Elvidge, J. A., Linstead, R. P., Orkin, B. A., Sims, P., Baer, H. & Pattison, D. B. (1950). *J. chem. Soc.* p. 2228.
Evans, W. C., Smith, B. S. W., Linstead, R. P. & Elvidge, J. A. (1951). *Nature, Lond.*, **168**, 772.
Gornall, A. G., Bardawill, C. H. & David, M. A. (1949). *J. biol. Chem.* **177**, 751.
Gross, S. R., Gafford, R. D. & Tatum, E. L. (1956). *J. biol. Chem.* **219**. 781.
Happold, F. C. & Key, A. (1932). *J. Hyg., Camb.*, **32**, 573.
Hestrin, S. (1949). *J. biol. Chem.* **180**, 249.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Katagiri, M. & Hayaishi, O. (1957). *J. biol. Chem.* **226**, 439.
Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc.* B, **124**, 397.
Kilby, B. A. (1951). *Biochem. J.* **49**, 671.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McDonald, D. L., Stanier, R. Y. & Ingraham, J. L. (1954). *J. biol. Chem.* **210**, 809.
Mason, H. S. (1957). *Advanc. Enzymol.* **19**, 79.
Ottey, L. & Tatum, E. L. (1957). *J. biol. Chem.* **229**, 77.
Ribbons, D. W. & Evans, W. C. (1959). *Biochem. J.* **73**, 21P.
Ribbons, D. W. & Evans, W. C. (1960). *Biochem. J.* **76**, 310.
Ryan, F. J., Beadle, G. W. & Tatum, E. L. (1943). *Amer. J. Bot.* **30**, 784.
Sistrom, W. R. & Stanier, R. Y. (1953). *J. Bact.* **66**, 404.
Sistrom, W. R. & Stanier, R. Y. (1954). *J. biol. Chem.* **210**, 821.
Sleeper, B. P. & Stanier, R. Y. (1950). *J. Bact.* **59**, 117.
Stanier, R. Y. (1948). *J. Bact.* **55**, 477.
Stanier, R. Y. (1950). *J. Bact.* **59**, 527.
Stanier, R. Y. & Ingraham, J. R. (1954). *J. biol. Chem.* **210**, 799.
Stanier, R. Y., Sleeper, B. P., Tsuchida, M. & McDonald, D. L. (1950). *J. Bact.* **59**, 137.
Tatum, E. L. & Gross, S. (1956). *J. biol. Chem.* **219**, 797.
Trippett, S., Stopher, D. A. & Dagley, S. (1960). *Biochem. J.* **76**, 9P.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. Minneapolis: Burgess Publishing Co.

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The Metabolism of Protocatechuic Acid by Certain Micro-organisms

A REASSESSMENT OF THE EVIDENCE FOR THE PARTICIPATION OF 2:6-DIOXA-3:7-DIOXO*BICYCLO*[3:3:0]OCTANE AS AN INTERMEDIATE

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A variety of microbial species are capable of the aerobic metabolism of protocatechuic acid through the β-oxoadipate pathway. Gross, Gafford & Tatum (1956), using [2:6-$^{14}C_2$]protocatechuic acid, showed that the carbonyl-C of β-oxoadipate produced by the *Neurospora crassa* enzyme system originated solely from C-6, whereas with certain *Pseudomonas* enzymes (strain, A3.12, Stanier) it was randomly derived from C-1 and C-6 of protocatechuic acid. The latter pathway clearly diverges from the former route after the *cis–cis*-β-carboxymuconic acid stage. Table 1 summarizes results showing the ability of cell-free systems of various micro-organisms, induced to protocatechuate, to form β-oxoadipate from likely precursors.

Elsden & Peel (1958) pointed out that the isotopic results of Gross *et al.* (1956) with the *Pseudomonas* system could be explained if an intermediate possessing a symmetrical structure was formed before β-oxoadipate, and suggested this might be 2:6-dioxa-3:7-dioxo*bicyclo*[3:3:0]octane (I). This dilactone was isolated by Landa & Eliasek (1956) from *Oospora* (*Geotrichum*?) cultures growing with catechol as carbon source, and had previously been synthesized by Elvidge *et al.* (1950). Ribbons & Evans (1959) repeated this synthesis, and found that both the synthetic dilactone and the compound isolated from the culture (kindly provided by Professor Landa) were converted into β-oxoadipate by crude extracts of a phthalic acid-oxidizing *Pseudomonas* sp., which also converted protocatechuate into the same product. The rate of production of β-oxoadipate from the dilactone under the conditions of this experiment, with extracts of cells grown with phthalate, was as rapid as that from protocatechuate, β-carboxymuconate or muconolactone; only the dilactone and muconolactone were rapidly attacked if the cells were grown with glucose as carbon source. This evidence appeared to support the contention that the dilactone or its open-chain counterpart, βγ-

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dihydroxyadipate, is the symmetrical intermediate preceding β-oxoadipate in the *Pseudomonas* pathway.

EXPERIMENTAL

Organisms and growth techniques. *Neurospora crassa* (SY4a) was a subculture of the original strain used by Gross *et al.* (1956) and was grown on the minimal medium described by them. Vanillic acid (0·02%, w/v) was added as an inducer of the enzymes for degrading protocatechuic acid. The phthalate-utilizing *Pseudomonas* was isolated and grown with phthalic acid as carbon source (Ribbons & Evans, 1960). *Vibrio* O1 (N.C.I.B. 8250) was grown with *p*-hydroxybenzoic acid as described by Cain (1961). *Nocardia erythropolis* was grown with *p*-nitrobenzoic acid as sole carbon and nitrogen sources (Cain, 1958). Each of these substrates induced enzymes capable of degrading protocatechuic acid in the respective organisms. *Pseudomonas fluorescens* (A3.12, Stanier) was grown as described by Sleeper, Tsuchida & Stanier (1950).

Cell-free extracts. Extracts of *Neurospora crassa* were made by grinding the mycelium with powdered glass (Cain, 1961). The preparation of extracts of the other micro-organisms has been previously described (Ribbons & Evans, 1960; Cain, 1961; Cain & Cartwright, 1960).

Chemicals. β-Carboxymuconic acid was synthesized biologically from protocatechuic acid, by using the purified protocatechuic acid oxidase from *Nocardia* (Cain & Cartwright, 1960), or the dialysed enzyme preparation from *Pseudomonas* (phthalate-utilizer) (Ribbons & Evans, 1960). β-Carboxymuconolactone was prepared as described by Gross *et al.* (1956). (+)-Muconolactone was isolated from incubation mixtures of protocatechuic acid and heat-treated *Vibrio* extracts (Cain, 1961). *cis–cis*-Muconic acid, (±)-muconolactone and the dilactone were synthesized according to the methods of Elvidge *et al.* (1950).

βγ-Dihydroxyadipic acid was synthesized by two different routes: (*a*) Ketipic ester (diethyl βγ-dioxoadipate), m.p. 76°, was prepared by the method of Wislicenus (1887) (cf. Franzen & Schmitt, 1925). The ester was reduced with aluminium amalgam and saponified with barium hydroxide, and the barium salt of βγ-dihydroxyadipic acid precipitated with ethanol according to the procedure of Pankoke (1925). (*b*) Δ^2-Dihydromuconic acid (m.p. 195°) was obtained from mucic acid by the method of Ruhemann & Elliot (1890). Bromination of this, followed by hydrolysis of the product with barium hydroxide afforded the barium salt of βγ-dihydroxyadipate, isolated by precipitation with ethanol as described by Limpricht (1873).

A third route to βγ-dihydroxyadipic acid is that of Przybytek (1884) starting from erythritol via the dichlorohydrin, the oxide and the cyanhydrin, followed by hydrolysis. In our hands the yields obtained were unsatisfactory, and since Pankoke (1925) asserts that his product is similar to that of Przybytek, the latter's synthesis was not persevered with.

Estimations. β-Oxoadipate and other estimations were performed as described previously (Cain, 1961).

The rate of hydrolysis of the dilactone was estimated by two different methods, thus: (i) by following with a Unicam SP. 500 spectrophotometer the increase in *E* at 220 mμ due to the formation of the stronger-absorbing (±)-muconolactone; or (ii) by following the formation of the liberated carboxyl group at increasing pH values, by titrating it with 0·1 N-NaOH in an Automatic Titrator (pH-Stat) (Radiometer Ltd., Copenhagen). The Automatic Titrator consisted of an electrometric titration assembly, an automatic titrator and a recording unit. The reaction cuvette was maintained at 30° by circulating water from a constant-temperature bath with a Circotherm unit (Braun, Melsungen, W. Germany) controlled by a contact thermometer.

RESULTS AND DISCUSSION

Extracts of *Vibrio* O1 and *Nocardia erythropolis*, adapted to protocatechuate, attacked the dilactone over a 2 hr. incubation period (Table 1), but the rate of attack on the dilactone by these extracts

Table 1. *Ability of cell-free systems of various micro-organisms, induced to protocatechuate, to produce β-oxoadipate from suspected precursors*

Reaction mixtures of each system in Warburg flasks contained: phosphate buffer, pH 7·0, 100 μmoles; cell-free extract as indicated (1·0 ml. = 3 mg. of protein); substrates, 5 μmoles, in side arm, as indicated; water to a final volume of 3·0 ml. Reactions were initiated by addition of substrate, and, after incubation for 2 hr. at 30°, a sample (2 ml.) from each flask was pipetted into new Warburg flasks and brought to pH 4·0 with 1 N-acetic acid. Decarboxylation of β-oxoadipate was initiated by addition of 0·1 M-4-aminoantipyrine (0·4 ml.). Control flasks contained reaction mixtures without substrate. The values recorded, which are expressed as μmoles of β-oxoadipate formed, are corrected for the controls. Temp., 30°. ×, Not tested; –, not attacked; +, rapidly attacked. [No figures are given by Gross *et al.* (1956) for *Pseudomonas* A3.12.]

	Extracts derived from				
Substrate	*Neurospora crassa* SY4 *a*	*Pseudomonas* A3.12 Stanier	*Pseudomonas* (phthalate-utilizer)	*Vibrio* O1	*Nocardia erythropolis*
Protocatechuic acid	4·6	+	4·9	4·8	5·0
β-Carboxymuconic acid	4·1	+	4·2	4·3	4·3
β-Carboxymuconolactone	4·9	–	0·4	0·3	0·2
(+)-Muconolactone	0·1	–	×	4·8	4·7
(±)-Muconolactone	0·2	–	2·5	2·4	2·3
cis–cis-Muconic acid	×	–	0·1	0	0·1
Dilactone (synthetic)	0·1	–	2·4	0·3	1·2
Dilactone (isolated from *Oospora*)	0·15	–	2·1	0·3	×

was very much slower than it was on protocatechuate, β-carboxymuconate or muconolactone. Extracts of unadapted cells of *Vibrio* O1 attacked neither muconolactone nor the dilactone (Cain, 1961).

The synthetic dilactone (m.p. 127°) was examined in an attempt to identify the probable lactone-like compound formed by *Nocardia* in the dissimilation of protocatechuate (Cain & Cartwright, 1960). The dilactone and muconolactone were readily distinguished in aqueous solution spectrophotometrically, since the former had no absorption peak down to 200 mμ whereas muconolactone absorbs maximally at 207 mμ (Cain, 1961); both therefore differ from the unknown compound of *Nocardia* ($E_{max.}$ at 215–218 mμ).

In their original description of the dilactone, Elvidge *et al.* (1950) merely stated that on titration at room temperature it consumed 1 equiv. of sodium hydroxide, but at 50° 2 equiv. was rapidly taken up. As no other information was given, we further investigated the hydrolysis properties at different pH values. When the dilactone was added to reaction mixtures buffered at pH 7·0 or higher, in cuvettes, an unexpected rapid increase in *E* at 220 mμ occurred. This change was non-enzymic, both boiled and boiled dialysed extracts giving the same result, which was finally traced to the pH of the solution. Figs. 1 and 2 show the rate of hydrolysis at various pH values measured spectrophotometrically, and by titration of the liberated carboxyl group in a pH-Stat, respectively. The hydrolysis product had the spectrum of, and showed chromatographic behaviour in solvents *C* and *D* (Cain, 1961) identical with that of an authentic sample of muconolactone; the hydrolysis product was also converted by crude extracts of *Vibrio* O1 and *Pseudomonas* (phthalate-utilizing strain) into β-oxoadipate, at a rate equivalent to that of muconolactone. The spontaneous hydrolysis of the dilactone over this pH range thus yields 4-hydroxyhex-2-enedioic 1→4-lactone (muconolactone, II). The dilactone possesses two similar asymmetric carbon atoms, and can thus exist as the (+),(−), *meso* and racemic forms. The synthetic compound (10 μmoles), after overnight hydrolysis at pH 7·8 in Warburg flasks, gave (±)-muconolactone, of which only the (+)-isomer was used by *Nocardia* or *Vibrio* extracts, as measured by the β-oxoadipate

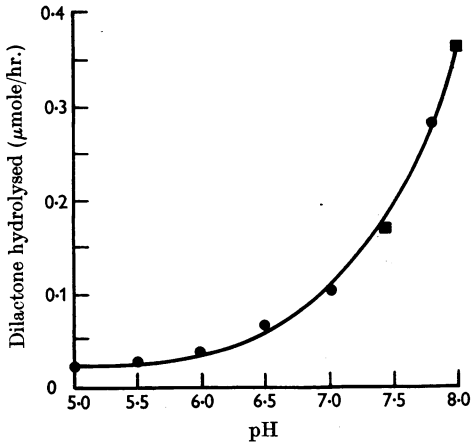


Fig. 1. Hydrolysis of the dilactone at different pH values recorded spectrophotometrically. Phosphate (●) and 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl (■) buffers were used. Hydrolysis rate was measured by extinction increment at 220 mμ [ε (dilactone) = 840; ε (muconolactone) = 4670] calculated per hour from the initial portion of the time–increment curves. Each cuvette contained: buffer, 200 μmoles; dilactone, 0·5 μmole; water to a final volume of 3 ml. Temp., 22°.

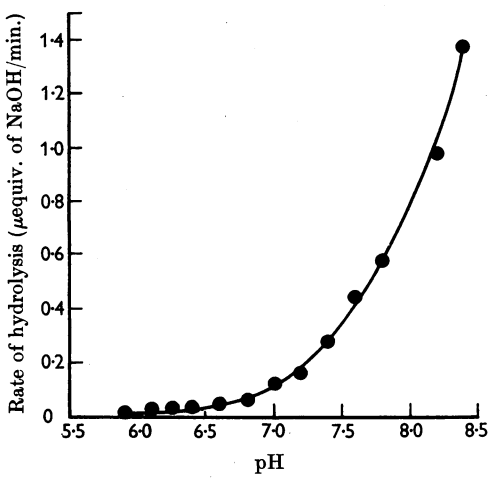
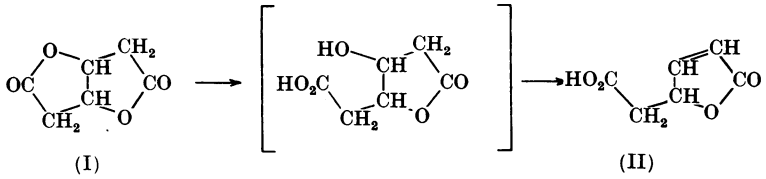


Fig. 2. Hydrolysis of the dilactone at different pH values recorded titrimetrically. The titration cup contained 4·5 ml. of a solution of the dilactone (20 μmoles/ml.). Hydrolysis at each pH was recorded graphically by the automatic addition of 0·1 N-NaOH. Temp., 30°.



(I) (II)

(5 μmoles) produced. The dilactone sample, originally isolated from catechol cultures by Landa & Eliasek (1956) after similar hydrolysis, was also only half-utilized. Moreover, the identity of the melting points of both synthetic and isolated materials (127°), which were undepressed on admixture of the materials, strongly supports the original view of Landa & Eliasek (1956), that their material was an artifact probably derived during isolation from muconolactone or *cis–cis*-muconic acid produced from catechol in their cultures.

Fig. 3 shows the relative rates of β-oxoadipate formation by extracts of *Pseudomonas* (phthalate-utilizing strain) at pH 6·25, acting on protocatechuate, muconolactone and the dilactone; at this pH, the rate of non-enzymic hydrolysis of the dilactone is low. It is apparent from this result that the spontaneous hydrolysis of the dilactone to muconolactone at higher pH values, and enzymic attack on the latter, are responsible for suspected attack on the dilactone itself, and the contention that the dilactone is an intermediate cannot be true. A similar result has since been obtained with *Nocardia* extracts at pH 6·6.

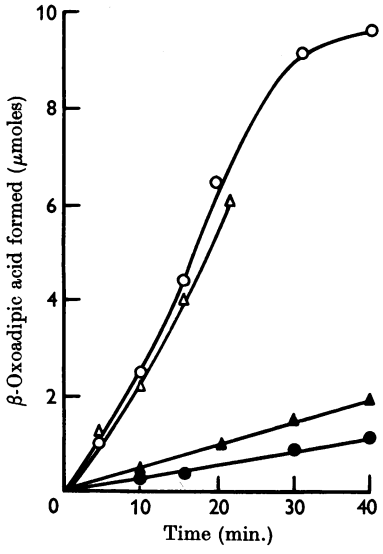


Fig. 3. Relative rates of β-oxoadipate formation by cell-free extracts of *Pseudomonas* sp. (phthalate-utilizing strain). Each Warburg flask contained: cell-free extract, 1 ml.; phosphate buffer, pH 6·25, 100 μmoles; water and substrate as indicated to a final volume of 3·0 ml. The amount of substrate was 10 μmoles (calculated for racemic mixtures of the dilactone and muconolactone was 20 μmoles). Atmosphere, air; temp., 30°. β-Oxoadipate was estimated by catalytic decarboxylation with 4-aminoantipyrine. One sample (2 ml.) from each flask, after various reaction times as indicated, was pipetted into a new Warburg flask containing 1 N-acetic acid (1 ml.) in the main compartment, and 0·1 M-4-aminoantipyrine (0·4 ml.) in the side arm. The β-oxoadipate formed was estimated immediately after equilibration at 30°. Substrates were: ○, protocatechuate; △, muconolactone; ●, dilactone. The rate of non-enzymic hydrolysis of the dilactone is also shown (▲).

The possibility that $\beta\gamma$-dihydroxyadipic acid is the symmetrical intermediate required from the tracer evidence of Gross *et al.* (1956) was also tested. Extracts from *P. fluorescens* A3.12 and *Pseudomonas* sp. (phthalate-utilizer) were incubated with two different preparations of this compound. No β-oxoadipate was detected after incubation with either extract.

The incubations were performed as follows. Each reductase tube contained: 0·2 M-phosphate buffer, pH 6·2, 1 ml.; cell-free extract, 1 ml. (containing about 15 mg. of protein); $\beta\gamma$-dihydroxyadipic acid, approx. 5 μmoles in 0·2 ml.; water to a final volume of 3·0 ml. Reactions were initiated by addition of substrate, and, after incubation for 20 min. at room temperature (approx. 20°), 1 ml. samples were saturated with ammonium sulphate; 2–3 drops of dilute sodium nitroprusside solution, followed by 0·5 ml. of conc. aq. ammonia soln. were added. An intense purple colour developed in 5 min. when β-oxoadipate was produced (Rothera, 1908). Control incubations, with no substrate, with protocatechuate and muconolactone as substrates, and with boiled extracts, were carried out simultaneously.

The negative results obtained with the two samples of $\beta\gamma$-dihydroxyadipic acid indicate that neither is an intermediate in the conversion of protocatechuate into β-oxoadipate by *P. fluorescens* (A3.12) or *Pseudomonas* sp. (phthalate-utilizer). $\beta\gamma$-Dihydroxyadipic acid possesses two asymmetric carbon atoms, and can therefore exist as the (+), (−), *meso* and racemic forms; the inability of these $\beta\gamma$-dihydroxyadipic acid preparations to act as precursors for β-oxoadipate in this enzyme system could be due to the samples' being biologically inactive forms of the compound. Pankoke (1925) maintained that his product was similar to that of Przybytek (1884) but different from that of Limpricht (1873); it is not known whether these correspond to the *meso* and racemic forms.

Protocatechuate degradation by extracts of *P. fluorescens* (A3.12) does not appear to proceed through the symmetrical intermediates, dilactone or $\beta\gamma$-dihydroxyadipic acid; extracts of *Pseudomonas* sp. (phthalate-utilizer) do not attack these substrates either, but do form β-oxoadipate from muconolactone; thus the pathway in this organism appears similar to that found for *Vibrio* O1 (Cain, 1961), and dissimilar to the *N. crassa* and *P. fluorescens* (A3.12) pathways. The tracer evidence of Gross *et al.* (1956) for *P. fluorescens* (A3.12) may not therefore be applicable to *Vibrio* O1 and *Pseudomonas* sp. (phthalate-utilizer).

SUMMARY

1. 2:6-Dioxa-3:7-dioxo*bicyclo*[3:3:0]octane ('the dilactone') hydrolyses spontaneously and non-enzymically to (±)-muconolactone; this hydrolysis is rapid above pH 7·0.

2. At pH 6·2–6·6, where the hydrolysis rate is low, extracts of *Pseudomonas* (phthalate-utilizer), *P. fluorescens* (A3.12) and *Nocardia* do not metabolize the dilactone at a rate in excess of its rate of hydrolysis.

3. The dilactone is now considered not to be an intermediate in protocatechuate metabolism by these micro-organisms.

4. Two samples of $\beta\gamma$-dihydroxyadipic acid failed to act as precursors of β-oxoadipate when used as substrates in the enzyme system which converts protocatechuate into β-oxoadipate.

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REFERENCES

Cain, R. B. (1958). *J. gen. Microbiol.* **19**, 1.
Cain, R. B. (1961). *Biochem. J.* **79**, 298.
Cain, R. B. & Cartwright, N. J. (1960). *Biochim. biophys. Acta*, **37**, 197.
Elsden, S. R. & Peel, J. L. (1958). *Annu. Rev. Microbiol.* **12**, 145.
Elvidge, J. A., Linstead, R. P., Orkin, B. A., Sims, P., Baer, H. & Pattison, D. B. (1950). *J. chem. Soc.* p. 2235.
Franzen, H. & Schmitt, F. (1925). *Ber. dtsch. chem. Ges.* **20**, 590.
Gross, S. R., Gafford, R. D. & Tatum, E. L. (1956). *J. biol. Chem.* **219**, 781.
Landa, S. & Eliasek, J. (1956). *Chem. Listy*, **50**, 1834.
Limpricht, H. (1873). *Liebigs Ann.* **165**, 267.
Pankoke, K. (1925). *Liebigs Ann.* **441**, 188.
Przybytek, S. (1884). *Ber. dtsch. chem. Ges.* **17**, 1091.
Ribbons, D. W. & Evans, W. C. (1959). *Biochem. J.* **73**, 21P.
Ribbons, D. W. & Evans, W. C. (1960). *Biochem. J.* **76**, 310.
Rothera, A. C. H. (1908). *J. Physiol.* **37**, 491.
Ruhemann, S. & Elliot, W. J. (1890). *J. chem. Soc.* p. 931.
Sleeper, B. P., Tsuchida, M. & Stanier, R. Y. (1950). *J. Bact.* **59**, 129.
Wislicenus, W. (1887). *Ber. dtsch. chem. Ges.* **20**, 590.

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Studies on Phospholipids

7. THE DISTRIBUTION OF COMPLEX PHOSPHOLIPIDS IN VARIOUS SPECIES AND TISSUES*

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Collins & Wheeldon (1957) have advanced evidence for the occurrence in rat liver, ox brain and egg yolk of 'complex' forms of amino-phospholipids and Collins (1959*b*) has described their separation and characterization by means of countercurrent distribution. The nature of these 'complex' phospholipids has been discussed by Collins (1960), who has presented evidence that they contain a phosphate triester group and at least two atoms of phosphorus/molecule. This paper is concerned with the distribution of these complex phospholipids in rat tissues and in other biological material.

* Part 6: Collins (1959*a*).

EXPERIMENTAL

Materials

Rats. The livers, brains, hearts, small intestines, kidneys, lungs, spleens and testes of twenty white rats (Wistar strain, body wt. 150–250 g.) were removed, quickly frozen and stored at −18° until required. For the additional experiments on the countercurrent distribution of the phospholipids animals were killed as required and the tissue was extracted as rapidly as possible.

Hen's eggs. These were not more than 24 hr. old.

Cabbage leaf. Extraction was carried out within 3 hr. of collection.

Influenza virus and hen's-egg allantoic membrane. These were received in a frozen-dried condition and were prepared