2. The isomers in the major dienoic acid fraction had *cis–cis* non-conjugated configurations, and the double bonds were largely at C-11 or C-12 and C-15 or C-16.

3. The monoenoic acids formed were largely *trans*, with the double bond predominantly at C-13 or C-14.

4. Rapid hydrogenation of [1-^{14}C]linoleic acid and [1-^{14}C]oleic acid occurred in the artificial rumen. The former gave a *trans* monoenoic acid as an intermediary.

5. The *trans* C_{18} monoenoic acids passing from the rumen were almost quantitatively absorbed in the ileum.

6. The unsaturated C_{18} acids present in the ileum digesta were hydrogenated in the caecum and colon, so that nearly all the acids in the excreta were saturated.

7. The hydrogenation of [1-^{14}C]linoleic acid by caecal and colon contents resulted again in the formation of substantial amounts of *trans* C_{18} monoenoic acids.

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REFERENCES

Ahrens, E. H., Insull, W., Hirsch, J., Stoffel, W., Petersen, M. L., Farquhar, J. W., Miller, T. & Thomasson, H. J. (1959). *Lancet*, i, 115.

Allison, M. J., Bryant, M. P., Keeney, M. & Katz, I. (1961). *Rep. U.S. Dep. Agric.* A.R.S. 44–98.

Beerthuis, R. K., Dijkstra, G., Keppler, J. G. & Recourt, J. H. (1959). *Ann. N.Y. Acad. Sci.* **72**, 616.

Burr, W. W., McPherson, J. C. & Tidwell, H. C. (1960). *J. Nutr.* **70**, 171.

Dawson, R. M. C., Ward, P. F. V. & Scott, T. W. (1964). *Biochem. J.* **90**, 9.

de Vries, B. (1962). *Chem. & Ind.* p. 1049.

de Vries, B. (1963). *J. Amer. Oil Chem. Soc.* **40**, 184.

Garton, G. A. (1960). *Nature, Lond.*, **187**, 511.

Garton, G. A. (1961). In *Digestive Physiology and Nutrition of the Ruminant*, p. 140. Ed. by Lewis, D. London: Butterworths.

Gompertz, S. M. & Sammons, H. G. (1963). *Clin. chim. Acta*, **8**, 591.

Hansen, R. P. (1963). *Nature, Lond.*, **198**, 995.

Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1953). *Biochem. J.* **53**, 374.

Hartman, L., Shorland, F. B. & McDonald, I. R. C. (1954). *Nature, Lond.*, **174**, 185.

James, A. T. (1963). *Analyst*, **88**, 572.

James, A. T., Webb, J. P. W. & Kellock, T. D. (1961). *Biochem. J.* **78**, 333.

Litchfield, C., Reiser, R. & Isbell, A. F. (1963). *J. Amer. Oil Chem. Soc.* **40**, 302.

McCarthy, R. D. (1962). In *Use of Radioisotopes in Animal Biology and Medical Sciences*, vol. 2, p. 151. New York: Academic Press Inc.

Ogilvie, B. M., McClymont, G. L. & Shorland, F. B. (1961). *Nature, Lond.*, **190**, 725.

Reiser, R. (1951). *Fed. Proc.* **10**, 236.

Rudloff, E. von (1956). *Canad. J. Chem.* **34**, 1413.

Sammons, H. G., Frazer, A. C., Gompertz, S. M. & Morgan, D. B. (1962). *Proc. 2nd World Conf. Gastroenterol., Munich* (in the Press).

Scheuerbrandt, G. & Bloch, K. (1962). *J. biol. Chem.* **237**, 2064.

Scholfield, C. R., Jones, E. P., Nowakowska, J., Selke, E. & Dutton, H. J. (1961). *J. Amer. Oil Chem. Soc.* **38**, 208.

Scott, T. W., Ward, P. F. V. & Dawson, R. M. C. (1964). *Biochem. J.* **90**, 12.

Shorland, F. B., Weenink, R. O. & Johns, A. T. (1955). *Nature, Lond.*, **175**, 1129.

Shorland, F. B., Weenink, R. O., Johns, A. T. & McDonald, I. R. C. (1957). *Biochem. J.* **67**, 328.

Sreenivasan, B., Nowakowska, J., Jones, E. P., Selke, E., Scholfield, C. R. & Dutton, H. J. (1963). *J. Amer. Oil Chem. Soc.* **40**, 45.

Biochem. J. (1964), **92**, 68

Preparation of Potassium 2-Deoxy-2-[^{35}S]sulphoamino-D-glucose

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Although it is well known that the biological activity of exogenous samples of the sulphated aminopolysaccharide heparin disappears rapidly *in vivo* (cf. Loomis, 1959, 1961), the sequence of events involved in the disposal of the injected polymer is still not fully understood. Up to the present, studies on the metabolic fate of heparin have been based on observations made after the injection of unlabelled biosynthetic preparations of the polymer (Wilander & Holmgren, 1938; Wilander, 1939; Jacques, 1939; Reinert & Winterstein, 1939; Copley & Schendorf, 1941; Piper, 1947;

Jaques, Napke & Levy, 1953), of preparations labelled biosynthetically with radioactive sulphate (Eiber, Danishefsky & Borelli, 1958; Danishefsky & Eiber, 1959; Loomis, 1961; Day, Green & Robinson, 1962) and of semi-chemically synthesized material prepared by the selective *N*-resulphation with trimethylamine–[^{35}S]sulphur trioxide of *N*-desulphated commercial heparin (Levy & Petracek, 1962). The earlier reports dealt mainly with the urinary excretion of the apparently unchanged polymer, but in the later work attention has been given to the tissue distribution of radioactivity and to the appearance of inorganic [^{35}S]sulphate, detectable in the tissues or in the urine, after the injection of the ^{35}S-labelled materials. A different approach has involved studies on the 'heparinase' system of human liver (Jaques, 1940; Jaques & Kerri-Szanto, 1952; Jaques & Cho, 1954; Cho & Jaques, 1956), reported as destroying the biological action of heparin, although the mode of action of the system is still in doubt (Schuytema & Cushing, 1958).

The difficulties of interpreting the results of such studies, in particular with regard to the mechanisms involved in the liberation of inorganic sulphate, are readily apparent when it is considered that a recently proposed structure for heparin (Durant, Hendrickson & Montgomery, 1962) suggests that a hexadecasaccharide period in the polymer, composed of alternating D-glucuronic and D-glucosamine moieties, would carry 25–26 ester-bound sulphate groups. It is postulated that seven of these are present as *N*-sulphate (sulphamate) groups on the hexosamine residues and at least seven as *O*-sulphate groups at position 2 of the uronic acid moieties. Of the remainder it is suggested that eight of the hexosamine moieties are *O*-sulphated at position 6 (cf. Nominé, Bucourt & Bertin, 1961; Hoffman & Meyer, 1962), while four or five of these moieties have extra *O*-sulphate groups at position 3. A further complicating feature with studies based on the use of the biosynthesized polymer is the knowledge that the connective-tissue aminopolysaccharide heparan sulphate, which is also believed to contain *O*-sulphate and *N*-sulphate groups, may occur as a contaminant in the preparations (see Jeanloz, 1963).

Previous reports (Lloyd, 1959, 1960, 1962*a*) have dealt with the development of methods suitable for the synthesis of the *O*-[^{35}S]sulphate esters of hexoses and *N*-acetylhexosamines for use in studies on the metabolism of naturally occurring compounds known to contain this type of residue (Lloyd, 1961*a*, *b*; Lloyd, 1962*b*). We now report a method for the preparation, by a direct esterification procedure, of 2-deoxy-2-[^{35}S]sulphoamino-D-glucose suitable for use as a model compound in studies on the biological activity of the sulphamate group as found in heparin and heparan sulphate.

EXPERIMENTAL AND RESULTS

Analytical methods

Paper chromatography and paper electrophoresis. The homogeneity of intermediates in the reaction and the identity of the final product was checked by paper chromatography and paper electrophoresis. Chromatograms were run on Whatman no. 1 filter paper by using downward irrigation with isobutyric acid–aq. 0·5 N-NH_3 soln. (5:3, v/v) as solvent for 20 hr. at 20°. The air-dried chromatograms were then sprayed with the *p*-anisidine reagent (Hough, Jones & Wadman, 1950) for reducing sugars or with 0·2% (w/v) ninhydrin in acetone for free hexosamines. Electrophoresis was carried out on Whatman no. 1 filter paper with 0·1 M-ammonium acetate–acetic acid solution (pH 4·0) as buffer at 400 v for 2 hr. Separated components were detected by heating the moist electrophoresis strip at 110° for 5 min., when reducing sugars appeared as brown areas. Alternatively, the air-dried electrophoresis strips were sprayed with *p*-anisidine or ninhydrin solutions as above.

Determination of radioactivity. For the determination of radioactivity samples of the ^{35}S-labelled materials were hydrolysed with 2·5 N-HCl (5 ml.) for 2 hr. at 100°. Liberated inorganic [^{35}S]sulphate was then precipitated as $Ba^{35}SO_4$ before plating and counting according to Lloyd (1961*b*). The radioactivities of the $Ba^{35}SO_4$ samples were measured with a D. 47 gas-flow counting system, in windowless operation, in conjunction with automatic scaling equipment (Nuclear–Chicago Corp., Ill., U.S.A.). Corrections were made for coincidence and decay.

Radioactive components were detected on paper chromatograms and paper-electrophoresis strips with a thin (1·4 mg./cm.^{2}) mica end-window Geiger–Müller tube in the C. 100 Actigraph chromatogram strip-scanner (Nuclear–Chicago Corp., Ill., U.S.A.).

Analyses. For the determination of ester sulphate and of hexosamine nitrogen, samples were hydrolysed by refluxing with 0·5 N-HCl for 2 hr. The liberated inorganic sulphate was determined titrimetrically by the method of Belcher, Gibbons & West (1954) and hexosamine nitrogen according to Tracey (1952). Total nitrogen was determined by semi-micro-Kjeldahl analysis and potassium by flame photometry. For the determination of loss in weight on drying, samples of crystalline products were dried at 82° *in vacuo* for 12 hr. However, as the dried samples were hygroscopic, analyses were performed on the hydrated materials and the appropriate corrections made.

Infrared spectroscopy. Infrared-absorption spectra were measured with the Perkin–Elmer Infracord spectrophotometer. Compounds were examined as mulls in Nujol (liquid paraffin).

Sulphation and purification procedures

Chemicals. D-Glucosamine hydrochloride was a commercial preparation (British Drug Houses Ltd.). The pyridine–sulphur trioxide reagent was prepared according to Baumgarten (1926). Initially, pyridine–[^{35}S]sulphur trioxide was prepared by using chloro[^{35}S]sulphonic acid of low specific activity, prepared in the laboratory by the method of Tudball (1962). In subsequent preparations chloro[^{35}S]sulphonic acid of high specific activity (total activity 21 mC; The Radiochemical Centre, Amersham, Bucks.) was used.

Preparation of 2-*deoxy*-2-[^{35}S]*sulphoamino*-D-*glucose*. D-Glucosamine hydrochloride (4·2 g.) was dissolved in water (25 ml.) and the pH of the solution adjusted to 9·5 by the addition of N-NaOH. Pyridine–[^{35}S]sulphur trioxide (0·956 g.) was added to the well-stirred solution over a period of 1 hr. at 24°. During the period of the addition, the pH of the mixture was maintained between 9 and 10 by the dropwise addition of N-NaOH. Particular care was exercized during the addition stage to minimize the formation of the red dye reported by Baumgarten (1926) as being formed from the pyridine–sulphur trioxide reagent by an excess of alkali (cf. Warner & Coleman, 1958). Samples of the resulting pale-yellow solution, examined by paper chromatography and paper electrophoresis, showed the presence of two reducing sugars. One of these was ninhydrin-positive and had chromatographic and electrophoretic mobilities identical with D-glucosamine. The other gave no reaction with ninhydrin and moved toward the anode on electrophoresis. When scanned for radioactivity, chromatograms and electrophoresis strips showed the presence of only two radioactive bands, one identical in mobility with inorganic sulphate and the other corresponding to the ninhydrin-negative reducing sugar.

The excess of D-glucosamine was removed by passing the sulphation mixture through a column of Dowex 50 (H^+ form; 50–100 mesh) at 4°. The acid eluate and washings were pooled and adjusted to pH 8·0 with satd. $Ba(OH)_2$ solution to precipitate inorganic $^{35}SO_4^{2-}$ ions. Precipitated $Ba^{35}SO_4$ was removed by centrifuging and excess of Ba^{2+} ions in the clear supernatant removed by passing the solution through a column of Dowex 50 (H^+ form; 50–100 mesh) at 4°. The acid eluate and washings were pooled and the solution was neutralized to pH 6·0 by stirring with an excess of solid Ag_2CO_3 (6 g.) to remove Cl^- ions. The precipitate was removed by centrifuging. The crude product was then converted into the potassium salt by passing the clear supernatant through a column of Dowex 50 (H^+ form; 50–100 mesh) and adjusting the pooled acid eluate to pH 7·2 with 0·2 N-KOH. The solution was concentrated to 10 ml. by rotary evaporation under reduced pressure at 37°. The product was precipitated by the addition of ethanol (80 ml.) and the precipitate collected, washed with absolute ethanol and ether and dried *in vacuo* over $CaCl_2$ at room temperature. The product, a pale-yellow amorphous powder (average yield 1·05 g.), was shown to consist of 2-deoxy-2-[^{35}S]sulphoamino-D-glucose contaminated with the red dye product.

Purification of the crude product. When the concentration of the contaminating dye was low it could be removed by a single treatment of an aqueous solution of the crude product with activated charcoal. However, in certain instances the yield of dye product was high because the pyridine–sulphur trioxide reagent in the sulphation step (see above) was added too rapidly. In such instances it was found necessary to use a modification of the method of Lloyd (1962*a*) to remove the unchanged dye.

An aqueous solution of the crude potassium salt (10 ml.; 1%, w/v) was added to a column (8 cm. × 1·5 cm.) of Dowex 1 (X8; OH^- form; 200–400 mesh) and allowed to drain into the resin bed under gravity flow. Under these conditions the red dye passed straight through the resin bed, which was then washed with water until the eluate was no longer alkaline. The column was then eluted with 0·04 N-H_2SO_4 at 2° and a flow rate of 1·5 ml./min.; the eluate was collected in 5 ml. fractions, the pH of each fraction being determined. When the pH of the column effluent had fallen to 2·2, each of the fractions was examined by paper electrophoresis and monitored for radioactivity. Fractions containing a single radioactive component were pooled and freed from traces of unlabelled SO_4^{2-} ions by neutralization with satd. $Ba(OH)_2$ solution. The $BaSO_4$ precipitate was removed by centrifuging. The product was converted into the potassium salt in the manner described above and the volume of the solution reduced to 10 ml. by rotary evaporation under reduced pressure at 37°.

To the aqueous solution, obtained after treatment with charcoal or Dowex 1, was added 4 vol. of ethanol in a dropwise manner to give a product consisting of colourless microcrystalline plates, which were collected by filtration and dried *in vacuo* over $CaCl_2$. The compound, potassium 2-deoxy-2-[^{35}S]sulphoamino-D-glucose monohydrate, had m.p. 171° (decomp.) and $[\alpha]_D^{23·5} + 50 \pm 1°$ (*c* 1·0 in water) (Found: C, 22·8; H, 4·8; total N, 4·3; hexosamine N, 4·3; SO_4^{2-} ion, 29·7; K, 12·7; acid equiv.wt. 316. $C_6H_{12}KNO_8S,H_2O$ requires C, 22·8; H, 4·4; N, 4·4; SO_4^{2-} ion, 30·5; K, 12·4%; acid equiv.wt. 315). On filter-paper chromatography and electrophoresis the product was found to be homogeneous and identical in mobility with 2-deoxy-2-sulphoamino-D-glucose prepared by a definitive synthetic route (Foster, Martlew, Stacey, Taylor & Webber, 1961). After hydrolysis in 0·5 N-HCl, inorganic [^{35}S]sulphate and D-glucosamine were the only products that were recognizable by paper chromatography and paper electrophoresis. The radioactivity of the material of high specific activity corresponded to 8·02 mc/m-mole when measured, after hydrolysis, as an infinitely thick plate of $Ba^{35}SO_4$ (cf. Lloyd, 1961*b*). It was necessary to store this product in the form of a dilute aqueous solution in the frozen state to minimize degradation due to 'self-irradiation' (A. G. Lloyd, unpublished results).

The infrared-absorption spectrum of potassium 2-deoxy-2-sulphoamino-D-glucose, together with those of potassium *N*-bis-(2-hydroxyethyl)sulphamate and L-*N*-sulphoserine prepared by the method of Warner & Coleman (1958) for comparison, is reproduced in Fig. 1.

DISCUSSION

The majority of methods used in the preparation of monosaccharide, oligosaccharide and polysaccharide models containing the sulphamate grouping fall into two main categories. First, there are the definitive syntheses, which, although employing sulphating agents under conditions where both *O*-sulphate and *N*-sulphate esters may be formed, are based on the use of suitably blocked *O*-acyl derivatives in which only the amino group is available for reaction (Wolfrom, Gibbons & Huggard, 1957; Foster *et al.* 1961; Onodera & Komano, 1962; Onodera, Kitaoka & Ochiai, 1962). Such methods obviously favour the formation of the required *N*-sulphate derivative. However, they suffer the disadvantage that the large number of chemical manipulations required in the preparative procedures is not ideally suited to the preparation

of radioactive derivatives where only small quantities of material, frequently associated with high specific activities, may be involved. These difficulties are accentuated by the relative ease with which intermediates in the syntheses undergo $O \rightarrow N$ acyl transformations, making the products of the esterification procedures difficult to purify (cf. Foster *et al.* 1961). On the other hand, the use of unsubstituted starting materials results in the formation of *N*-sulphate compounds that may also be accompanied by varying degrees of *O*-sulphation (see Meyer & Schwartz, 1950; Wolfrom, Shen & Summers, 1953; Ricketts, 1953; Doczi, Fischman & King, 1953; Coleman, McCarty, Warner, Willy & Flokstra, 1953; Wolfrom & Shen-Han, 1959). The sulphation conditions, based on those of Warner & Coleman (1958), used in the present study permit the selective sulphation of the amine group under conditions where *O*-sulphation is unlikely to occur. Thus it is well known that a primary prerequisite of *O*-sulphation reactions is the maintenance of strictly anhydrous conditions (cf. Lloyd, 1959, 1960, 1962*a*). The preparation of 2-deoxy-2-sulphoamino-D-glucose under conditions similar to those presently reported has already been attempted by Foster *et al.* (1961), who recorded that the products of reaction of the reaction were difficult to purify and did not investigate the technique further. The method of purification which has been developed offers no serious problems, especially if the content of dye product is minimized, and is suitable for use in the preparation of material of high specific radioactivity.

The infrared spectra of potassium 2-deoxy-2-sulphoamino-D-glucose, potassium *N*-bis-(2-hydroxyethyl)sulphamate and potassium L-*N*-sulphoserine differ substantially from those reported for the *O*-sulphate esters of hexoses, *N*-acetylhexosamines, amino alcohols and hydroxylated amino acids (Lloyd & Dodgson, 1959, 1961; Lloyd, Tudball & Dodgson, 1961*b*). Thus both *N*-bis-(2-hydroxyethyl)sulphamate and L-*N*-sulphoserine exhibit characteristic strong absorption at 3320 cm.$^{-1}$, attributable to vibrations involving the unsubstituted hydroxyl group. Such absorption is absent from the spectra of the corresponding *O*-sulphate esters. A common feature of the spectra of all three *N*-sulphate derivatives is the presence of strong absorption in the range 1150–1250 cm.$^{-1}$. Although *O*-sulphate esters absorb in a similar range, maximal absorption usually occurs at 1240 cm.$^{-1}$. It is a characteristic feature of the *N*-sulphate compounds that the frequency of maximum absorption occurs at 1200 cm.$^{-1}$. In consequence, absorption in this range is tentatively assigned to vibrations involving S–O linkages within the N–SO_3^- system. The observed shift in the frequency of maximum absorption from 1230 cm.$^{-1}$ in the spectrum of heparin, to which both *O*-sulphate and *N*-sulphate groups would contribute, to 1240 cm.$^{-1}$ in the spectrum of *N*-desulphated heparin, to which only *O*-sulphate groups would contribute (Foster *et al.* 1961), is in agreement with this suggestion. Support for the specificity of the *N*-sulphation procedure used in the preparation of 2-deoxy-2-sulphoamino-D-glucose, and the unreactivity of the hydroxyl groups under these conditions, is obtained from the observation of the absence of absorption from the range 820–850 cm.$^{-1}$. It is known that the *O*-sulphate-substituted pyranose ring absorbs strongly at 820 cm.$^{-1}$, 830 cm.$^{-1}$ or 850 cm.$^{-1}$ according to the spatial distribution of the ester group on the ring (cf. Lloyd & Dodgson, 1959, 1961; Lloyd, Dodgson, Price & Rose, 1961*a*; Lloyd *et al.* 1961*b*; Lloyd, Dodgson & Price, 1963). An attempt to resolve the problem of the contribution in the infrared of the N–S bond by comparing the spectra of 2-deoxy-2-sulphoamino-D-glucose, containing the N–S system, and 2-deoxy-2-[^{35}S]sulphoamino-D-glucose, containing both N–S and N–^{35}S systems, was unsuccessful as no duplication of bands or band shifts attributable to the mass differences were detectable.

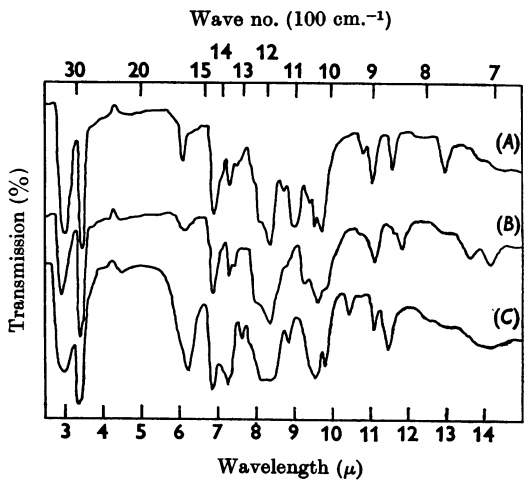

Fig. 1. Infrared spectra of *N*-sulphate esters. (*A*) Potassium 2-deoxy-2-sulphoamino-D-glucose monohydrate. (*B*) Potassium *N*-bis-(2-hydroxyethyl)sulphamate. (*C*) Potassium L-*N*-sulphoserine. Spectra are displaced vertically to facilitate comparison.

SUMMARY

1. A method has been developed for the preparation of potassium 2-deoxy-2-[^{35}S]sulphoamino-D-glucose of high specific radioactivity suitable for use as a model compound in studies on the biological stability of the *N*-sulphate group as found in heparin and heparan sulphate.

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REFERENCES

Baumgarten, P. (1926). *Ber. dtsch. chem. Ges.* **59**, 1166.

Belcher, R., Gibbons, D. & West, T. S. (1954). *Chem. & Ind.* p. 127.

Cho, M. H. & Jaques, L. B. (1956). *Canad. J. Biochem. Physiol.* **34**, 799.

Coleman, L. L., McCarty, L. P., Warner, D. T., Willy, R. F. & Flokstra, J. H. (1953). *Abstr. Pap. Amer. chem. Soc.* **123**, 19L.

Copley, A. L. & Schendorf, J. G. (1941). *Amer. J. Physiol.* **133**, 562.

Danishefsky, I. & Eiber, H. B. (1959). *Arch. Biochem. Biophys.* **85**, 53.

Day, M., Green, J. P. & Robinson, J. D. (1962). *Brit. J. Pharmacol.* **18**, 625.

Doczi, J., Fischman, A. & King, J. A. (1953). *J. Amer. chem. Soc.* **75**, 1512.

Durant, G. J., Hendrickson, H. R. & Montgomery, R. (1962). *Arch. Biochem. Biophys.* **99**, 418.

Eiber, H. B., Danishefsky, I. & Borelli, F. J. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 672.

Foster, A. B., Martlew, E. F., Stacey, M., Taylor, P. J. M. & Webber, J. M. (1961). *J. chem. Soc.* p. 1204.

Hoffman, P. & Meyer, K. (1962). *Fed. Proc.* **21**, 1064.

Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.

Jaques, L. B. (1939). *Amer. J. Physiol.* **125**, 98.

Jaques, L. B. (1940). *J. biol. Chem.* **133**, 445.

Jaques, L. B. & Cho, M. H. (1954). *Biochem. J.* **58**, xxv.

Jaques, L. B. & Kerry-Szanto, E. (1952). *Canad. J. med. Sci.* **30**, 353.

Jaques, L. B., Napke, E. & Levy, S. W. (1953). *Circulation Res.* **1**, 321.

Jeanloz, R. W. (1963). In *Comprehensive Biochemistry*, vol. 5, p. 262. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.

Levy, L. & Petracek, F. J. (1962). *Proc. Soc. exp. Biol., N.Y.*, **109**, 901.

Lloyd, A. G. (1959). *Nature, Lond.*, **183**, 109.

Lloyd, A. G. (1960). *Biochem. J.* **75**, 478.

Lloyd, A. G. (1961*a*). *Nature, Lond.*, **190**, 914.

Lloyd, A. G. (1961*b*). *Biochem. J.* **80**, 572.

Lloyd, A. G. (1962*a*). *Biochem. J.* **83**, 455.

Lloyd, A. G. (1962*b*). *Biochim. biophys. Acta*, **58**, 1.

Lloyd, A. G. & Dodgson, K. S. (1959). *Nature, Lond.*, **184**, 549.

Lloyd, A. G. & Dodgson, K. S. (1961). *Biochim. biophys. Acta*, **46**, 116.

Lloyd, A. G., Dodgson, K. S. & Price, R. G. (1963). *Biochim. biophys. Acta*, **69**, 496.

Lloyd, A. G., Dodgson, K. S., Price, R. G. & Rose, F. A. (1961*a*). *Biochim. biophys. Acta*, **46**, 108.

Lloyd, A. G., Tudball, N. & Dodgson, K. S. (1961*b*). *Biochim. biophys. Acta*, **52**, 413.

Loomis, T. A. (1959). *Proc. Soc. exp. Biol., N.Y.*, **101**, 447.

Loomis, T. A. (1961). *Proc. Soc. exp. Biol., N.Y.*, **106**, 490.

Meyer, K. H. & Schwartz, D. E. (1950). *Helv. chim. acta*, **33**, 1651.

Nominé, G., Bucourt, R. & Bertin, D. (1961). *Bull. Soc. chim. Fr.* p. 561.

Onodera, K., Kitaoka, S. & Ochiai, H. (1962). *J. org. Chem.* **27**, 156.

Onodera, K. & Komano, T. (1962). *J. org. Chem.* **27**, 1069.

Piper, J. (1947). *Acta pharm. tox., Kbh.*, **3**, 373.

Reinert, M. & Winterstein, A. (1939). *Arch. int. Pharmacodyn.* **62**, 427.

Ricketts, C. R. (1953). *Research*, **6**, 175.

Schuytema, E. C. & Cushing, I. B. (1958). *Fed. Proc.* **17**, 307.

Tracey, M. V. (1952). *Biochem. J.* **52**, 265.

Tudball, N. (1962). *Nature, Lond.*, **196**, 580.

Warner, D. T. & Coleman, L. L. (1958). *J. org. Chem.* **23**, 1133.

Wilander, O. (1939). *Skand. Arch. Physiol.* **81**, Suppl. 15, 1.

Wilander, O. & Holmgren, H. (1938). *Acta med. scand.* **94**, 258.

Wolfrom, M. L., Gibbons, R. A. & Huggard, A. J. (1957). *J. Amer. chem. Soc.* **79**, 5043.

Wolfrom, M. L., Shen, T. M. & Summers, C. G. (1953). *J. Amer. chem. Soc.* **75**, 1519.

Wolfrom, M. L. & Shen-Han, T. M. (1959). *J. Amer. chem. Soc.* **81**, 1764.