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Biochem. J. (1964), **93**, 487

The Isolation of *S*-Sulphoglutathione from the Small Intestine of the Rat

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(Received 21 February 1964)

Administered inorganic [^{35}S]sulphate is incorporated into sulphated mucopolysaccharides by mammalian intestine *in vivo* (Crevier & Bélanger, 1954; Kent, Whitehouse, Jennings & Florey, 1956). The initial reaction, the activation of sulphate to form adenosine 3'-phosphate 5'-sulphatophosphate (Robbins & Lipmann, 1957), has been shown to occur in mucosal extracts (Kent & Pasternak, 1958; Varandani, Wolf & Johnson, 1960), and adenosine 3'-phosphate 5'-sulphatophosphate itself has been isolated from mouse intestine (Pasternak, 1960). In the course of that isolation it was noted that other ^{35}S compounds of low molecular weight were formed by mammalian intestine and one of these has now been identified as *S*'[^{35}S]-sulphoglutathione. Waley (1959) has previously reported the isolation of *S*-sulphoglutathione from mammalian lens.

MATERIALS AND METHODS

S-Sulphocysteine. The sodium salt of *S*-sulphocysteine was prepared by the procedure outlined by Segel & Johnson (1963). The crystalline material gave an infrared-absorption spectrum similar to that published by those authors; $[\alpha]_D^{25} -93 \pm 1^\circ$ (c, 1.0) in water (Found: C, 15.0; H, 3.65; N, 6.5; S, 21.9; Na, 9.4. Calc. for $\text{C}_3\text{H}_6\text{NO}_2\text{S}_2\text{Na} \cdot \text{H}_2\text{O}$: C, 14.9; H, 3.35; N, 5.8; S, 26.6; Na, 9.5%). The material yielded a single orange-red spot with ninhydrin reagent after paper chromatography in solvents *A*, *B* and *C* (see below).

S-Sulphoglutathione. *S*-Sulphoglutathione was prepared by a modification of the method described by Waley (1959). Oxidized glutathione (350 mg.; Sigma Chemical Co., St Louis 18, Mo., U.S.A.) was shaken at 37° with 10 ml. of 1.0M-sodium sulphite-0.01M-cupric sulphate adjusted to pH 7.0 with nitric acid. After 12 hr. the reaction mixture

had changed from colourless to deep blue and the solution was then applied at 4° to a column (20 cm. $\times$ 1.5 cm. diam.) of Dowex 50 (X4; H^+ form; 200-400 mesh). The column was washed with 20 ml. of water and the total eluate was neutralized with an aqueous suspension of barium hydroxide. The barium sulphate was removed by centrifugation and the barium salt of *S*-sulphoglutathione precipitated with 5 vol. of ethanol. The white precipitate was collected by centrifugation, dissolved in water, filtered through an Oxoid membrane filter (Oxo Ltd., London) and reprecipitated with ethanol five times. The final precipitate (250 mg., 36% yield) was dried under vacuum over anhydrous calcium chloride at room temperature. The barium salt of *S*-sulphoglutathione had $[\alpha]_D^{20} -36 \pm 6^\circ$ (c 0.17) in water (Found: C, 19.8; H, 3.85; N, 7.3; S, 11.95; Ba, 23.4. Calc. for $\text{C}_{10}\text{H}_{15}\text{N}_3\text{O}_6\text{S}_2\text{Ba} \cdot 4\text{H}_2\text{O}$: C, 20.2; H, 3.9; N, 7.1; S, 10.8; Ba, 23.1%). The first sulphur analysis gave a very low value (5.6%). That quoted was obtained by iodine titration of reduced material. It yielded only one ninhydrin-positive spot after paper electrophoresis in 0.1M-sodium acetate buffer, pH 5.6, and after paper chromatography in solvents *A*, *B* and *C* (see below). It was hydrolysed by 1.0N-hydrochloric acid (at 100° for 30 min.) to yield inorganic sulphate. The pure material gave a positive reaction for free thiol with sodium nitroprusside only after acid hydrolysis.

2,4-Dinitrophenyl-*S*-sulphoglutathione. The barium salt of *S*-sulphoglutathione (6 mg.) in 2.0 ml. of aq. 1% (v/v) triethylamine was mixed with a solution containing 5 ml. of ethanol, 0.05 ml. of 1-fluoro-2,4-dinitrobenzene and 0.05 ml. of triethylamine. The mixture was shaken at 37° for 2 hr. and the reaction terminated by the addition of 4.0 ml. of 2.0N-formic acid and 10 ml. of ether. The aqueous phase which separated was extracted with ether and the DNP derivative of *S*-sulphoglutathione so obtained was purified by paper electrophoresis on Whatman 3MM paper in 0.1M-sodium acetate buffer, pH 5.6, followed by preparative paper chromatography on Whatman 3MM paper in solvent *B*. The yellow material was eluted from the paper in a few millilitres of water and stored in the dark at -20° .

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Rats. Albino Wistar rats that had been starved overnight were used. The animals were stunned by a blow on the head and decapitated.

Ion-exchange chromatography. Dowex 1 (X4; 200–400 mesh) and Dowex 50 (X8; 200–400 mesh) (V. A. Howe and Co. Ltd., London) were used in these experiments. Ammonium formate buffers (1.0M and 2.0M with respect to formate) were prepared by dissolving AnalR ammonium formate (British Drug Houses Ltd.; 63 g. and 126 g. respectively) in water to a final volume of 1.0 l. after adjusting the solution to pH 7.0 with AnalR formic acid or AnalR ammonia solution.

Gradient elution was achieved by using a 2 l. mixing vessel (initially containing water) into which the concentrated eluent was fed from a separating funnel. The flow rate through the column was adjusted to 25 ml./hr./cm.² of column area, and 25 ml. fractions were collected with an LKB 3400 Radi-Rac automatic fraction collector (Gallenkamp and Co. Ltd.).

Paper chromatography. Ascending and descending paper chromatography was performed on Whatman paper no. 1 or 3 MM. The following solvents were used: *A*, isobutyric acid–1.0N-ammonia–0.1M-EDTA (125:75:4, by vol.); *B*, butan-1-ol–acetic acid–pyridine–water (15:3:10:12, by vol.); *C*, pyridine–acetic acid–water (7:1:2, by vol.); *D*, 2-methylbutan-2-ol saturated with 0.1M-potassium phthalate buffer, pH 6.0 (Blackburn & Lowther, 1951); *E*, 1.5M-sodium phosphate buffer, pH 6.0 (Levy, 1954).

Paper electrophoresis. Paper electrophoresis was performed at 4° on Whatman 3MM paper strips clamped between glass plates coated with silicone grease. The strips were subjected to 13 v/cm. for 2 hr. in 0.1M-sodium acetate buffer, pH 5.6, or for 6 hr. in acetic acid–formic acid–water (10:1:40, by vol.).

Detection on paper. Ninhydrin (0.2% in acetone) and isatin (0.2% in acetone) were used to detect amino compounds. Radioactive compounds were detected by radioautography by using Kodirex X-ray film (Kodak Ltd., London). Reducing sugars were detected with alkaline silver nitrate reagent (Smith, 1958), and nucleotides were detected by examination under ultraviolet light.

Assay of radioactivity. Radioactivity samples (0.05–0.5 ml. containing 50–1000 counts/min.) were plated on aluminium planchets (4.5 cm.²) and dried at 105° for 45 min. to yield almost infinitely thin specimens (<1 mg./cm.²). The radioactivity was measured in a lead castle (background about 9 counts/min.) with a G.E.C. Geiger-Müller end-window tube and registered on a type D657 scaling unit (Panax Equipment Ltd., Redhill, Surrey). This apparatus gave a counting efficiency of approx. 5% (about 10⁶ counts/min./mc).

Chemical analyses. Inorganic sulphate was measured by the barium chloroanilate method of Lloyd (1959). Ester sulphate was measured by the same method after acid hydrolysis in 5.7N-hydrochloric acid at 110° for 20 hr. Free α -amino acid grouping was determined with ninhydrin (Moore & Stein, 1954), with tyrosine as standard. Protein was estimated by the biuret reaction (Layne, 1957), with crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne) as a standard.

Amino acid analyses were carried out with an automatic amino acid analyser (Evans Electroelenium Ltd., Essex), kindly operated by Dr D. B. Hope. The sample (approx. 2 mg.) was hydrolysed in 2.0 ml. of constant-boiling hydro-

chloric acid (5.7N; three times glass-distilled) in a sealed glass tube at 110° for 17 hr. in the absence of air (0.05 mm. Hg).

Thiol and disulphide groups were detected by titration with 4.8 mM-phenylmercuric hydroxide. The titration was performed at 37° under nitrogen in 3.5 ml. of 0.05M-sodium sulphite buffer, pH 7.0, and followed polarographically with a dropping-mercury electrode (–0.45 v) as described by Allison & Cecil (1958).

Infrared-absorption spectra were determined in a Perkin-Elmer Infracord split-beam recording spectrophotometer. Samples were examined as mulls in Nujol (liquid paraffin) or in the form of a pellet in potassium bromide. All the samples were dried at room temperature over anhydrous calcium chloride.

Optical rotation was measured in a Hilger Standard polarimeter (Hilger and Watts Ltd., London). A cell 20 cm. in length was used, and all measurements were made in water.

Microanalyses were performed by Weiler and Strauss (Microanalytical Laboratory, Oxford) or by Dr A. Bernhardt (Microanalytisches Laboratorium, Mülheim, Germany).

RESULTS

Fifty-three starved rats were given intraperitoneal injections of carrier-free sodium [³⁵S]sulphate (SJS1; The Radiochemical Centre, Amersham, Bucks.); each animal of body wt. 100–160 g. received 0.1–0.5 mc in 0.5 ml. of water. Then 2 hr. after the injection the animals were killed and the small intestines removed and chilled in ice. The intestines were dried on filter paper, rinsed clean of faecal matter, weighed and immediately blended in 9 vol. of ice-cold 5% (w/v) trichloroacetic acid. The protein precipitate was removed by centrifugation and the trichloroacetic acid was removed from the supernatant solution by ether extraction at 0°. The aqueous extract was neutralized with conc. ammonia solution, filtered through HyFlo Super-Cel (L. Light and Co. Ltd.) and combined with a similar extract prepared from the small intestines of 351 rats that had not been injected with [³⁵S]sulphate. The method of preparation of the extract is outlined in Scheme 1. The total extract was diluted to 30 l. with water and applied to two columns (30 cm. × 3.5 cm. diam.) of Dowex 1 (formate form). Each column was washed with 500 ml. of water and eluted with a concentration gradient of ammonium formate. Two separate peaks of radioactivity were eluted from each column: a large inorganic sulphate peak and a second peak containing a radioactive sulphate ester (Fig. 1).

The fractions containing the radioactive sulphate ester were combined and adjusted to pH 4.0 with conc. formic acid. The solution was then applied to a column (20 cm. × 1.5 cm. diam.) of acid-washed Nuchar C charcoal (West Virginia Pulp and Paper Co., New York, U.S.A.) at 4°. The ammonium formate was washed through with 500 ml. of water

Scheme 1. Isolation and purification of the sulphate ester.

Fig. 1. Ion-exchange chromatography of the aqueous extract of rat small intestine on a column (30 cm. $\times$ 3.5 cm. diam.) of Dowex 1 (X4; formate form). The column was eluted with a gradient of ammonium formate buffer; the mixing vessel contained 2 l. of water to which 2 l. of 1.0M buffer, followed by 2 l. of 2.0M buffer, were slowly added. The first peak of radioactivity was inorganic sulphate and the second was S-sulphogluthathione. The start of the 2M-ammonium formate buffer is indicated by the arrow.

and the radioactive sulphate ester was then eluted with 500 ml. of 0.2N-ammonia in aq. 50% (v/v) ethanol. The ammonia and the ethanol were removed by rotary evaporation at 20°. The volume was reduced to 50 ml. and the solution was mixed with 50 ml. of 2.0M-barium perchlorate, pH 7.0, at 0°. Nucleotides that were precipitated were removed by centrifugation, and the barium salt of the sul-

phate ester was precipitated from the supernatant solution with 500 ml. of ethanol. After 15 hr. at -20° , the barium salt was collected by centrifugation and redissolved in 100 ml. of water.

A sample (25 ml.) of the barium salt so obtained was further purified by passage through a column (10 cm. $\times$ 1.5 cm. diam.) of Dowex 50 (H^+ form) followed by two reprecipitations from 80% (v/v) ethanol as the barium salt. The material was then freeze-dried and dried under vacuum over anhydrous calcium chloride; $[\alpha]_D^{20} -26.5 \pm 2.5^\circ$ (c 0.42 in water).

Another sample (10 ml.) of the crude barium salt was purified by preparative paper electrophoresis on Whatman 3MM paper in acetic acid-formic acid-water (10:1:40, by vol.). The radioactivity moved as a single band that was detected on the dry papers by radioautography. The band was eluted from the paper in a few millilitres of water and was subjected to chemical analysis.

Chemical and physical properties of the sulphate ester. The sulphate ester behaved as a single radioactive compound during paper chromatography and paper electrophoresis. It reacted with ninhydrin reagent on paper, producing a purple colour typical of amino compounds, and it reacted with isatin reagent on paper at 110° to produce a pink-blue colour. It failed to give a positive reaction on paper with alkaline silver nitrate and it had no ultraviolet absorption.

Samples of the purified sulphate ester were completely hydrolysed to inorganic sulphate by 1.0N-

hydrochloric acid at 100° for 60 min. and by 0.4N-barium hydroxide at 125° for 12 hr. Acid hydrolysis in 0.01N-hydrochloric acid at 120° for 30 min. caused very little breakdown to inorganic sulphate.

Table 1 shows the results obtained by chemical analysis of the purified sulphate ester. It contained only small quantities of reducing sugar, amino sugar, sialic acid, ester phosphate, nucleotide base, disulphide bonding or free thiol groups. It gave a positive reaction with the ninhydrin reagent of Moore & Stein (1954), and colorimetric determination indicated that for each ester sulphate moiety there was one free amino group. It gave a positive biuret reaction and quantitative estimation of the peptide linkage gave an equivalent weight of about 410. The weight of the free acid gave a value of approx. 600 in terms of its sulphate content.

It was apparent that the sulphate ester was a small peptide, and quantitative amino acid analysis of the purified material indicated the presence of three amino acids: glycine, glutamic acid and cyst(e)ine. Acid hydrolysis of the compound in 5.7N-hydrochloric acid in a sealed tube containing air (overnight at 110°) yielded glycine, glutamic acid and cysteic acid. The cysteic acid was identified by paper chromatography and paper electrophoresis and was found to be free of radioactivity.

N-Terminal amino acid. The DNP derivative of the sulphate ester was prepared as described in the Materials and Methods section. After paper chromatography in solvent *B* a sample of the DNP derivative (0.5 μ mole) was hydrolysed in 5.7N-hydrochloric acid at 100° for 15 hr., and the hydrochloric acid was removed over anhydrous calcium chloride in a vacuum desiccator. The residue was dissolved in 0.5 ml. of water and samples of the solution were subjected to descending paper chromatography on Whatman 3MM paper in solvents *D* and *E*. The DNP derivative of the *N*-terminal amino acid was identified as DNP-glutamic acid by

comparison with synthetic DNP-glutamic acid (Fraenkel-Conrat, Harris & Levy, 1955).

C-Terminal amino acid. A sample of the purified sulphate ester (8 μ moles) was incubated for 24 hr. at 37° with 0.5 mg. of crystalline carboxypeptidase (Sigma Chemical Co.), 40 μ moles of tris-hydrochloric acid buffer, pH 7.5, and 0.05 ml. of toluene in a final volume of 1.0 ml. The reaction was terminated by heating the mixture for 1 min. in a boiling-water bath and the protein was removed by centrifugation. The supernatant solution was analysed by paper chromatography in solvents *B* and *C* (Fig. 2). Glycine was identified as the *C*-terminal amino acid and was the only ninhydrin-positive compound formed when the DNP derivative of the sulphate ester was incubated with carboxypeptidase in the same way.

Peptidase digestion. A sample of the sulphate ester (8 μ moles) was incubated for 24 hr. at 37° with 20 mg. of a crude peptidase preparation (L. Light and Co. Ltd.), 40 μ moles of tris-hydrochloric acid buffer, pH 7.5, 5 μ moles of magnesium chloride, 2 μ moles of manganese chloride and 0.05 ml. of toluene in a final volume of 1.0 ml. (Smith, 1951). The reaction was terminated by heating the mixture for 2 min. in a boiling-water bath and the protein was removed by centrifugation. The supernatant solution was analysed by paper chromatography in solvents *B* and *C* and the products of the reaction were detected by radioautography and with ninhydrin reagent. The radioactive product of this reaction was indistinguishable from *S*-sulphocysteine by paper chromatography in the two solvents employed. The same product was obtained when the sulphate ester was exhaustively digested with carboxypeptidase and then digested with the peptidase (Fig. 2).

Comparison with S-sulphogluthathione. The sulphate ester was subjected to co-chromatography with authentic *S*-sulphogluthathione in solvents *A*, *B* and *C*. In each system the two compounds moved to the same position and the radioactivity of the sulphate ester coincided exactly with the ninhydrin colour produced by a large excess of the synthetic material.

The DNP derivative of the sulphate ester could not be separated from the DNP derivative of *S*-sulphogluthathione by paper chromatography in solvent *B* or by paper electrophoresis. The radioactivity of the DNP derivative of the ester coincided exactly with the yellow colour produced by an excess of the synthetic DNP-*S*-sulphogluthathione.

The infrared-absorption spectrum of the purified barium salt of the sulphate ester was similar to that of authentic barium *S*-sulphogluthathione with characteristic absorption bands at 1650, 1580, 1410, 1310, 1225 and 1030 cm.⁻¹.

Table 1. *Chemical analysis of the radioactive sulphate ester*

The analytical results are given as equivalent to 1.00 μ -mole of ester sulphate (27 000 counts/min. of ³⁵S).

1.06 μ moles of free α -amino acid grouping (ninhydrin)
0.82 μ mole of DNP-peptide (ultraviolet absorption)
0.41 mg. of peptide (biuret reaction)
0.74 μ mole of glutamic acid (amino acid analysis)
0.84 μ mole of glycine (amino acid analysis)
0.31 μ mole of $\frac{1}{2}$ cystine (amino acid analysis)
0.38 μ mole of ammonia (amino acid analysis)
< 0.4 μ mole of reducing sugar (Park & Johnson, 1949)
< 0.23 μ mole of amino sugar (Levy & McAllan, 1959)
< 0.01 μ mole of sialic acid (Aminoff, 1961)
< 0.02 μ mole of free thiol group
< 0.02 μ mole of disulphide bond
< 0.07 μ mole of ester phosphate (Taussky & Shorr, 1953)
< 0.27 μ mole of nucleotide base (ultraviolet absorption)

Fig. 2. Paper chromatography (Whatman 3MM paper; solvent C) of the products of enzymic digestion of *S*-sulphogluthathione from rat small intestine. The solvent front had run off the end of the paper. White spots represent ninhydrin-positive spots and black spots represent radioactive spots. The ninhydrin-positive bands in (4) and (5) are not shown because they were masked by contaminating material from the enzyme preparation. (1) Glutamic acid marker; (2) *S*-sulphocysteine marker; (3) *S*-sulphogluthathione from rat small intestine; (4) *S*-sulphogluthathione from rat small intestine after carboxypeptidase digestion and subsequent peptidase digestion; (5) *S*-sulphogluthathione from rat small intestine after peptidase digestion; (6) *S*-sulphogluthathione from rat small intestine after carboxypeptidase digestion; (7) glycine marker.

DISCUSSION

The present results establish the identity of the radioactive compound as *S*-sulphogluthathione. A very low yield of cystine (Table 1) was obtained after amino acid analysis, but an equally low recovery was obtained when an authentic sample of *S*-sulphogluthathione was hydrolysed in the same way. The formation of ammonia, however, was unique to the material isolated from rat intestine

and it is probable that the ammonia was present as a contaminant despite precautions taken to remove it.

The only radioactive product after hydrolysis in concentrated acid was inorganic sulphate; the cysteine sulphur atom was not radioactive since the cysteine acid was free of radioactivity. This evidence suggests that *S*-sulphogluthathione may be formed *in vivo* by the addition of a sulphate or sulphite moiety to a preformed cysteinyl or cystinyl residue respectively.

Two hours after injection of radioactive sulphate, at least 14 % of the radioactivity extracted from the small intestine was in the form of *S*-sulphogluthathione. The concentration of inorganic sulphate in the intestine was 1.2–3.3 $\mu\text{moles/g. wet wt. of tissue}$. The concentration of *S*-sulphogluthathione was 0.16 $\mu\text{mole/g.}$, representing about 10 % of the total sulphate and about 4 % of the total glutathione (4.0 $\mu\text{moles/g.}$; Leaf & Neuberger, 1947) within the tissue.

The incorporation of radioactive inorganic sulphate into *S*-sulphogluthathione and the presence of this compound within the intestine indicate that mammalian tissues can in effect reduce inorganic sulphate to inorganic sulphite. This is a consequence of reaction (1), which proceeds readily and non-enzymically at pH 7, giving rise to an equilibrium mixture of the reactants. Radioactive sulphite

formed in this way could then react with protein disulphide bonds and give rise to labelled *S*-sulpho-proteins, and this type of reaction may play a part in the observed uptake of ^{35}S from inorganic [^{35}S]sulphate into the serum proteins of the rat (Dziewiatkowski & Di Ferrante, 1957).

SUMMARY

1. Extracts of rat small intestine were prepared in 5 % trichloroacetic acid 2 hr. after the intraperitoneal injection of sodium [^{35}S]sulphate.

2. Ion-exchange chromatography of these extracts yielded a radioactive sulphuric acid ester that has been identified as *S*-sulphogluthathione.

3. The concentration of *S*-sulphogluthathione in the small intestine is approx. 0.16 $\mu\text{mole/g. wet wt. of tissue}$.

We are indebted to Mr P. M. Bayley for help with the thiol determinations and to the Rockefeller Foundation and the National Institutes of Health, U.S. Public Health Service (Grant no. A-3369) for financial support. H. C. R. thanks the University of Western Australia for a Hackett Studentship and the Commonwealth Scientific and Industrial Research Organization for an Overseas Postgraduate Studentship.

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Studies on Essential Fatty Acid Deficiency

EFFECT OF THE DEFICIENCY ON THE LIPIDS IN VARIOUS RAT TISSUES AND THE INFLUENCE OF DIETARY SUPPLEMENTATION WITH ESSENTIAL FATTY ACIDS ON DEFICIENT RATS

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(Received 19 March 1964)

The composition of the lipids present in a variety of animal tissues has been the subject of numerous reports (Rieckehoff, Holman & Burr, 1949; Gray & Macfarlane, 1958, 1961; Macfarlane, 1961; Getz, Bartley, Stirpe, Notton & Renshaw, 1961; Collins, 1963). The pattern of distribution between the cellular compartments has also been investigated for rat liver (Klein & Johnson, 1954; Macfarlane, Gray & Wheeldon, 1960; Getz & Bartley, 1961; Getz, Bartley, Stirpe, Notton & Renshaw, 1962). Though certain differences exist in the proportions of the principal lipid fractions and of the constituent fatty acids present in different organs and species, a broad similarity of pattern can be recognized. In the tissue phospholipids the incorporation of certain polyunsaturated fatty acids of the linoleic acid series appears to be of special importance for their functional role.

The effect of dietary deficiency of essential fatty acids on the composition of the mixed lipids of a variety of rat tissues was studied by Rieckehoff *et al.* (1949) and by Widmer & Holman (1950). These workers used the alkaline-isomerization technique

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and spectrophotometric characterization of fatty acids used by Holman & Burr (1948). Supplementation of the diet with corn oil or linoleate produced an increase in the dienoic acid and polyenoic acid content of the lipids of heart, liver and kidney when compared with that of deficient animals, together with a decrease of their trienoic acid content. On the other hand, the inclusion of corn oil or linoleate produced little or no effect in the polyenoic acid content of brain, skeletal muscle, skin or depot fat. Supplementation with stearate or oleate was ineffective in raising the tissue content of polyenoic acids. More recently, Mohrhauer & Holman (1963*a, b*), using gas-liquid chromatography, have confirmed these findings. After the administration of graded doses of purified linoleate, linolenate or arachidonate to rats on a diet deficient in essential fatty acids, they recorded a detailed pattern of the constituent fatty acids in the mixed lipids of liver, depot fat and erythrocytes. The present paper extends the investigation of the effect of dietary deficiency of essential fatty acids by fractionating the lipids of a number of tissues into neutral lipids, 'cardiolipin' and other phosphatides, and examining by gas-liquid chromatography the changes in