Methods for investigating cholesterol and associated lipids

By J. A. LOVERN, *Department of Scientific and Industrial Research, Food Investigation Organization, Torry Research Station, Aberdeen*

According to the nature of the investigation, so will the experimental techniques vary. I shall attempt to review very briefly all the major steps in lipid investigation, but clearly I can deal only with the principles involved and not with the experimental details. Moreover, certain exceptions to the generalized statements will have to be ignored. Special emphasis will be laid on cholesterol, but many of the procedures will apply to lipids in general.

Extraction of lipids

Quantitative extraction of lipids often involves disrupting lipid-protein or lipid-carbohydrate complexes, which are themselves usually insoluble in organic solvents. There is a further requirement; the extraction procedure should not permit or promote lipid degradation, e.g. by oxidation or enzyme action. Autolysis during drying and extraction has led to publication of unsound results, e.g. in most of the reports of the occurrence of phosphatidic acids in plant tissues (Hanahan & Chaikoff, 1947, 1948; Acker & Ernst, 1954; Smith, 1954*a*).

The lipid-freeing powers of lower aliphatic alcohols are well known. Similar properties are possessed to greater or less degree by a number of organic solvents, e.g. acetone (Delage, 1935), tetrahydrofuran (Cremer, 1949) and methylal (Delsal, 1944*a*; Schjeide, 1955). Methanol and ethanol are the most commonly used lipid-freeing agents but, being poor solvents for many lipids, they are commonly used in association with some other solvent. Examples of commonly used mixed solvents are ethanol-ether, ethanol-benzene and methanol-chloroform. The ratio of components and temperature of extraction are not generally critical (Boyd, 1936). Some of these lipid-freeing solvents are also good extractants for tissue water and permit lipid extraction direct from moist tissue. Due regard must, of course, be paid to the presence of any lipids with special characteristics of solubility.

Cholesterol and its esters are among the more easily extracted and readily-soluble lipids. They are not so firmly bound in complexes as many other lipids (cf. Lovern, 1955). The risk of rapid enzymic hydrolysis of cholesterol esters during extraction seems small, and the esters seem less liable to rapid atmospheric oxidation than some other lipids, e.g. lecithin. Thus extraction difficulties should not arise in work solely on cholesterol and its esters.

Purification of lipid extracts

The crude extract always contains non-lipid impurities, varying in amount and nature with the tissue. Often the impurities are substances normally insoluble in the solvents used, but readily soluble in the presence of lipids, e.g. amino-acids,

https://doi.org/10.1079/PNS19560010 Published online by Cambridge University Press

urea and sodium chloride are soluble in light petroleum containing phospholipids (Christensen, 1939; Folch & Van Slyke, 1939*a*; Olley & Lovern, 1953). Their presence is probably not serious in work concerned only with cholesterol, but may be fatal to a detailed study of the total lipids of a tissue. The worst interference will arise in work with phospholipids, or the complex glycolipids of seeds or certain micro-organisms.

In general the contaminants are water-soluble substances and most purification procedures utilize this property. The crude lipid extract, dissolved in an organic solvent, is washed with water. Either great care is taken to avoid emulsions, e.g. by dialysis (Sinclair, 1948) or by a very gentle washing procedure (Folch, Ascoli, Lees, Meath & Le Baron, 1951), or emulsions are deliberately produced and then broken (MacLean, 1914; McKibbin & Taylor, 1949). Alternatively, aqueous extraction of the tissue may precede extraction of lipids, which have been prevented from emulsifying with the water (e.g. Folch & Van Slyke, 1939*b*; Johnson & Dutch, 1951).

Chromatography has recently been used to remove non-lipid impurities. Thus a cellulose column was used to purify the phospholipids of egg yolk (Lea & Rhodes, 1953) and of rubber latex (Smith, 1954*b*). Paper chromatography has indicated (Bode & Ludwig, 1954) that reported lipid-peptide complexes may only be mixtures. It is unlikely that any procedure will remove all impurities in all instances, and there is sometimes selective loss of lipids, e.g. of gangliosides and diphosphoinositide by water washing (Folch *et al.* 1951) and of certain types of inositol lipids by chromatography on cellulose (Garcia, Lovern & Olley, 1956).

Separation of mixed lipids

No method has yet been devised for the quantitative separation of all types of lipids from the total extract. If pure lipid samples are required, the yield is far from quantitative and the sample may be unrepresentative, e.g. in its fatty acids. If quantitative recovery is the aim, concentrates of the lipids are all that can be expected for many of them.

Many separation procedures are based on slight differences of solubility in various solvents. There is here an inherent risk of fractionation by fatty-acid composition, most lipids being mixtures of homologues. So far as cholesteryl esters are concerned, there has been no success in attempts to separate them from triglycerides, waxes and the like, by differential solubility (e.g. Clément, Clément-Champougny & Louedec, 1954) even if counter-current fractionation is used (Lovern & Olley, 1953). Preferential enzymic hydrolysis of triglycerides, leaving cholesteryl esters intact, was claimed by Kelsey & Longenecker (1941) but could not be confirmed by later workers (Clément *et al.* 1954).

Cholesteryl esters can be separated fairly well from triglycerides by chromatography. With alumina, recovery of cholesteryl esters uncontaminated by the more strongly adsorbed triglycerides is claimed, but quantitative elution of the triglyceride fraction is impossible (Borgström, 1952*b*) or has not even been attempted (Clément *et al.* 1954). Borgström (1952*b*) has claimed adequate separation of these

https://doi.org/10.1079/PNS19560010 Published online by Cambridge University Press

two lipid classes, with quantitative recovery of both, by the use of silicic acid but Lovern (unpublished) found much less sharp separation when each lipid is a whole range of homologues of differing fatty-acid composition. Borgström's cholesteryl ester was entirely palmitate. The quantitative recovery of uncontaminated cholesteryl esters is essential if their metabolic role is to be studied, since their fatty-acid composition is reported to be characteristic (Kelsey & Longenecker, 1941; Clément *et al.* 1954).

The total fractionation of the mixed lipids of a tissue extract is a most complicated procedure, which will not be discussed here. Both free and esterified cholesterol are readily separated from phospholipids and cerebrosides by differential solubility, e.g. in acetone, and direct chromatography of the total extract likewise can usually effect such separation (e.g. Fillerup & Mead, 1953; Borgström, 1952*a*). Free cholesterol can, of course, be determined without isolating it, and isolation of a pure sample in 'tracer' metabolic studies is simple. One difficulty with cholesteryl esters is that several types of associated lipids, e.g. wax esters and free aliphatic alcohols, found in some tissue extracts, have not so far been adequately studied, e.g. their separation by chromatography has not been followed, as with the commoner triglyceride components.

Analytical procedures

Many papers have been published on the determination of cholesterol, and the findings of various workers are frequently contradictory. I shall try to assess some of the outstanding points in dispute.

Free cholesterol may be assayed either gravimetrically or colorimetrically, esterified cholesterol either directly by a colour reaction or, after saponification, as the free sterol. I shall not discuss gravimetric determination beyond mentioning that various authors (e.g. Kelsey, 1939; Paget & Pierrart, 1939*a*; Folch, Schneider & Van Slyke, 1940) have emphasized that substances other than free cholesterol may be precipitated with digitonin or be entrained in the precipitate. The colorimetric assays are far more widely used, even when precipitation of the digitonide is a stage in the method.

The determination of free cholesterol, when cholesteryl esters are also present, requires its precipitation as the digitonide. The speed with which this operation can be completed varies enormously according to the technique used and the state of the lipids, e.g. Schoenheimer & Sperry (1934) noted much slower precipitation of free cholesterol in blood-lipid extracts than of total cholesterol after saponification. In the presence of aluminium hydroxide or aluminium ions, precipitation can be complete within 5 min (Brown, Zlatkis, Zak & Boyle, 1954). Saponification of cholesteryl esters must be very carefully performed if subsequent precipitation as digitonide is proposed, otherwise low results will be obtained (e.g. Okey, 1930; Delsal, 1944*b*; Sperry, 1955), although colorimetric assay is apparently not so sensitive to saponification (Delsal, 1944*b*). Although some workers (e.g. Schoenheimer & Sperry, 1934) report cholesteryl esters as very easily saponifiable, others (e.g.

https://doi.org/10.1079/PNS19560010 Published online by Cambridge University Press

Okey, 1930; Noyons & Polano, 1940) claim that they are very resistant to alkali. My own experience is that it all depends on the degree of unsaturation of the fatty acids, the more saturated derivatives being increasingly resistant to ethanolic KOH.

The Liebermann-Burchard reaction is probably still the most widely used in colorimetric assay of cholesterol. It is always found that colour develops more rapidly with the esters than with the free sterol, but whereas some workers (e.g. Sperry & Brand, 1943) find a higher maximum colour density with the esters, others find the same maximum (e.g. Paget & Pierrart, 1939*b*; Saifer & Kammerer, 1946). Since the Liebermann-Burchard reaction measures the net effect of two consecutive reactions, the relative rates of which vary with the temperature (Hoffman, 1940), and probably with the solvent (Sheftel, 1944; Saifer & Kammerer, 1946), it seems unlikely that free cholesterol and its esters will give a common maximum under all experimental conditions. Some workers measure the colour at its maximum intensity (Noyons, 1938; Saifer & Kammerer, 1946), others employ rigid conditions of development (e.g. Sperry & Brand, 1943).

Two other colorimetric reactions of cholesterol do not suffer from the main drawback of the Liebermann-Burchard method, i.e. the transient nature of the colour and its great dependence on time, temperature and solvent. One (Rose, Schattner & Exton, 1941) employs the Tschugaeff reaction, the production of a red colour with an acetyl chloride—zinc chloride—acetic acid reagent. The colour is completely stable at room temperature for a long time, certainly over an hour. I have found this reaction to give a colour directly proportional to the cholesterol present, whether it is free, esterified or as digitonide (Lovern, 1955). Hence the uncertainty attaching to saponification followed by digitonin precipitation can be avoided, except with highly coloured lipids where purification by digitonin is desirable. The sensitivity is some fifteen times that of the Liebermann-Burchard reaction.

The use of a ferric chloride—acetic acid—sulphuric acid reagent has recently been reported (Zlatkis, Zak & Boyle, 1953). It yields a colour stable for very long periods, and the sensitivity is as high as that of the Tschugaeff reaction. I have found that here also the colour is directly proportional to the cholesterol present, whether free, esterified or as digitonide. However, I have found the reaction less specific than the Liebermann-Burchard and Tschugaeff reactions, and with certain lipid preparations it has given falsely high values. Where preliminary tests show that it can be used, it is the simplest method yet devised.

Metabolic studies

The use of 'labelled' compounds has thrown much light on the metabolism of many types of lipid, including cholesterol. Information can be obtained in this way about (a) metabolic rates and (b) metabolic routes. The rate at which a particular label, e.g. an isotope, accumulates in or disappears from a lipid in a tissue gives information of the first kind, and the relative levels of labelling with time in a series of possible metabolic intermediates can indicate which of them may be intermediary in the reaction in question and in what order.

https://doi.org/10.1079/PNS19560010 Published online by Cambridge University Press

With cholesterol, labels widely employed have been isotopes of hydrogen and carbon, generally in the form of heavy water or radioactive acetate. Administered thus to an animal, the isotope accumulates in its cholesterol, the rate varying widely in different tissues. If the labelled substance is maintained at a steady level in the animal's system until equilibrium is reached in the tissue cholesterol, the so-called 'turnover time' or, if desired, the 'half life time' of it can be determined (e.g. Rittenberg & Schoenheimer, 1937; Pihl, Bloch & Anker, 1950; Popják & Beeckmans, 1950). Unfortunately, constant-level administration of labelled acetate does not maintain a constant level of isotope in the animal's total store of acetate (Pihl *et al.* 1950), so that the method lacks precision. Measurement of rate of isotope depletion in the tissue cholesterol after cessation of administration of labelled acetate (which need not be to equilibrium level) is no more precise, since a whole series of animals must be used in such work and there is no assurance that all have reached the same initial level of cholesterol labelling. Nevertheless, smooth curves are reported (Landon & Greenberg, 1954).

Only one administration of labelled precursor is often given with such substances as lipid phosphorus. If the subsequent time curve of label enrichment in a tissue lipid is followed, it can be interpreted only by reference to a similar curve for the immediate precursor of the lipid. This is difficult enough for phospholipid phosphorus (cf. Lovern, 1955), but on present knowledge is impossible for cholesterol.

In the study of metabolic routes a substance is usually rejected as an intermediate if it shows a higher level of labelling than its presumed precursor. This conclusion is strictly valid only if alternative metabolic pathways are excluded. If radioactive acetate is administered to an animal, radioactivity is present in all sorts of compounds. If the activities of cholesterol and cholic acid are compared in such an animal, cholic acid usually shows the higher specific activity (Staple & Gurin, 1954), but this is no proof that cholesterol is not converted into cholic acid—indeed administration of labelled cholesterol, rather than acetate, shows that some cholesterol is so converted (e.g. Zabin & Barker, 1953).

If a series of compounds shows a steadily diminishing specific activity at any particular time, these may, but do not necessarily, form a direct metabolic sequence. If one compound is the immediate precursor of another, the time course of the specific activities of the two compounds must show a characteristic relationship (Zilversmit, Entenman & Fishler, 1943). Further, if one substance is the sole or major precursor of another, the relative total amounts metabolized in a given time must be commensurate.

Probably the most valuable use of isotopic labelling has been in the study of the derivation of various atoms in the cholesterol skeleton from certain administered groupings, e.g. the methyl and carboxyl carbons respectively of acetate (Cornforth, Hunter & Popják, 1953). By such means, including studies of postulated intermediates, the mechanism of cholesterol synthesis in the animal is steadily being elucidated (*cf.* Bloch, Little, Huang, Zabin, Würsch & Langdon, 1954; Cornforth, 1955).

https://doi.org/10.1079/PNS19560010 Published online by Cambridge University Press

This paper was prepared as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

Acker, L. & Ernst, G. (1954). *Biochem. Z.* **325**, 253.
Bloch, K., Little, H. N., Huang, R. L., Zabin, I., Würsch, J. & Langdon, R. G. (1954). *Harvey Lect.* **48**, 68.
Bode, F. & Ludwig, U. M. (1954). *Klin. Wschr.* **32**, 1097.
Borgström, B. (1952*a*). *Acta physiol. scand.* **25**, 101.
Borgström, B. (1952*b*). *Acta physiol. scand.* **25**, 111.
Boyd, E. M. (1936). *J. biol. Chem.* **114**, 223.
Brown, H. H., Zlatkis, A., Zak, B. & Boyle, A. J. (1954). *Analyt. Chem.* **26**, 397.
Christensen, H. N. (1939). *J. biol. Chem.* **129**, 531.
Clément, G., Clément-Champougny, J. & Louedec, A. (1954). *Arch. Sci. physiol.* **8**, 233.
Cornforth, J. W. (1955). *Rev. pure appl. Chem.* **4**, 275.
Cornforth, J. W., Hunter, C. D. & Popják, G. (1953). *Arch. Biochem. Biophys.* **42**, 481.
Cremer, H. D. (1949). *Klin. Wschr.* **27**, 755.
Delage, B. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 923,
Delsal, J. L. (1944*a*). *Bull. Soc. Chim. biol., Paris*, **26**, 99.
Delsal, J. L. (1944*b*). *Bull. Soc. Chim. biol., Paris*, **26**, 239.
Fillerup, D. L. & Mead, J. F. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 574.
Folch, J., Ascoli, I., Lees, M., Meath, J. A. & Le Baron, F. N. (1951). *J. biol. Chem.* **191**, 833.
Folch, J., Schneider, H. A. & Van Slyke, D. D. (1940). *J. biol. Chem.* **133**, xxxiii.
Folch, J. & Van Slyke, D. D. (1939*a*). *J. biol. Chem.* **129**, 539.
Folch, J. & Van Slyke, D. D. (1939*b*). *Proc. Soc. exp. Biol., N.Y.*, **41**, 514.
Garcia, M. D., Lovern, J. A. & Olley, J. (1956). *Biochem. J.* **62**, 99.
Hanahan, D. J., & Chaikoff, I. L. (1947). *J. biol. Chem.* **169**, 699.
Hanahan, D. J. & Chaikoff, I. L. (1948). *J. biol. Chem.* **172**, 191.
Hoffman, W. S. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 38.
Johnson, R. M. & Dutch, P. R. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 662.
Kelsey, F. E. (1939). *J. biol. Chem.* **127**, 15.
Kelsey, F. E. & Longenecker, H. E. (1941). *J. biol. Chem.* **139**, 727.
Landon, E. J. & Greenberg, D. M. (1954). *J. biol. Chem.* **209**, 493.
Lea, C. H. & Rhodes, D. N. (1953). *Biochem. J.* **54**, 467.
Lovern, J. A. (1955). *The Chemistry of Lipids of Biochemical Significance*, 1st ed. London: Methuen & Co.
Lovern, J. A. & Olley, J. (1953). *Biochem. J.* **54**, 128.
McKibbin, J. M. & Taylor, W. E. (1949). *J. biol. Chem.* **178**, 17.
MacLean, H. (1914). *J. Path. Bact.* **18**, 490.
Noyons, E. C. (1938). *Biochem. Z.* **298**, 391.
Noyons, E. C. & Polano, M. K. (1940). *Biochem. Z.* **303**, 415.
Okey, R. (1930). *J. biol. Chem.* **88**. 367.
Olley, J. & Lovern, J. A. (1953). *Biochem. J.* **54**, 559.
Paget, M. & Pierrart, G. (1939*a*). *Bull. Soc. Chim. biol., Paris*, **21**, 528.
Paget, M. & Pierrart, G. (1939*b*). *Bull. Soc. Chim. biol., Paris*, **21**, 535.
Pihl, A., Bloch, K. & Anker, H. S. (1950). *J. biol. Chem.* **183**, 441.
Popják, G. & Beeckmans, M. L. (1950). *Biochem. J.* **47**, 233.
Rittenberg, D. & Schoenheimer, R. (1937). *J. biol. Chem.* **121**, 235.
Rose, A. R., Schattner, F. & Exton, W. G. (1941). *Amer. J. Path. Tech. Suppl.* **5**, 19.
Saifer, A. & Kammerer, O. F. (1946). *J. biol. Chem.* **164**, 657.
Schjeide, O. A. (1955). *J. biol. Chem.* **214**, 315.
Schoenheimer, R. & Sperry, W. M. (1934). *J. biol. Chem.* **106**, 745.
Sheftel, A. G. (1944). *J. Lab. clin. Med.* **29**, 875.
Sinclair, R. G. (1948). *J. biol. Chem.* **174**, 343.
Sperry, W. M. (1955). *Fed. Proc.* **14**, 284.
Sperry, W. M. & Brand, F. C. (1943). *J. biol. Chem.* **150**, 315.
Smith, R. H. (1954*a*). *Biochem. J.* **56**, 240.
Smith, R. H. (1954*b*). *Biochem. J.* **57**, 130.
Staple, E. & Gurin, S. (1954). *Biochim. biophys. Acta*, **15**, 372.
Zabin, I. & Barker, W. F. (1953). *J. biol. Chem.* **205**, 633.
Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.
Zlatkis, A., Zak, B. & Boyle, A. J. (1953). *J. Lab. clin. Med.* **41**, 486.

https://doi.org/10.1079/PNS19560010 Published online by Cambridge University Press