Short Communications

Changes in Thiol Content of the Hamster Cheek Pouch after X-Irradiation and some Speculations on the Relationship between Phospholipids and Protein Thiol Groups

By DWIGHT B. MCNAIR SCOTT, A. B. REISKIN and ANNA M. PAKOSKEY

Departments of Physiology and Radiology, School of Medicine, and Interdisciplinary Cancer Research Unit, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

(Received 29 June 1964)

The primary target of irradiation of biological material is still uncertain. In an attempt to investigate the effect on thiol groups, cheek pouches of albino Syrian hamsters were exposed to 5000 r. of X-rays while the rest of the body was shielded. In one series control pouches were everted and sham-irradiated, and in the other series the control pouches were shielded while the other pouch was being irradiated. At chosen times after irradiation the pouches were removed under light Nembutal anaesthesia. The pouches taken at 215 and 236 hr. appeared inflamed and the wet weight was greater than 500 mg. The pouches taken at 500 and 732 hr. and one pouch taken at 612 hr. were small, weighing less than 300 mg., two as little as 180 mg. The other pouches listed in Table 1 did not look abnormal and weighed between 260 and 400 mg. The appearance of stained sections under the microscope was similar to the descriptions by Fulton,

Lutz & Kagan (1956) at different times after X-irradiation.

The pouches were minced, ground with alumina and extracted with tris. Samples of the extract were incubated for 20 min. with glucose 6-phosphate and NADP⁺. The protein of these samples and of the untreated samples was precipitated by sulphosalicyclic acid. The thiol contents of the acid-soluble supernatant fluids and of the protein pellets were titrated with silver nitrate amperometrically (Morris, Scott & Reiskin, 1961). In other samples of the extracts, certain enzyme activities were investigated.

Some of the results of the amperometric titrations, expressed as μ moles of thiol group/g. of protein of the extract, are shown in Table 1. The times chosen for this Table were those with a maximum or minimum value in a cycle.

From the changes in the shielded controls, it was

Table 1. *Concentrations of thiol groups in extracts of pouches*

Results are given as μ moles of thiol group/g. of protein.

	Time	Acid-soluble thiol groups		Protein thiol groups	
		Direct	Reduced	Direct	Reduced
Controls (means $\pm$ S.D. of 27)		17.8 $\pm$ 6	29 $\pm$ 6	59 $\pm$ 8	60 $\pm$ 8
Shielded controls	14 min.	18.5	22.5	41.5	47.0
	30 min.	25.0	35.8	69.5	74.0
	24 hr.	28.8	43.4	64.6	65.0
	71 hr.	17.6	28.2	48.0	52.5
	95 hr.	18.9	37.0	71.0	58.0
	215 hr.	18.3	38.0	83.0	81.0
	236 hr.	8.2	25.4	45.0	47.0
	500 hr.	12.6	23.0	44.0	48.0
	732 hr.	15.4	37.5	52.0	60.5
Irradiated pouches (means of 3)	14 min.	20.0	26.7	46.0	50.0
	30 min.	17.4	26.8	50.5	55.6
	2 hr.	25.0	32.3	57.0	64.0
	6 hr.	24.7	31.0	70.0	69.0
	24 hr.	23.7	36.0	55.0	59.0
	48 hr.	22.2	33.0	69.0	70.0
	95 hr.	7.8	29.7	56.0	61.5
	198 hr.	14.2	21.7	44.3	50.5
Inflammation	215 hr.	6.3	24.3	64.0	59.2
	236 hr.	2.8	13.8	42.8	45.7
	500 hr.	5.3	17.8	37.0	41.4
Atrophy	732 hr.	10.0	24.0	44.0	57.0

concluded that the changes in thiol concentration found in the irradiated pouches were not the primary reaction, but that there was an 'abscopic' reaction (Mole, 1953). Even in our early samples, taken at 30 min. after the beginning of irradiation, a humoral reaction had produced an increase of thiol content in all the fractions in the shielded pouch. Since the concentrations were higher in the shielded pouches than in the irradiated pouches, it was surmised that in the irradiated pouch there may have been an immediate decrease in the thiol content, perhaps especially in the protein with a very fast reaction, from humoral sources to repair the damage. We also found an increase of 50 % in the activity of glutathione reductase at 14 min. in the irradiated pouch and an increase of 250 % in the shielded pouch at 30 min. This would reduce glutathione that had been oxidized by exchange with disulphide groups formed by the oxidizing action of the X-rays on the protein, since only GSSG is a substrate for the reductase (Pihl, Eldjarn & Bremer, 1957). The fast reduction of GSSG could prevent the detection of any significant decrease in GSH in the irradiated pouches. The protein thiol contents were low at 14 min. Deakin, Ord & Stocken (1963) reported that, if glutathione reductase were inhibited by the presence of Zn^{2+} ions, they could find a decrease in the thiol content in thymus nuclei immediately after irradiation.

For a long time many workers postulated that the primary target of ionizing radiation is the thiol group (Barron, 1955; Markakis & Tappel, 1960). Until the work of Deakin *et al.* (1963), it has been difficult to obtain direct evidence *in vivo*, although indirect evidence of oxidation of the sulphur and its excretion as taurine in the urine after irradiation has been reported frequently (Kay & Entenman, 1954; Angel & Noonan, 1961; Aebi, Bernays, Fluckiger, Schmidli & Zuppinger, 1955).

Others have concluded that the primary target of both ultraviolet and X-irradiation was the unsaturated bond of the fatty acids in phospholipids (Mead, 1952; Dubouloz & Fondarai, 1953; Belokonskii & Rusev, 1959; Clubb & Wills, 1959). Howton (1963) reported that X-irradiation of unsaturated fatty acids in the absence of oxygen produced saturated acids, hydroxy acids and dimers. When oxygen was present, peroxides were formed and these started a chain of autoxidation and free-radical formation. The electron holes were finally transferred to thiol or disulphide groups or other antioxidants, thus stopping the chain. Gordy, Ard & Shields (1955) found that irradiation of proteins that contain any cystine show a nuclear-magnetic-resonance spectrum only of irradiated cysteine. They concluded that, wherever the X-ray hit, the electron vacancy so produced was passed along the protein chain to the sulphur atom.

It seems possible that a combination of the two theories might unite the two suggested targets as an addition reaction of the unsaturated group of the fatty acid and the thiol group of a protein. Radiation damage might have oxidized both groups with the breaking of the bond. Addition of a thiol group across a double bond is well known and is very firm in the compound of $\text{R}\cdot\text{SH}$ with *N*-ethylmaleimide (Friedmann, Marrian & Simon-Reuss, 1949) or acrylonitrile (Weil & Seibles, 1961). The bonds of

in these compounds are very strong, but, if the bond were separated from a carbonyl group by five or more methylene groups in a chain, we might expect the bond to be less firm and perhaps easily exchangeable, or the unsaturation to be easily reformable on very mild treatment.

Boyland (1964) reported that the K region designated by Pullman is the most reactive area of carcinogens and that this had been missed previously just because the sulphur bonds were so labile that they were broken by acid to give the original compound. Thus at the K region epoxides were formed most easily, and these reacted with glutathione or water to form less phenolic compounds than dihydrodihydroxy- or dihydrohydroxy-cysteine derivatives. These last compounds were unstable in acid and produce the original hydrocarbon and di-*N*-acetylcystine. Thus the K region would be acting as a catalyst for the oxidation of thiol groups. Perhaps the double bonds of the unsaturated fatty acids of phospholipids are analogous to the K region of the carcinogenic hydrocarbons, with the result that GSSG is produced catalytically. Some GSSG would be reduced by NADPH and glutathione reductase but some would be hydrolysed. We might expect a depletion of both GSH and NADP^+ with time. Table 1 shows the depletion of GSH as early as 95 hr. after irradiation. Similarly the GSH was depleted after painting with carcinogen (Morris *et al.* 1961).

Should irradiation hit anywhere on the molecule, the effect might then be visualized as travelling to the sulphur, breaking the bond and allowing any oxygen present both to oxidize sulphur and to form peroxide on the double bond of the phospholipid. The repair process then would not only need to reduce the sulphur and to re-form the unsaturated bond, but probably also to increase synthesis of phospholipid. This last reaction has been reported (Johnson & Mier, 1962). If the phospholipid-protein complex in any membrane were broken by irradiation, a change in the permeability might be expected. Such changes have been reported (Henri

& Mayer, 1904; Goldfeder, 1963) in various kinds of membranes.

We are indebted to Dr M. L. Mendelsohn of the Department of Radiology, School of Medicine, University of Pennsylvania, for his help and advice. This study was supported by Grant CA4170 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

Aebi, H., Bernays, L., Fluckiger, H., Schmidli, B. & Zuppinger, A. (1955). *Helv. physiol. acta*, **13**, C49.
 Angel, C. R. & Noonan, T. R. (1961). *Radiat. Res.* **15**, 298.

Barron, E. S. G. (1955). *Ann. N.Y. Acad. Sci.* **59**, 574.
 Belokonskii, I. & Rusev, G. (1959). *Biofizika*, **4**, 204.
 Boyland, E. (1964). *Brit. med. Bull.* **20**, 121.
 Clubb, M. E. & Wills, E. D. (1959). *Biochem. J.* **71**, 16P.
 Deakin, H., Ord, M. G. & Stocken, L. A. (1963). *Biochem. J.* **89**, 296.

Dubouloz, P. & Fondarai, J. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 819.

Friedmann, E., Marrian, D. H. & Simon-Reuss, I. (1949). *Brit. J. Pharmacol.* **4**, 105.

Fulton, G. P., Lutz, B. R. & Kagan, R. (1956). *Circulation Res.* **4**, 133.

Goldfeder, A. (1963). *Trans. N.Y. Acad. Sci.* **II**, **26**, 215.

Gordy, W., Ard, W. B. & Shields, H. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 983.

Henri, V. & Mayer, A. (1904). *C.R. Acad. Sci., Paris*, **138**, 521.

Howton, D. R. (1963). *Radiat. Res.* **20**, 161.

Johnson, B. E. & Mier, P. (1962). *Nature, Lond.*, **194**, 101.

Kay, R. E. & Entenman, C. (1954). *Fed. Proc.* **13**, 520.

Markakis, P. & Tappel, A. L. (1960). *J. Amer. chem. Soc.* **82**, 1613.

Mead, J. F. (1952). *Science*, **115**, 470.

Mole, R. H. (1953). *Brit. J. Radiol.* **26**, 234.

Morris, A. L., Scott, D. B. McN. & Reiskin, A. B. (1961). *Cancer Res.* **21**, 1352.

Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.

Weil, L. & Seibles, T. S. (1961). *Arch. Biochem. Biophys.* **95**, 470.

Biochem. J. (1964), **93**, 3c

The Estimation of Plasma Renin Concentration in the Dog

By J. J. BROWN, D. L. DAVIES, A. F. LEVER, J. I. S. ROBERTSON and M. TREE

Medical Unit, St Mary's Hospital, London, W. 2

(Received 8 July 1964)

Previous papers have described in detail techniques for the estimation of renin in the plasma of the rabbit (Lever, Robertson & Tree, 1964) and man (Brown, Davies, Lever, Robertson & Tree, 1964a). In each species the method is in principle the same, consisting of an extraction of renin from plasma, incubation under standard conditions with a substrate prepared from ox serum and determination of the velocity of angiotensin formation. It is necessary to free all components of the incubation mixture from angiotensinase activity; in addition, the prepared substrate is required to be free from renin and the extracted renin to be free from substrate. A standard renin preparation of each species is also necessary, free from both substrate and angiotensinase activity.

The method of extraction of renin from dog plasma, and the techniques of incubation and assay, are identical with those for the estimation of renin in rabbit plasma (Lever *et al.* 1964). The main differences concern the preparation of a standard renin from dog kidneys.

Renin and substrate units are defined in similar terms to those used by Lever *et al.* (1964) and Brown *et al.* (1964a). One unit of dog renin is taken as the quantity that, when incubated with

prepared ox-serum substrate at a concentration of 30 substrate units/ml., in a total volume of 5 ml., at pH 5.7 and 37°, forms 0.1 µg. of angiotensin/ml. of incubation mixture/hr.

Renin preparation. Dog kidneys (250 g.) were minced, stirred with 500 ml. of 95 % (v/v) ethanol at 8° for 12 hr. and filtered through muslin, and the filtrate was discarded. The retained material was dried on filter paper at room temperature and then stirred with 2.5 l. of 0.9 % NaCl at 8° for 12 hr. The solution was filtered first through muslin and then through a filter bed of Celite 545 (Johns Manville Co.). (NH₄)₂SO₄ was added to the final filtrate to give a 40 % (w/v) concn., the solution was left at 8° for 12 hr. and then filtered through Whatman no. 50 paper, and the filtrate was discarded. The precipitate was redissolved in 160 ml. of water. Angiotensinase was eliminated by acidification to pH 2.7 for 1 hr. at 22° with N-HCl, then adjustment to approx. pH 6 with N-NaOH and removal of the resultant precipitate by filtration through Whatman no. 1 paper. This renin solution was dialysed against 0.15 M-phosphate-saline buffer, pH 5.7 (Lever *et al.* 1964), before use.

Extraction of renin from dog plasma. The technique employed was that described for renin

extraction from rabbit plasma (Lever *et al.* 1964), which differs only in detail from the method for human plasma (Brown *et al.* 1964a).

Protein estimation. Protein concentration was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Standard dog renin. The standard dog renin had a specific activity of 9 units/mg. of protein.

At a concentration of 30 units/ml. this solution was free from angiotensinase and substrate by the criteria defined by Lever *et al.* (1964).

Renin extracted from plasma. The extract prepared from 20 ml. of dog plasma, reconstituted in 2.5 ml. of buffer, had a protein concentration of 22 mg./ml. This was similar to the results previously obtained with rabbit and human plasma extracts.

The enzyme extracted from dog plasma was indistinguishable from dog renin by the same physicochemical criteria as were applied to the identification of rabbit and human plasma extracts (Lever *et al.* 1964; Brown *et al.* 1964a). Further, the reactions of dog renin and the dog plasma extract with ox-serum substrate were similarly influenced by substrate concentration (Fig. 1), pH and temperature. The optimum pH range, from pH 5.7 to 6.2, was narrower than that of human renin, and similar to that of rabbit renin. The incubation products of both dog renin and dog plasma extract resembled the synthetic Asp₍₁₎-β-amide Val₍₅₎ octapeptide angiotensin physicochemically and pharmacologically, and all were rapidly destroyed by trypsin, chymotrypsin, carboxypeptidase and leucine aminopeptidase (Brown *et al.* 1964a).

Thirty-five consecutive dog plasma extracts were free from angiotensinase and endogenous

substrate by the criteria applied to the rabbit by Lever *et al.* (1964).

The mean recovery of added renin was 32 % [s.d. ± 6.6 % recovery (16 experiments)]. Similar proportionate recoveries were obtained from 10 and 20 ml. starting plasma volumes.

The method was adequate for the estimation of renin in the peripheral venous plasma of the normal dog, and for measuring the rise in plasma renin concentration after a 23 ml./kg. haemorrhage (Brown, Davies, Lever, Robertson & Verniory, 1964b).

Discussion. The method described here is concerned solely with the estimation of renin concentration in plasma. This is achieved by incubating the extracted enzyme in a system from which all other variables have been removed as far as possible. A method of this kind has to be distinguished from techniques that are a measure of 'renin activity' (Boucher, Veyrat, de Champlain & Genest, 1964). The distinction is important, since several factors other than renin concentration probably influence the activity of the renin-angiotensin system *in vivo*. Skinner, McCubbin & Page (1963a, b) estimated renin activity in the dog by incubating separated plasma (in some experiments after the addition of EDTA to inhibit normal plasma angiotensinase). Increases in renin activity were found in renal venous plasma in response to changes in renal arterial pressure. However, the experimental procedure used might have caused alterations in circulating renin substrate. Thus, though Skinner *et al.* (1963a) regarded their results as indicative of changes in plasma renin concentration, they rightly pointed out (Skinner *et al.* 1963b) that differences in substrate concentration (which was not measured in their experiments) might have led to misinterpretation. Similar disadvantages are associated with the method of Boucher *et al.* (1964). These techniques cannot properly be used to measure plasma renin concentration.

We are grateful to Professor W. S. Peart for encouragement and advice. This work was partly supported by a grant from the Wellcome Trust.

Fig. 1. Relationship between ox-serum substrate concentration and initial velocity of reaction. ●, Dog renin; ○, dog plasma extract. Substrate concn. ([S]) is given in units/ml. of incubation mixture; initial velocity (v) is given in μ mg./ml./hr. for renin and in μ mg./ml./20 hr. for plasma extract.

- Boucher, R., Veyrat, R., de Champlain, J. & Genest, J. (1964). *Canad. med. Ass. J.* **90**, 194.
 Brown, J. J., Davies, D. L., Lever, A. F., Robertson, J. I. S. & Tree, M. (1964a). *Biochem. J.* **93** (in the Press).
 Brown, J. J., Davies, D. L., Lever, A. F., Robertson, J. I. S. & Verniory, A. (1964b). *Nephron*, **1** (in the Press).
 Lever, A. F., Robertson, J. I. S. & Tree, M. (1964). *Biochem. J.* **91**, 346.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Skinner, S. L., McCubbin, J. W. & Page, I. H. (1963a). *Science*, **141**, 814.
 Skinner, S. L., McCubbin, J. W. & Page, I. H. (1963b). *Circulation Res.* **13**, 336.

Isolation of Deoxyribonucleic Acid and Ribosomal Ribonucleic Acid from *Escherichia coli*

By K. S. KIRBY

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3

(Received 8 July 1964)

Separation of RNA from DNA without the use of enzymes from bacteria has proved troublesome, partly because bacteria have no nucleus and DNA is held in the chromosome by proteins different from those in mammalian chromosomes.

The present method depends on the insolubility of DNA in the presence of phenolphthalein diphosphate and phenol (Kirby, 1961) and the ability to precipitate ribosomal RNA free from polysaccharide and transfer RNA (s-RNA) by *m*-cresol in the presence of 20 % (w/v) sodium benzoate.

Escherichia coli (26 g. centrifuged wet wt.), which had been growing in the exponential phase, were stirred in a blender with a solution of disodium phenolphthalein diphosphate, pH 6 (8 %), and sodium dodecyl sulphate (1 %) for 2–3 min. There was considerable frothing and the mixture was transferred to a beaker and mixed with 300 ml. of a phenol-*m*-cresol-8-hydroxyquinoline-water mixture (500:70:0.5:55, by wt.). Stirring was continued for 2 hr. at 20° and the mixture was centrifuged at 9000 rev./min. for 20 min. at 5° in an MSE 17 centrifuge. There was a white sediment at the bottom containing the DNA; RNA remained in the supernatant phase, which was treated as follows. The phase was separated and extracted (for 20 min.) with 0.5 vol. of the same phenol-*m*-cresol-8-hydroxyquinoline-water mixture as above. After a further centrifuging and separation, the aqueous phase (160 ml.) was mixed with NaCl (4.8 g.), sodium benzoate (32 g.) and *m*-cresol (20 ml.). The mixture was centrifuged at 2000 rev./min. for 10 min. at 5°. A clear gel was present at the bottom of the centrifuge bottle, and this was washed twice with a water-sodium benzoate-NaCl-*m*-cresol mixture (100:20:3:10, by wt.), and once with 75 % (v/v) ethanol containing NaCl (1 %), but it still contained a small amount of DNA. This was completely removed by washing three times with 3M-sodium acetate, pH 6, and centrifuging at 5°. The product was washed with 75 % (v/v) ethanol and then ethanol, and centrifuged (at 5°) and dried. The yield was 25 mg.; the base composition was G:A:C:U, 32.2:25.0:22.8:20.0 (cf. Midgeley, 1962). The sedimentation profile in 0.15M-NaCl-0.015M-trisodium citrate is shown in Fig. 1 and shows the usual two peaks and virtual absence of DNA.

DNA was extracted from the white sediment by four extractions with sodium benzoate-NaCl-water (20:3:100, by wt.) (twice with 25 ml. and twice with 15 ml.); insoluble material was removed by sedimenting each time at 12000 rev./min. for 15 min. at 5°. DNA was precipitated from the combined extracts by adding ethanol (0.66 vol.). The DNA was dissolved in 0.1M-sodium acetate, pH 6.0 (60 ml.), and the solution centrifuged at 30000 rev./min. for 2 hr. at 2° in a Spinco model L centrifuge. DNA was in the supernatant fluid that was poured off from sedimented polysaccharide, the solution was made 3M with respect to NaCl and DNA was precipitated by the addition of 0.33 vol. of propan-2-ol. The fibrous precipitate was washed successively with 75 % (v/v) ethanol containing NaCl (1 %), 75 % (v/v) ethanol and ethanol, and then dried. The yield was 50 mg.; the average $S_{20,w}$ was 20.6s; the base composition was G:A:C:T, 25.8:24.6:24.3:25.3. DNA can be prepared from *E. coli* by extraction with 5 % 4-aminosalicylate-1 %

Fig. 1. Sucrose-density-gradient centrifugation of ribosomal RNA, from *E. coli*, in 0.15M-NaCl-0.015M-trisodium citrate, pH 7.0, for 16 hr. at 24000 rev./min. in a Spinco model L centrifuge.

sodium dodecyl sulphate–90% phenol (unpublished work; see Lawley & Brookes, 1963) but it is necessary to remove RNA by degradation with ribonuclease. The present method gives a complete separation of DNA and ribosomal RNA, and presumably could be applied to other bacteria susceptible to lysis by dodecyl sulphate. The nature of the DNA that was not rendered insoluble by phenolphthalein diphosphate but was removed from ribosomal RNA by extraction with 3M-sodium acetate has not yet been investigated.

The author thanks Dr Myrna Guest for the *E. coli*, Mr P. A. Edwards for determining the sedimentation

distribution of DNA and Mr E. A. Cook for technical assistance. This investigation was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by Research Grant no. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service.

Kirby, K. S. (1961). *Biochim. biophys. Acta*, **47**, 18.

Lawley, P. D. & Brookes, P. (1963). *Biochem. J.* **89**, 127.

Midgeley, J. E. M. (1962). *Biochim. biophys. Acta*, **61**, 513.

Biochem. J. (1964), **93**, 6c

Synthesis of [6-methyl-¹⁴C]Megestrol Acetate (17 α -Acetoxy-6[¹⁴C]-Methylpregna-4,6-diene-3,20-dione)

By G. COOLEY and A. E. KELLIE

Chemical Research Laboratory, British Drug Houses Ltd., London, N. 1, and Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W. 1

(Received 17 July 1964)

During recent years, extensive use has been made of steroids of high progestational activity for the control of ovulation and the regulation of menstrual periods. Employed as oral contraceptives, appreciable quantities of such steroids may be ingested by women (during the years of fertility) to inhibit ovulation, and it is clearly desirable to know something of the distribution of these compounds in the tissues and also of their metabolism and excretion. Investigations of this character are greatly facilitated by the introduction of a radioactive atom into the compound studied, and the present paper deals with such an introduction (of a 6-methyl-¹⁴C group) into megestrol acetate [17 α -acetoxy-6-methylpregna-4,6-diene-3,20-dione (III) (cf. Ellis, Kirk, Petrow, Waterhouse & Williamson, 1960)], an ovulation inhibitor in current use.

A route to megestrol acetate from 17 α -acetoxy-3-methoxypregna-3,5-dien-20-one (I) reported by Burn, Ellis, Feather, Kirk & Petrow (1962), in which a Vilsmeier reagent prepared from dimethylformamide and carbonyl chloride was employed, formed the basis of the synthesis described below (see Scheme 1).

The preparation of dimethyl[¹⁴C]formamide from [¹⁴C]formic acid has been reported by Szmuszkovicz & Thomas (1961), but in our hands this method did not give dimethylformamide of sufficiently good quality for use in the synthesis of megestrol acetate. A satisfactory sample of dimethyl[¹⁴C]formamide was prepared from ethyl [¹⁴C]formate as described below.

Preparation of dimethyl[¹⁴C]formamide. Ethyl [¹⁴C]formate (0.367 ml., 4.66 m-moles) was transferred by Pasteur pipette to a pear-shaped 3-

Scheme 1. Synthesis of [6-methyl-¹⁴C]megestrol acetate (III) from 17 α -acetoxy-3-methoxypregna-3,5-dien-20-one (I) and dimethyl[¹⁴C]formamide via 17 α -acetoxy-6[¹⁴C]-methylenepregn-4-ene-3,20-dione (II).

socket flask (25 ml.) and washed in with a further 0.4 ml. of unlabelled ethyl formate. The flask was fitted with a water condenser, and dimethylamine was led into the flask through a capillary tube that dipped below the surface of the ethyl formate. Excess of dimethylamine from the third socket was trapped in conc. HCl. During the initial stages of the passing of dimethylamine through the mixture, a mild increase in temperature (to 35–37°) occurred and the passage of the amine was continued until the temperature had returned to normal (approx. 1 hr.). During this phase of the preparation, the volume of the contents of the flask increased to about 4 ml. Benzene (3 ml.) was added and the pear-shaped flask arranged for distillation. A paraffin bath was adjusted so that the oil level remained below that of the liquid in the distilling flask, and the contents were slowly distilled until the benzene and the dimethylamine (in solution) had been removed (the temperature at the still-head rose to 80° and then began to fall; residual volume approx. 1 ml.). Ethylene dichloride (4 ml.) was added and the distillation resumed until the temperature, which rose to 80°, had begun to subside (residual vol. approx. 0.8 ml.).

Preparation of 17 α -acetoxy-6[¹⁴C]-methylenepregn-4-ene-3,20-dione (II). The dimethylformamide (approx. 0.8 ml.) was transferred quantitatively by Pasteur pipette to a round-bottomed flask (25 ml.) with ethylene dichloride (5.9 ml.). The solution was chilled in an ice bath and stirred magnetically while a solution of 10 % (w/v) carbonyl chloride in redistilled ethylene dichloride (5.03 ml.) was added dropwise during 10 min. A white gelatinous precipitate formed at this stage. After 15 min. a solution of 17 α -acetoxy-3-methoxypregna-3,5-dien-20-one (I) (1.5 g.) in redistilled ethylene dichloride (4.4 ml.) containing 1 drop of pyridine was added, and the mixture, while chilled, was stirred for 2 hr. The ice bath was then removed and the stirring continued for 1 hr.

A solution of K₂CO₃ (2.95 ml.; 5.5 g. in 20 ml. of water) was added and the mixture stirred for 10 min. The contents of the flask were transferred to a separating funnel (25 ml.) and the ethylene dichloride layer was returned to the flask. Sodium borohydride solution was prepared by adding 500 mg. to a solution of 1 ml. of 10N-NaOH in chilled methanol (40 ml.). Of this solution 5.8 ml. was added dropwise to the ethylene dichloride layer, and the mixture was stirred for 1 hr. Sulphuric acid (0.59 ml. of 18N-H₂SO₄) was added, followed 15 min. later by sodium acetate solution [2.95 ml. of 50 % (w/v) sodium acetate trihydrate]. This mixture was transferred to a round-bottomed flask (100 ml.) and distilled *in vacuo* until no

organic solvent remained, the level of liquid being maintained constant by the slow addition of water during distillation. After cooling, the water was removed by fine Pasteur pipette and the contents of the flask were washed three times with hot water. The final product was recrystallized, after treatment with charcoal, from methylene chloride-methanol mixture. The first yield (972 mg.; m.p. 210–213°; m.p. after recrystallization from methanol 235–237°) gave an infrared-absorption spectrum (1800–800 cm.⁻¹) identical with that of a reference standard of the unlabelled 6-methylene compound (m.p. 238–244°); $\nu_{\text{max}}^{\text{CS}_2}$ 1738, 1718, 1673, 1247 and 906 cm.⁻¹. Both radioactive and unlabelled compounds had λ_{max} 260 m μ , and had identical mobilities (R_f 0.70) on paper chromatograms in the system light petroleum (b.p. 80–100°)-methanol-water (5:4:1, by vol.). A further 179.4 mg. (m.p. 208–210°) was obtained from the mother liquor.

Preparation of 17 α -acetoxy-6[¹⁴C]-methylpregna-4,6-diene-3,20-dione ([6-methyl-¹⁴C]megestrol acetate) (III). The 6-methylene compound (II) (972 mg.) dissolved in ethanol was refluxed with sodium acetate (0.495 g.) and 5 % palladium-charcoal (150 mg.) with stirring for 16 hr. The product after filtering was evaporated to dryness under vacuum and the residue was washed three times with hot water. Recrystallization of this material from methanol gave crystals, m.p. 214.5–216.5° (681 mg.), the infrared-absorption spectrum of which proved identical with that of a reference sample of megestrol acetate (m.p. 214–216°); $\nu_{\text{max}}^{\text{CS}_2}$ 1737, 1717, 1664, 1255 and 1246 cm.⁻¹. Standard and radioactive compounds absorbed in the ultraviolet region at λ_{max} 287 m μ (log ϵ – 4.4) and had identical mobilities (R_f 0.66) on paper chromatograms in the system quoted above. A further yield of 90.2 mg. (m.p. 212–214°) was obtained by concentrating the mother liquor.

The first and second crops were separately dissolved in 10 % (v/v) methanol in benzene (1 mg./ml.) and were stored under nitrogen at 4°. The specific activities of the two samples were identical: 1.03 mc/m-mole (2.68 μ C/mg.).

The interest and support of Sir Charles Dodds, Bt., in this work is gratefully acknowledged. A. E. K. is indebted to the Medical Research Council for a grant to purchase the ethyl [¹⁴C]formate used in this work. We thank Mr J. W. Holsman for valuable technical assistance.

Burn, D., Ellis, B., Feather, P., Kirk, D. N. & Petrow, V. (1962). *Chem. & Ind.* p. 1907.

Ellis, B., Kirk, D. N., Petrow, V., Waterhouse, B. & Williamson, D. M. (1960). *J. chem. Soc.* p. 2828.

Szmuszkovicz, J. & Thomas, R. C. (1961). *J. org. Chem.* **26**, 960.

Synthesis of [1,2-³H₂]Medroxyprogesterone Acetate (17 α -Acetoxy-6 α -methyl[1,2-³H₂]pregn-4-ene-3,20-dione) and [1,2-³H₂]Megestrol Acetate (17 α -Acetoxy-6-methyl[1,2-³H₂]pregna-4,6-diene-3,20-dione)

By G. COOLEY and A. E. KELLIE

Chemical Research Laboratory, British Drug Houses Ltd., London, N. 1, and Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W. 1

(Received 17 July 1964)

The technique of double isotope dilution has been successfully applied for the determination of steroid-hormone concentrations in peripheral plasma (Hudson, Coghlan, Dulmanis, Wintour & Ekkel, 1963; Riandel *et al.* 1963; Woolever & Goldfien, 1963). One advantage of the method of Riandel *et al.* (1963), which depends on the formation of [³⁵S]thiosemicarbazones, is that it permits the use of [1,2-³H₂]steroids as internal standards. These compounds can be prepared at high specific activity by the procedure of Osinski & Vanderhaege (1960); the [1,2-³H₂]steroids testosterone, progesterone and the corticosteroid hormones are already available (from the U.S. Public Health Service, Bethesda 14, Md., U.S.A.). The progestational compounds, medroxyprogesterone acetate [17 α -acetoxy-6 α -methylpregn-4-ene-3,20-dione (II) (cf. Babcock *et al.* 1958)] and megestrol acetate [17 α -acetoxy-6-methylpregna-4,6-diene-3,20-dione (III) (cf. Ellis, Kirk, Petrow, Waterhouse & Williamson, 1960)] are suitable for determination as [³⁵S]thiosemicarbazones and, unlike progesterone, form only the monothiosemicarbazone at the 3-position. In each case these derivatives can be modified chemically by hydrolysis of the 17 α -acetate group and by the formation of the corresponding 2',4'-diacetylthiosemicarbazone derivatives without loss of the label. [1,2-³H₂]Medroxyprogesterone acetate and [1,2-³H₂]megestrol acetate, necessary for the application of the double isotope derivative procedure, have not so far been reported. The

syntheses of these compounds, at high specific activity, are described below (see Scheme 1).

Preparation of 17 α -acetoxy-6 α -methyl[1,2-³H₂]pregn-4-ene-3,20-dione ([1,2-³H₂]medroxyprogesterone acetate) (II). 17 α -Acetoxy-6 α -methylpregna-1,4-diene-3,20-dione (I) (*Fr. Patent M975, 1962*) (384 mg., 1 m-mole) was reduced at The Radiochemical Centre, Amersham, Bucks., with carrier-free tritium in the presence of 5% palladium-charcoal catalyst. Approx. 15.5 ml. of tritium (40c) was absorbed and the product, which consisted of a mixture of starting material, the required 4-en-3-one (medroxyprogesterone acetate), the 1-en-3-one and the saturated 3-one, was separated by partition chromatography on Celite 535 (80 g.; 60 cm. $\times$ 2 cm. internal diam.) in the system light petroleum (b.p. 80–100°)–methanol–water (5:4:1, by vol.). Identification of the compounds was made by paper chromatography, with the same system, against the corresponding unlabelled standard compounds. Fractions containing medroxyprogesterone acetate were combined and the residue was recrystallized from acetone–light petroleum (b.p. 60–80°). A first yield of 46.2 mg. (m.p. 198–201°; literature, 205–208°; λ_{max} , 238 m μ) was obtained and a further 28.7 mg. (m.p. 196–200°) by concentration of the mother liquor.

The latter sample (28.7 mg.) was diluted with 14.35 mg. of unlabelled medroxyprogesterone acetate and the combined sample was purified by adsorption chromatography on alumina [50 g.;

Scheme 1. Syntheses of [1,2-³H₂]medroxyprogesterone acetate (II) and [1,2-³H₂]megestrol acetate (III) from 17 α -acetoxy-6 α -methylpregna-1,4-diene-3,20-dione (I).

35 cm. $\times$ 2 cm. internal diam.; gradient 3 % (v/v) ethanol in benzene into benzene]. The radioactive peak was located by counting minute samples (0.4 μ g.) from each column fraction, and the homogeneous character of the material was established by infrared-absorption spectroscopy of the fractions in the 1800–800 cm^{-1} region. Fractions containing medroxyprogesterone acetate were combined, evaporated to dryness and dissolved in benzene containing 10 % (v/v) of methanol (500 ml.). A sample from this solution, after recrystallization from acetone–light petroleum (b.p. 60–80°), had m.p. 203–205°. Examined by infrared-absorption spectroscopy, this showed close agreement with data from a pure sample of the unlabelled compound, m.p. 205–208° (mixed m.p. 203–205°), $\nu_{\text{max}}^{\text{CS}_2}$ 1737, 1717, 1673, 1255 and 1246 cm^{-1} . Both radioactive and unlabelled compounds gave λ_{max} 238 $\text{m}\mu$ in the ultraviolet region, and they had identical mobilities (R_f 0.59) in the system light petroleum (b.p. 80–100°)–methanol–water (5:4:1, by vol.). The extinction at 238 $\text{m}\mu$ corresponded to a yield of 35.7 mg. with a specific activity of $95 \pm 3.5 \mu\text{C}/\mu\text{g}$. (35.3–38.0 c/m-mole).

Preparation of 17 α -acetoxy-6-methyl[1,2- $^3\text{H}_2$]-pregna-4,6-diene-3,20-dione ([1,2- $^3\text{H}_2$]megestrol acetate) (III). Medroxyprogesterone acetate (46.2 mg. of the 1,2- $^3\text{H}_2$ -labelled compound and 23.1 mg. of the unlabelled compound) (II) was refluxed with chloranil (69 mg.) and *p*-nitrophenol (7 mg.) in butan-2-ol (1.5 ml.) for 8 hr. The reaction was carried out, with magnetic stirring, in a pear-shaped flask (5 ml.) that just dipped into a paraffin bath maintained at 125–135°. After cooling, the contents were transferred by Pasteur pipette with methylene chloride (2 $\times$ 4 ml.) to a stoppered B14 tube. The solution was washed with 0.35N-KOH (4 $\times$ 3 ml.) and subsequently with water until neutral. The methylene chloride solution was dried and the solvent removed. The yellow–brown residue, dissolved in methanol (3 $\times$ 1 ml.), was transferred to a conical flask (5 ml.) and concentrated to approx. 1.5 ml. On cooling, yellow crystals separated (27 mg.) with m.p. 208–210° and $\nu_{\text{max}}^{\text{CS}_2}$ 1737, 1717, 1659, 1255 and 1244 cm^{-1} (cf. megestrol acetate: m.p. 214–216°; $\nu_{\text{max}}^{\text{CS}_2}$ 1737, 1717, 1664, 1255 and 1243 cm^{-1}). Maximum extinction in the ultraviolet region was at 288 $\text{m}\mu$ and corresponded to a yield of 22.9 mg. of megestrol acetate. Paper chromatography of the product in the system light petroleum (b.p. 80–100°)–methanol–water (5:4:1,

by vol.) showed the presence of an impurity (approx. 5 %) that also absorbed ultraviolet light and that was more polar (R_f 0.50) than megestrol acetate (R_f 0.66).

Preliminary work on the preparation of unlabelled megestrol acetate from medroxyprogesterone acetate by the chloranil reaction showed the presence of a similar impurity that could be separated from the former compound by partition chromatography on Celite 535. For this reason the radioactive megestrol acetate (27 mg.) was further purified on a column of Celite 535 (60 g.; 46 cm. $\times$ 2 cm. internal diam.) in the light petroleum–methanol–water system. Counting of samples (0.5 μ g.) from each column fraction showed the presence of one major radioactive component. The corresponding fractions were combined and evaporated to dryness, and the residue was dissolved in benzene containing 10 % (v/v) of methanol (500 ml.). A sample from this solution recrystallized from methanol had m.p. 212–214° and gave an infrared-absorption spectrum identical with that of a reference sample of megestrol acetate, m.p. 214–216° (mixed m.p. 212–214°); $\nu_{\text{max}}^{\text{CS}_2}$ 1737, 1717, 1664, 1255 and 1243 cm^{-1} . Both radioactive and unlabelled compounds had λ_{max} 287 $\text{m}\mu$, and both had identical mobilities (R_f 0.66) on paper chromatograms; no trace of a second component could be found when 50 μ g. was tested. The extinction at 287 $\text{m}\mu$ corresponded to a yield of 16.8 mg. with a specific activity of $92 \pm 5.5 \mu\text{C}/\mu\text{g}$. (33.3–37.4 c/m-mole). The solution was stored under nitrogen at 4°.

The interest and support of Sir Charles Dodds, Bt., in this work is gratefully acknowledged. A. E. K. is indebted to the Medical Research Council for a grant to cover the cost of the reduction with carrier-free tritium. We thank Mr J. W. Holsman for valuable technical assistance.

- Babcock, J. C., Gutsell, E. S., Herr, M. E., Hogg, J. A., Stucki, J. C., Barnes, L. E. & Dulin, W. E. (1958). *J. Amer. chem. Soc.* **80**, 2904.
 Ellis, B., Kirk, D. N., Petrow, V., Waterhouse, B. & Williamson, D. M. (1960). *J. chem. Soc.* p. 2828.
 Hudson, B. J., Coghlan, A., Dulmanis, M., Wintour, M. & Ekkel, I. (1963). *Aust. J. exp. Biol. med. Sci.* **41**, 235.
 Osinski, P. & Vanderhaege, H. (1960). *Rec. Trav. chim. Pays-Bas*, **79**, 216.
 Riondel, A., Tait, J. F., Gut, M., Tait, S. A. S., Joachim, E. & Little, B. (1963). *J. clin. Endocrin.* **23**, 620.
 Woolever, C. A. & Goldfien, A. (1963). *Int. J. appl. Radiat. Isotopes*, **14**, 163.

The Ability of Actinomycin D to Increase the Clearing-Factor Lipase Activity of Rat Adipose Tissue

By G. R. EAGLE and D. S. ROBINSON

*Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry,
University of Oxford*

(Received 21 July 1964)

Present evidence suggests that the uptake of tri-glyceride fatty acids from the blood by the extra-hepatic tissues of the body is dependent on prior hydrolysis of the triglycerides by the enzyme, clearing-factor lipase, and that the extent of uptake by a particular tissue is determined by the activity of the enzyme in that tissue (see Robinson, 1964). Recent observations on the clearing-factor lipase of rat adipose tissue are consistent with this view. Thus in the fed animal the activity of the enzyme in adipose tissue is high and triglyceride fatty acids are taken up from the blood and stored there, whereas in the starved animal the clearing-factor lipase activity of the tissue falls to a low level and deposition of triglyceride fatty acids from the blood ceases. The activity of the enzyme in the male epididymal fat body, for instance, falls from 25–40 units in fed animals to 4–7 units in animals that have been starved for 48 hr., where 1 unit of activity is equivalent to the release by the enzyme of 1 μ mole of free fatty acid/fat body/hr. from its triglyceride substrate.

The low activity of the enzyme in epididymal fat bodies from starved rats is increased when the bodies are incubated *in vitro* at 37° in a medium containing heparin, glucose, insulin, dialysed serum, amino acids and salts (Salaman, 1964). The increase, which occurs at an initial rate sufficient to re-establish the tissue activities found in the fed animal in 12–18 hr., is abolished if puromycin (0.5 mg./ml.) is present in the incubation medium, a finding compatible with its being the result of the synthesis of new enzyme in the tissue. Since actinomycin D is known to inhibit DNA-dependent RNA synthesis, it was decided to test the effect of this substance on this increase in adipose-tissue enzyme activity. A description of the findings forms the subject of this communication.

Salaman (1964) measured the increase in tissue enzyme activity by two methods. In one, ether-dried powders of tissue and medium combined were prepared after incubation, and the activity of the enzyme in aqueous homogenates of these was compared with that in similar preparations made from the tissue before its incubation. In the second method, the time-course of the rise in enzyme activity was followed by assaying the enzyme in the incubation medium at different

time-intervals. In the presence of heparin, clearing-factor lipase is extracted from the tissue and the amount extracted is proportional to the total tissue content. Both of these procedures were used in the present study.

The results of Expt. 1 (Table 1) show that, under the conditions used by Salaman (1964), the increase in medium enzyme activity reaches a plateau level after 6 hr. If actinomycin D is present, however, enzyme continues to appear in the medium at a linear rate for at least 9 hr. The total activity of tissue and medium combined is also greater at 9 hr. in the presence of actinomycin D than in its absence. Thus, when fat bodies from two starved rats were incubated for 9 hr. with and without actinomycin D, by using the paired-fat-body technique, the mean activities of ether-dried powders prepared from the tissue and medium combined after the incubation were 11.3 units in the absence and 26.9 units in the presence of actinomycin D.

In Salaman's (1964) studies the extent of the increase in clearing-factor lipase activity was markedly decreased when glucose and insulin were omitted from the incubation medium. The results of Expt. 2 (Table 1) show, however, that the increase in enzyme activity produced by actinomycin D is independent of the presence of glucose and insulin.

The addition of puromycin to the medium prevents the increase in activity brought about by actinomycin D as well as that which occurs in the presence of glucose and insulin.

Further evidence that the action of actinomycin D can be separated from that of glucose and insulin was obtained in an experiment with fed animals. The clearing-factor lipase activity of epididymal fat bodies from such animals is not increased further when they are incubated in the medium described by Salaman (1964). When the fat bodies of two fed rats were incubated for 9 hr. in such a medium to which actinomycin D (5 μ g./ml.) had been added, however, the mean clearing-factor lipase activity of ether-dried powders prepared from the tissue and medium combined was 43 units, as compared with a mean initial activity of 25 units for the contralateral fat bodies.

An action of actinomycin D on adipose-tissue clearing-factor lipase activity has also been demon-

Table 1. *Effect of actinomycin D on the clearing-factor lipase activity of epididymal adipose tissue from starved rats*

Expt. 1. Six epididymal fat bodies from six male starved (48 hr.) rats (120 g. body wt.) were incubated at 37° and pH 7.4 in 15 ml. of the medium described by Salaman (1964). This contained glucose (36 mg.), insulin (0.18 unit), heparin (36 units), casein hydrolysate (18 mg. of Bacto-Casamino Acids), NaHCO_3 (22.5 mg.) and 7.2 ml. of serum (from fed male rats) that had been dialysed against 0.85% NaCl soln. for 24 hr. The volume was made up to 15 ml. with a salt soln. so that the medium contained NaCl at physiological concentration (0.85%) and the other salts of serum at approximately half their physiological concentration. The six contralateral fat bodies were incubated in an identical medium except for the addition of actinomycin D (75 $\mu\text{g.}$). The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). Samples (1 ml.) of the media were taken at intervals for the assay of clearing-factor lipase by techniques described by Salaman (1964) and Robinson (1964), the media being replenished on each occasion with fresh medium. Each value in the Table has been corrected for the amount of enzyme removed for the previous assays. Expt. 2. Conditions were as in Expt. 1 except that one fat body from each rat was incubated separately in 2.5 ml. of the medium described and a single assay of the medium was carried out at 9 hr. Glucose, insulin, actinomycin D and puromycin (0.5 mg./ml.) were present or absent as shown. The values given represent, in each case, the mean activities of two experiments.

Time of incubation (hr.)	Expt. 1		Expt. 2			Medium clearing-factor lipase activity at 9 hr. (μ moles of free fatty acids produced/fat body/hr.)
	Medium clearing-factor lipase activity (μ moles of free fatty acids produced/fat body/hr.)		Present in medium			
	Without actinomycin	With actinomycin	Glucose and insulin	Actinomycin	Puromycin	
1	0.8	0.5	—	—	—	1.5
3	4.8	3.3	+	—	—	6.5
5	9.3	8.0	—	+	—	12.3
7	9.9	12.0	+	+	—	16.3
9	9.4	15.6	+	—	+	1.8
			—	+	+	0.9

strated *in vivo*. Thus, when 200 $\mu\text{g.}$ of actinomycin D was injected intraperitoneally into each of four starved male rats (100 g. body wt.), the mean activity of the enzyme in ether-dried powders prepared from the epididymal fat bodies 12 hr. later was 17.6 units, as compared with a mean activity of 5.8 units in four control starved animals that were injected with 0.85% NaCl solution.

At the concentrations used in this study actinomycin D has been shown to prevent DNA-dependent synthesis of RNA in mammalian cells (Reich, Franklin, Shatkin & Tatum, 1962; Franklin, 1963). Evidence that inhibition of RNA synthesis was occurring in rat adipose tissue under the conditions of the present experiments was obtained by including 2 μC of [8- ^{14}C]adenine sulphate (10 mc/-m-mole) (The Radiochemical Centre, Amersham, Bucks.) in each of the incubation media in an experiment similar to that of Expt. 1 (Table 1). Ether-dried powders were prepared from the fat bodies and medium combined after incubation for 6 hr. and were washed exhaustively with 5% (w/v) trichloroacetic acid solution at 2°. After the trichloroacetic acid had been removed and the powders had been dried with acetone and ether, the RNA was hydrolysed in 0.3N-KOH at 37° for 16 hr. Protein and DNA were precipitated by acidification to pH 1.5 with perchloric acid at 0° and removed by centrifugation

before the radioactivity in the supernatant solution, which contained the soluble RNA nucleotides, was measured. When actinomycin D was included in this incubation medium the incorporation of adenine was inhibited by 90%.

These results show that actinomycin D, at concentrations that inhibit DNA-dependent RNA synthesis, causes a marked and sustained rise in the activity of the enzyme, clearing-factor lipase, in rat epididymal fat bodies, both *in vivo* and *in vitro* and with tissues from both starved and fed animals. If, as the puromycin findings suggest, this increase in activity is due to the synthesis of new enzyme in the tissue, the observations are most readily explained if synthesis of clearing-factor lipase occurs on a stable RNA template and its destruction is mediated through the action of an unstable actinomycin-sensitive form of RNA.

Gifts of actinomycin D from Merck, Sharp and Dohme, Rahway, N.J., U.S.A., and of puromycin from American Cyanamid Co., Pearl River, N.Y., U.S.A., are gratefully acknowledged.

- Franklin, R. M. (1963). *Biochim. biophys. Acta*, **72**, 555.
 Reich, E., Franklin, R. M., Shatkin, A. J. & Tatum, E. L. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1238.
 Robinson, D. S. (1964). *Advanc. Lipid Res.* **1**, 133.
 Salaman, M. R. (1964). D.Phil. Thesis: University of Oxford.