Szent-Györgyi, A. G. (1960). In *Structure and Function of Muscle*, p. 1. Ed. by Bourne, G. H. New York: Academic Press Inc.
Szent-Györgyi, A. G. & Cohen, C. (1957). *Science*, **126**, 697.
Tsao, T.-C. (1953). *Biochim. biophys. Acta*, **11**, 368.
Tsao, T.-C. & Bailey, K. (1953). *Biochim. biophys. Acta*, **11**, 102.
Tsao, T.-C., Bailey, K. & Adair, G. S. (1951). *Biochem. J.* **49**, 27.
Umbreit, W. W. & Bond, V. S. (1936). *Industr. Engng Chem. (Anal.)*, **8**, 276.
Velick, S. F. (1956). *Biochim. biophys. Acta*, **20**, 228.
Weber, A. (1956). *Biochim. biophys. Acta*, **19**, 345.
Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.

Biochem. J. (1963) **89**, 561

The Action of Enzymes on Human α-Lipoprotein

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Two types of structure have been proposed for human α-lipoprotein. Gurd (1960) and Cook & Martin (1962) suggested that the protein maintained its integrity as a protein, the lipid being bound to its surface. On the other hand, Vandenheuvel (1962) concluded that the lipid could be sandwiched between sheets of peptide.

Investigations on whole plasma and partially purified preparations have indicated that both peptidases and phospholipases could attack α-lipoprotein (Canal & Girard, 1962; Condrea, de Vries & Mager, 1962). Using purified horse α-lipoprotein, Krumwiede (1958) studied the effects of these enzymes on the extraction of lipids from the complex. To obtain information on the lipoprotein structure, we have made a more detailed study of the effects of enzymic digestion. A preliminary account of this work has been published (Ashworth & Green, 1963*a*).

EXPERIMENTAL

Materials

Crystalline trypsin was obtained from British Drug Houses Ltd., Poole, Dorset, and crystalline α-chymotrypsin from L. Light and Co. Ltd., Colnbrook, Bucks. *Naja naja* venom was obtained from the London Zoo through the courtesy of Dr H. G. Vevers and was used without purification as a source of phospholipase A (EC 3.1.1.4). Phospholipase D (EC 3.1.4.4) was either from British Drug Houses Ltd. or prepared from Savoy cabbage ('stage 3 precipitate') as described by Davidson & Long (1958). The commercial preparation was used as a 0·1% solution. The 'stage 3 precipitate' was dissolved in water to give a solution containing 160 milliunits/ml. The enzyme units used are as recommended by the Commission on Enzymes of the International Union of Biochemistry (1961).

Human α-lipoprotein was prepared as described by Ashworth & Green (1963*b*), and purified by flotation in the ultracentrifuge at a density of 1·21 (Green, Oncley & Karnovsky, 1960). For experiments with trypsin and chymotrypsin, it was dissolved in 0·16M-phosphate buffer (NaH_2PO_4–Na_2HPO_4), pH 7·65. For all other experiments, it was left in the NaCl–KBr solution used in its preparation.

Alumina (grade O) was from Peter Spence and Co., Widnes, Lancs., and silicic acid (analytical reagent; 100 mesh) from Mallinckrodt Chemical Works. Ether was dried over sodium and distilled before use.

Methods

Paper electrophoresis. This was performed as described by Green (1962).

Extraction and titration of fatty acids. These were carried out by the procedure of Davis (1947).

Determination of choline. This was carried out by the method of Shapiro (1953).

Extraction of total lipids. This was performed as described by Ashworth & Green (1963*b*).

Other analyses. All other analyses were performed as described by Ashworth & Green (1963*b*).

Ether extraction. The aqueous solution was added to twice its own volume of diethyl ether in a 15 ml. glass-stoppered tube and left at room temperature with intermittent shaking for 20 min., centrifuged to separate the phases and the ether phase removed with a capillary pipette. This procedure was carried out three times, the extracts being combined for analysis.

Separation of esterified and unesterified sterols. Chromatography on columns of silicic acid or alumina was used. Silicic acid columns consisted of a mixture of 3 g. of silicic acid and 2 g. of Celite, and alumina columns of 5 g. of alumina (partially deactivated to Brockmann grade 3). In each case, columns 1 cm. in diameter were prepared from suspensions of the adsorbent in light petroleum (b.p. 40–60°). The lipid was applied to the column in ether–light petroleum (1:24, v/v) and sterol esters were eluted with 100 ml. of this mixture. Unesterified sterols were eluted with 100 ml. of diethyl ether.

Enzyme studies. Trypsin and chymotrypsin reaction mixtures contained 12·5–20 mg. of α-lipoprotein and

0·17 mg. of enzyme in 2 ml. of phosphate buffer. Controls containing no enzyme were set up simultaneously and the vessels incubated at 37° for 2–4 hr. Samples (0·5 ml.) were removed for examination by paper electrophoresis and for determination of trichloroacetic acid-soluble nitrogen. For the latter, an equal volume of 5% (w/v) trichloroacetic acid solution was added to the sample and the mixture kept for 30 min. The precipitate was removed by centrifuging and the nitrogen content of the supernatant was determined. The remaining volume of the reaction mixture (1·0 ml.) was first extracted with ether as described above and then subjected to total lipid extraction.

Phospholipase D reaction mixtures contained 1 ml. of enzyme, 0·5 ml. of lipoprotein solution (7–11 mg. of lipoprotein), 0·25 ml. of M-$CaCl_2$ and 0·75 ml. of buffer (Davidson & Long, 1958). Ether was added to each tube; the amount was not important, as identical results were obtained with volumes varying between 1 drop and 1 ml. 'No-enzyme' and 'no-substrate' controls were set up at the same time, and incubation was carried out at 26° for 2–4 hr. The reaction was stopped by the addition of sufficient EDTA (0·25 M) to remove all the Ca^{2+} ions.

RESULTS

Extensive hydrolysis of the protein moiety of α-lipoprotein occurred with both peptidases. After digestion with trypsin 65·3% of the nitrogen remained soluble in trichloroacetic acid, and after digestion with chymotrypsin 65·7%. As with purified β-lipoproteins (Banaszak & McDonald, 1962) a significant amount (13%) of the nitrogen of the intact α-lipoprotein was not precipitated by trichloroacetic acid.

As reported by Ashworth & Green (1963*a*) phospholipase A did not attack the lipoprotein under the conditions used. However, more recent observations indicate that the enzyme is inhibited by the high concentration of salts present in the reaction mixture, so the significance of this result is uncertain. The results of Marinetti (1961) and Condrea *et al.* (1962), who used respectively whole plasma and plasma from which β-lipoproteins had been removed, suggested that all of the lecithin present in α-lipoprotein can be attacked by this enzyme.

The other phospholipase studied, phospholipase D, readily attacked α-lipoprotein. It was calculated, by assuming that 80% of the total phospholipid was lecithin (Nelson & Freeman, 1960), that 87·6% of the choline present was liberated. As EDTA was added before ether, hydrolysis could not proceed during extraction.

Electrophoresis. In all enzyme experiments, the solutions remained clear throughout incubation and there was no sign of aggregation of the lipoprotein residues. Paper electrophoresis of α-lipoprotein after digestion with trypsin or chymotrypsin (Fig. 1) showed no discrete bands. No material staining with Naphthalene Black 12B could be detected. Staining with Sudan Black showed that the lipid was spread out over a wide zone extending from the origin to the normal α_2-globulin position.

Lipid extraction. Human α-lipoprotein is a stable complex, fairly resistant to extraction with non-polar solvents. Avigan (1957) found that 29% of the sterol and 1% of the phospholipid were removed by mild ether extraction. The extraction technique used in the present investigation gave very similar results (Table 1). Trypsin, chymotrypsin and phospholipase D all affect lipid-binding, since phospholipid and more sterol appeared in the ether extracts after their action. Although the two peptidases broke down the protein to approximately the same extent, trypsin had a much more marked effect on lipid binding. Thus, after digestion by trypsin, more than twice as much phospholipid and extra sterol (i.e. the proportion in excess of that extracted from the control) were removed as after digestion with chymotrypsin. A similar finding was made by

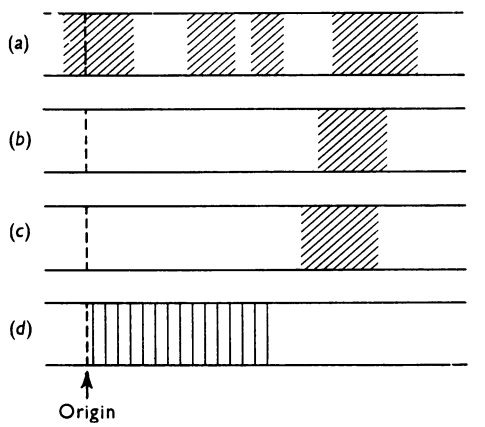

Fig. 1. Paper electrophoresis of: (*a*) human plasma (Naphthalene Black stain); (*b*) human α-lipoprotein (Naphthalene Black stain); (*c*) human α-lipoprotein (Sudan Black stain); (*d*) trypsin-digested human α-lipoprotein (Sudan Black stain). Details are given in the text.

Table 1. *Ether extraction of human α-lipoprotein after enzymic digestion*

Experimental details are given in the text. Results are expressed as means ± S.E.M.

Enzyme	No. of experiments	Sterol extracted (% of total)	Phospholipid extracted (% of total)
None	13	31·2±3·1	0
Trypsin	4	64·7±5·5	52·8±3·8
Chymotrypsin	9	43·5±4·7	23·0±5·1
Phospholipase D	4	78·5±5·5	50·9±1·7

Banaszak & McDonald (1962) with human β-lipoproteins. Phospholipase D had a greater effect than either of the proteolytic enzymes, nearly 80% of the sterol and 50% of the phospholipid being extracted after treatment of α-lipoprotein with this enzyme.

The importance of phospholipids in the maintenance of lipoprotein structure can be demonstrated by plotting the percentages of sterol and phospholipid extracted in each experiment (Fig. 2). Apart from the loosely bound sterol, there is a relationship between the two, suggesting that they are packed together in the complex. No such relationship can be demonstrated between the extent of protein hydrolysis and the amount of either lipid extracted. Phospholipid is more firmly bound in the complex than sterol. In only one experiment was more than 60% of the phospholipid removed, although 90% of the sterol could be extracted. Even when most of the lecithin had been converted into phosphatidic acid, no more than 57% could be extracted.

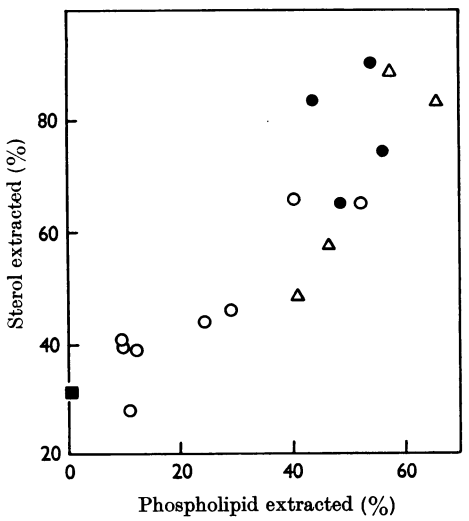

Fig. 2. Relationship between the amounts of sterol and phospholipid in ether extracts of enzyme-treated α-lipoprotein. Experimental details are given in the text. ■, Untreated α-lipoprotein; ○, chymotrypsin-treated α-lipoprotein; △, trypsin-treated α-lipoprotein; ●, phospholipase D-treated α-lipoprotein.

Table 2. *Extraction of esterified and unesterified sterol from α-lipoprotein preparations*

Experimental details are given in the text.

	Unesterified sterol (% of total)			
	Untreated α-lipoprotein		Trypsin-digested α-lipoprotein	
	Intact	Residue from ether extraction	Ether extract	Residue from ether extraction
Expt. 1	31·4	33·9	38·7	36·3
Expt. 2	33·3	40·5	36·2	40·2

Esterified and unesterified sterol. All plasma lipoproteins contain both unesterified sterol and sterol esters, with at least 65% in the esterified form (Gurd, 1960). As it seemed probable that the binding of the two forms would depend on the structure of the lipoprotein, their relative proportions in extracts of normal and trypsin-treated α-lipoprotein were measured (Table 2). The proportion of unesterified sterol was the same (within experimental error) in the original lipoprotein, its ether extract and the ether extract of trypsin-digested α-lipoprotein. Thus there was no preferential removal of either form of sterol, even when 60% of the total was extracted.

DISCUSSION

Most of the protein present in human α-lipoprotein can be removed without causing the residues to be precipitated from solution or to agglutinate. The low extraction of lipids after chymotrypsin digestion (Table 1) suggests that much of the protein is not involved in lipid-binding. The residue from such digestion does not stain with Naphthalene Black 12B, although β-lipoproteins, which contain much less protein, do stain at the same concentration. This finding and the lipid-staining pattern shown in Fig. 1 are consistent with a residual structure possessing a surface largely made up of lipid molecules. All complexes with predominantly lipid surfaces (phospholipid micelles, chylomicrons, low-density β-lipoproteins) tail markedly during electrophoresis on paper.

α-Lipoprotein differs from β-lipoproteins in being much more readily attacked by the two peptidases. Banaszak & MacDonald (1962) and Bernfeld & Kelley (1963) found that peptidases had very little effect on β-lipoproteins.

As nearly all of the lecithin present can be hydrolysed by phospholipase D, it is probable that the hydrophilic ends of the molecules are exposed at the surface. The results of Marinetti (1961) and Condrea *et al.* (1962) support this view. However, the action of phospholipase C is puzzling. Williams (1954) found that this enzyme had no effect on α-lipoprotein in whole plasma, and Krumwiede (1958) found it did not affect the extraction of lipid from horse α-lipoprotein. It is difficult to reconcile these results with the marked effect of phospholipase D on lipid extraction, but they do indicate differences between α-lipoprotein on the one hand and

β-lipoproteins and chylomicrons on the other (Williams, 1954; Robinson, 1955).

The results given in Fig. 2 indicate that, although one-third of the sterol is held loosely in the complex, the remainder, both esterified and unesterified, is bound with the phospholipid. Here again α-lipoprotein differs from β-lipoproteins. Hayashi, Lindgren & Nichols (1959) found that triglycerides and sterol esters were more readily removed from low-density β-lipoproteins than were unesterified sterols or phospholipids.

These results are more in keeping with the type of structure proposed by Gurd (1960) in which the surface is made up of both peptide and lipid and the structure maintained by both lipid–protein and lipid–lipid links. Previous results on the binding of lipids and dyes by α-lipoprotein (Ashworth & Green, 1963*b*) also lend support to this view. The marked differences in behaviour between α-lipoprotein on the one hand and β-lipoproteins and chylomicrons on the other cannot easily be reconciled with an α-lipoprotein structure in which a hydrophobic core is stabilized by a surface of phospholipid and peptide. This means that sterol esters (and a small amount of triglyceride) must be present with phospholipid and unesterified sterol in some ordered arrangement. Previous experimental work on artificial films and micelles of mixed lipids has mainly involved unesterified sterols. Vandenheuvel (1962) has, however, considered in some detail how both forms of sterol and triglycerides could be packed with phospholipids in lipoproteins.

SUMMARY

1. Gentle extraction with ether removed 31 % of the total sterol but no phospholipid from human α-lipoprotein.

2. After digestion with chymotrypsin, 65 % of the nitrogen was rendered trichloroacetic acid-soluble. Ether extracts then contained 44 % of the sterol and 23 % of the phospholipid.

3. Trypsin breaks down the protein to the same extent as chymotrypsin but has more effect on lipid binding, 65 % of the sterol and 53 % of the phospholipid appearing in the ether extracts.

4. There was no preferential extraction of either esterified or unesterified sterol from intact or trypsin-digested α-lipoprotein.

5. Phospholipase D liberated 88 % of the choline present in the lipoprotein and, after such treatment, ether extracts contained 79 % of the sterol and 51 % of the phospholipid.

6. Paper electrophoresis of trypsin-digested α-lipoprotein revealed no protein-staining zones. Lipid was spread in a wide zone between the origin and the normal α_2-globulin position.

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REFERENCES

Ashworth, L. A. E. & Green, C. (1963*a*). *Biochem. J.* **87**, 11P.

Ashworth, L. A. E. & Green, C. (1963*b*). *Biochim. biophys. Acta*, **70**, 68.

Avigan, J. (1957). *J. biol. Chem.* **226**, 957.

Banaszak, L. J. & McDonald, H. J. (1962). *Biochemistry*, **1**, 344.

Bernfeld, P. & Kelley, T. F. (1963). *Fed. Proc.* **22**, 527.

Canal, J. R. & Girard, M. L. (1962). *C.R. Acad. Sci., Paris*, **255**, 2306.

Commission on Enzymes of the International Union of Biochemistry (1961). *Report of the Commission on Enzymes of the International Union of Biochemistry*, p. 8. New York, London, Oxford and Paris: Pergamon Press Ltd.

Condrea, E., de Vries, A. & Mager, J. (1962). *Biochim. biophys. Acta*, **58**, 389.

Cook, W. H. & Martin, W. G. (1962). *Biochim. biophys. Acta*, **56**, 362.

Davidson, F. M. & Long, C. (1958). *Biochem. J.* **69**, 458.

Davis, B. D. (1947). *Arch. Biochem.* **15**, 351.

Green, C. (1962). *Biochem. J.* **84**, 50P.

Green, C., Oncley, J. L. & Karnovsky, M. L. (1960). *J. biol. Chem.* **235**, 2884.

Gurd, F. R. N. (1960). In *Lipide Chemistry*, p. 260. Ed. by Hanahan, D. J. New York: John Wiley and Sons Inc.

Hayashi, S., Lindgren, F. & Nichols, A. (1959). *J. Amer. chem. Soc.* **81**, 3793.

Krumwiede, E. (1958). *Proc. Soc. exp. Biol., N.Y.*, **99**, 151.

Marinetti, G. V. (1961). *Biochim. biophys. Acta*, **46**, 468.

Nelson, G. J. & Freeman, N. K. (1960). *J. biol. Chem.* **235**, 578.

Robinson, D. S. (1955). *Quart. J. exp. Physiol.* **40**, 112.

Shapiro, B. (1953). *Biochem. J.* **53**, 663.

Vandenheuvel, F. A. (1962). *Canad. J. Biochem. Physiol.* **40**, 1299.

Williams, G. R. (1954). *Biochim. biophys. Acta*, **13**, 72.