrates for the mollusc-enzyme glycosulphatase has also been described (Lloyd, 1959*c*; Dodgson, 1961; Dodgson & Lloyd, 1961).

Although definitive synthetic routes may be used for the preparation of monosaccharide 6-*O*-sulphate esters for use in this type of study, the fractionation of the products of direct sulphation to give the 6-*O*-sulphate offers a more convenient method of isolation, particularly for radioisotope-labelled material. Both chromatography and electrophoresis on cellulose columns have been used for this purpose in orientation experiments, but suffer several technical disadvantages for routine preparative work. The present general method involving the elution of monosulphate and disulphate fractions from Dowex 1 anion-exchange resin with dilute sulphuric acid provides a useful solution to these problems, though the method may not be used directly to yield the 6-*O*-sulphate ester from the products of sulphation prepared by using excess of sulphating agent as in procedure II. However, a preliminary fractionation on Dowex 1 resulting in the separation of the monosulphate components from contaminating parent and disulphate compounds greatly facilitates the subsequent preparation of the 6-*O*-sulphate ester free from contaminating isomeric monosulphate compounds by cellulose-column chromatography. The ion-exchange procedure has also proved valuable in the isolation of the monosulphate products of D-fucose and L-fucose.

SUMMARY

1. A method is described for the preparation of the 6-*O*-sulphate esters of D-glucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine by the fractionation of the products of the direct sulphation of the corresponding monosaccharides on Dowex 1 anion-exchange resin.

2. The method has also been used for the isolation of the monosulphate and disulphate fractions after the direct esterification of D-fucose and L-fucose.

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Studies on Cholesterol Esterases of the Small Intestine and Pancreas of Rats

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The literature on cholesterol esterases (this term is used because, as demonstrated here, the hydrolysis and synthesis of cholesteryl esters are brought about by two separate enzymes) has been assembled by Korzenovsky, Diller, Marshall & Auda (1960). This literature reveals: (1) that the cholesterol esterases of the pancreas and small intestine of rats may be identical (Swell, Byron & Treadwell, 1950; Hernandez, Chaikoff & Kiyasu, 1955) or may not be identical (Byers & Friedman, 1955); (2) that the hydrolytic and synthetic activities may be different (Nieft & Deuel, 1949; Hernandez & Chaikoff, 1957); (3) that the bile salts may function as cofactors for the hydrolytic and synthetic processes (Swell, Flick, Field & Treadwell, 1953; Korzenovsky *et al.* 1960). In the present report we have (1) produced evidence that the enzymes of the pancreas and small intestine of rats are of independent origin, (2) demonstrated the separation of the hydrolytic and synthetic activities from rat pancreas, and (3) produced evidence for the coenzymic functions of the bile salts.

EXPERIMENTAL

Materials

Sodium taurocholate (British Drug Houses Ltd.) was purified according to Harold & Chaikoff (1954). Deoxycholic acid and dehydrocholic acid were gifts from Miles-Ames Research Division, Elkhard, Ind., U.S.A., and sodium glycocholate from Professor C. R. Treadwell of George Washington University School of Medicine, Washington, D.C., U.S.A. Crystalline cholesterol, and its stearate and oleate, were from British Drug Houses Ltd. Cholesteryl laurate and palmitate were prepared according to Page & Rudy (1930). Cholesteryl linoleate was prepared enzymically (Murthy, Mahadevan, Seshadri Sastry & Ganguly, 1961*b*). Crystalline soya-bean trypsin inhibitor was from Sigma Chemical Co., U.S.A. The sources of the rest of the materials were as described by Ganguly, Krishna Murthy & Mahadevan (1959) and Mahadevan, Murthy, Krishna Murthy & Ganguly (1961).

Preparation of substrates

For the study of cholesterol esterases, cholesterol has been dispersed by a variety of methods. In this study cholesteryl esters, purified by passing through an alumina column, were dissolved in ethanol to give a concentration of 10 μmoles of the sterol/ml. of solvent. Free cholesterol was similarly dissolved, but 20 μmoles of oleic acid/ml. of ethanol were added; 0·5 ml. of the ethanolic solution was used/5 ml. of reaction mixture, which gave a final concentration of 10% of ethanol. It is shown in Table 1 that the ethanolic dispersion is as efficient as the albumin–bile salt dispersion (Yamamoto, Goldstein & Treadwell, 1949) and

Table 1. *A comparison of three dispersions of cholesterol*

Assay conditions with the ethanolic dispersions were as described in the Experimental section. The dispersions prepared according to Yamamoto *et al.* (1949) and Hernandez & Chaikoff (1957) were incubated with 1 ml. of a water extract of acetone-dried rat pancreas (containing 10 mg. of protein) for 1 hr. at 37°, after which the reactions were stopped by addition of ethanol. The free and esterified cholesterol were separated by chromatography and estimated as described in the Experimental section.

Method of dispersion	Cholesterol (μm-moles/mg. of protein/hr.)	
	Liberated	Esterified
Ethanolic dispersion	883	670
Yamamoto *et al.* (1949)	875	687
Hernandez & Chaikoff (1957)	763	610