The spray-dried enzymic hydrolyzate (50 g.) was also extracted with three successive 250-ml. portions of boiling 95% ethanol. From the combined extracts a cold alcohol-soluble fraction was isolated and evaporated to dryness. Paper chromatographic separation on Whatman No. 3 paper resulted in concentrating the 4-NBP positive material along a band at R_f 0.4–0.6. This zone was cut from the paper sheet and eluted with the propanol–water mixture. The eluate was evaporated and rechromatographed on Whatman No. 1 paper to yield five ninhydrin-positive spots, one of which (R_f 0.50) was 4-NBP, ultraviolet and sulfur positive and phosphorus negative. The concentrated eluate inhibited the growth of *Saccharomyces pastorianus*[27] in exactly the same manner as did the S-dichlorovinyl derivatives of L-cysteine and L-glutathione.

Colorimetric Correlation of Sulfhydryl Activity in Old and New Soybeans.—The dye, 2,6-dichlorophenol-indophenol, was used to estimate the free sulfhydryl group activity in old and new beans in accordance with the principle of Basford and Huennekens.[28] In a typical study 5% aqueous slurries of Clark variety soybeans (1956 and 1957 crops, *i.e.*, approximately 14 months and 2 months after harvesting) were prepared under nitrogen in a high-speed homogenizer and subjected to colorimetric assay with the dye. Thiol activity of a 5-ml. aliquot from each homogenate was abolished by 10-minute interaction, preferably in a glass-stoppered reaction flask, with 2.5 ml. of 0.005 molar *p*-chloromercuribenzoic acid (*p*-CMB) in a slight excess of alkali. Two milliliters of aqueous dye solution (0.004 molar) was added to each flask. After 6 minutes 5 ml. of *p*H 4 acetate buffer and 10 ml. of xylene were added in rapid succession. After vigorous shaking, the sample was centrifuged to separate the layers. The xylene layer was isolated, dried with anhydrous Na_2SO_4 and read in a colorimeter at 500 mμ against a blank provided by substituting water for dye in the assay. A measure of the normal dye decolorizing activity of the homogenate was obtained by substituting water for *p*-CMB in the assay. Thus, devoid of preliminary treatment with the thiol blocking reagent, *p*-CMB, soybean homogenates from both the 1956 and 1957 crops produced identical colorimetric changes with the dye. Where *p*-CMB treatment of the homogenate preceded reaction with the dye, a 79% decrease in bleaching action was observed with the homogenate of the 1957 soybeans in contrast with only 60% inhibition from the 1956 soybeans.

Acknowledgments.—Elementary analyses were performed by C. H. Van Etten and Clara McGrew.

PEORIA, ILLINOIS

(27) L. L. McKinney, A. C. Eldridge and J. C. Cowan, THIS JOURNAL, **81**, in press (1959).

(28) R. E. Basford and F. M. Huennekens, *ibid.*, **77**, 3873 (1955).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Studies on *myo*-Inositol Phosphates of Natural Origin

BY FRANCES LANE PIZER AND CLINTON E. BALLOU

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myo-Inositol monophosphates previously obtained by enzymic and basic hydrolysis of phytin have been prepared as pure crystalline cyclohexylamine salts. These two compounds have been shown to be identical with each other and with the *myo*-inositol 2-phosphate first prepared by Iselin. A *myo*-inositol monophosphate has been obtained as a crystalline salt from a base hydrolysis of soybean phosphoinositide. It is optically active and is shown to be *myo*-inositol 1-phosphate. This suggests that the ester linkage in the phosphoinositide is to position 1 of the *myo*-inositol ring, a structural feature that has long been in question. Chromatographic analysis of base hydrolysates of phosphoinositides from beef heart, liver and wheat germ indicates that the same linkage is present in the lipid from these different sources. *myo*-Inositol 1- and 2-phosphate have been shown to be readily interconverted by acid-catalyzed phosphate group migration, while the 5-phosphate does not undergo such migration. The action of dicyclohexylcarbodiimide (DCC) on *myo*-inositol 1- and 2-phosphate produces the same cyclic 1,2-phosphate. Base hydrolysis of this cyclic phosphate yields mostly the 1-phosphate, which explains why the major inositol phosphate component in the base hydrolysis of the phosphoinositide is the 1-phosphate. Treatment of the 5-phosphate with DCC leads to unstable cyclic phosphate intermediates, with the eventual formation of the 1,2-phosphate.

myo-Inositol monophosphates have been obtained by alkaline,[1] acid[2] and enzymic[3] hydrolysis of *myo*-inositol hexaphosphate, by acid and alkaline hydrolysis of phospholipids[4] and by synthesis.[5,6] Only in the case of one synthetic compound[5] has the structure been defined (as *myo*-inositol 2-phosphate),[7] although it has been claimed[8] that the product obtained by enzyme hydrolysis of phytic acid[3] is identical with the Iselin phosphate, and that made by alkaline hydrolysis of phytic acid is different.

We have now prepared *myo*-inositol monophosphates by several of the above mentioned procedures and have attempted to interrelate them structurally. All compounds were obtained as crystalline cyclohexylamine salts. The monophosphates prepared by the methods of Iselin,[5] Carter[3] and Desjobert[1] were found to be identical by infrared analysis, rate of periodate oxidation, chromatographic properties, melting point and microscopic appearance of the cyclohexylamine salts. Thus, accepting the Iselin compound as being *myo*-inositol 2-phosphate (from mode of synthesis), it follows that the Carter and Desjobert compounds have the same structure.[9]

The *myo*-inositol phosphate prepared from soybean phosphoinositide by alkaline hydrolysis gives a cyclohexylamine salt that differs from the 2-phos-

(1) A. Desjobert, *Bull. soc. chim. biol.*, **36**, 1293 (1954).

(2) R. J. Anderson, *J. Biol. Chem.*, **12**, 447 (1912); **18**, 441 (1914).

(3) M. H. McCormick and H. E. Carter, *Biochem. Preps.*, **2**, 65 (1952); S. Posternak and T. Posternak, *Helv. Chim. Acta*, **12**, 1165 (1929).

(4) D. W. Woolley, *J. Biol. Chem.*, **147**, 581 (1943); J. N. Hawthorne and E. Chargaff, *ibid.*, **206**, 27 (1954); J. M. McKibbin, *ibid.*, **220**, 537 (1956).

(5) B. Iselin, THIS JOURNAL, **71**, 3822 (1949).

(6) K. Horiuchi, *J. Biochem. (Japan)*, **14**, 163 (1931–1932).

(7) The *myo*-inositol ring was numbered as in I when Iselin named his substance the 5-phosphate. The presently accepted way for numbering the inositol ring, shown in II, makes Iselin's compound the 2-phosphate.

I

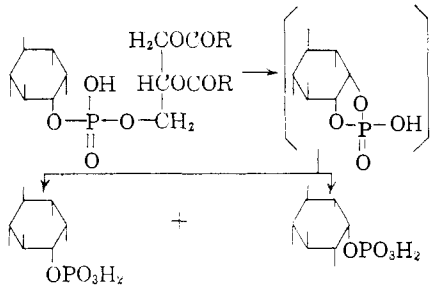
II

(8) P. Fleury, A. Desjobert and J. Lecocq, *Bull. soc. chim. biol.*, **36**, 1301 (1954).

(9) Drs. D. M. Brown and G. E. Hall of the University Chemical Laboratory, Cambridge, have informed us that they have come to the same conclusion.

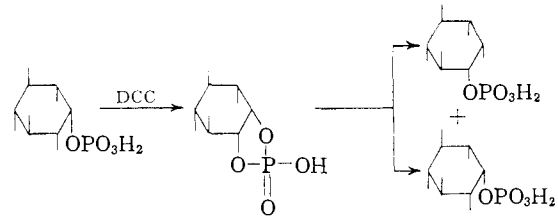
phate and the 5-phosphate[10] on the basis of its infrared spectrum, chromatographic properties and crystalline form. In addition, the soybean monophosphate is optically active, $[\alpha]^{25}_{589}$ + 3.4° (*c* 3, *p*H 9), −9.8° (*c* 3, *p*H 2). Thus, it must be either the 1(3)- or 4(6)-phosphate, the numbers in parentheses being the equivalent, but enantiomeric positions. That it is not a mixture of the two is suggested by the fine definition of character of the infrared spectrum and by the chromatographic homogeneity.

On chromatographic investigation of the crude mother liquors from the alkaline hydrolysis of soybean phosphoinositide, we were able to detect a small amount of *myo*-inositol 2-phosphate. Since the alkaline hydrolysis of the phosphoinositide (which by now is almost certainly defined as a diacyl-glycerol *myo*-inositol phosphate diester)[4,11] probably proceeds by way of a cyclic phosphate intermediate involving two adjacent hydroxyl groups in the *myo*-inositol,[12] in order to yield *myo*-inositol phosphate as one component, one would expect at least two different monophosphates to result. This is actually the case, and since one of the products is the 2-phosphate, the cyclic intermediate must involve position 1 and position 2.[13]



The choice as to which of these two positions is involved in the linkage in the phosphoinositide is suggested by the fact that one of the soybean monophosphates obtained on hydrolysis is optically active. This would result from cyclization and reopening of a phosphate group originally on position 1, since the intermediate cyclic phosphate would be asymmetric if the starting diester were asymmetric. Such would not be the result from a compound with the phosphate originally on the 2-position (thus having a plane of symmetry) unless the asymmetry of the glycerol portion were able to exert a directive influence during the reaction so that an asymmetric cyclic 1,2-phosphate was produced.

The major product from the alkaline hydrolysis of soybean phosphoinositide is the 1-phosphate, and since the cyclic 1,2-phosphate is a proposed intermediate, it would seem that the cyclic phosphate must open in alkali to give mainly the 1-phosphate. This was actually shown to be so. *myo*-Inositol 2-phosphate was treated with dicyclohexylcarbodiimide in the usual way for preparing cyclic phosphates.[14,15] This produced a new substance, isolated as a crystalline salt, with the chromatographic properties and analysis of a cyclic phosphate. On mild acid or base hydrolysis, it gave two monophosphates.[26] Both were isolated as pure crystalline salts. One was identical with the starting 2-phosphate, while the other had the same chromatographic properties, periodate oxidation rate and infrared spectrum as the optically active soybean compound. However, it was optically inactive.



These results would be expected if the 2-phosphate were converted to the racemic (±)-*myo*-inositol 1-phosphate by forced migration. Since the cyclic phosphate has the stability of a 5-membered ring,[15] it must be the (±)-*myo*-inositol 1,2-phosphate. As only two monophosphates were produced on hydrolysis, and one is identical with the starting compound, it is apparent that the phosphate group did not migrate around the ring. Alkaline hydrolysis of the cyclic phosphate gave about the same proportion of the two products as was obtained by alkaline hydrolysis of the soybean phosphoinositide (about 80–90% 1-phosphate to 10-20% 2-phosphate) indicating that the linkage to the 2-position of the *myo*-inositol 1,2-phosphate intermediate is the more readily attacked.

Confirmation of the structure of the soybean inositol phosphate was obtained by synthesis of (±)-*myo*-inositol 1-phosphate by phosphorylation of 1,4,5,6-tetra-O-acetyl-*myo*-inositol[16] with diphenylphosphorochloridate. The reaction apparently takes place with cyclization and phenol elimination, in a manner similar to that described by Kilgour and Ballou for a related reaction,[17] since the major phosphorylation product was obtained from the reaction mixture as a water-soluble salt. This was unblocked by saponification. The synthetic substance was primarily one isomer, different from the 2-phosphate although a trace of the latter was formed, and was identical with the soybean phosphate in all respects except the optical rotation.

We have also investigated the acid-catalyzed phosphate group migration in the soybean inositol phosphate, *myo*-inositol 2-phosphate and 5-phos-

(10) *myo*-Inositol 5-phosphate was synthesized by Dr. S. J. Angyal while working in this department on a sabbatical leave during the winter of 1957. It was prepared by phosphorylation of *myo*-inositol 1,2,3,4,6-tetra-O-acetate. Dr. Angyal very kindly supplied a sample of this compound for a reference in our studies. The synthesis of the 5-phosphate, a compound with a plane of symmetry, will be described elsewhere. The infrared spectrum in the region of 625–1000 cm.$^{-1}$ showed the following characteristics: 957 (strong split peak), 923 (strong), 897 (strong), 815 (medium), 715 (medium broad).

(11) D. J. Hanahan and J. N. Olley, *J. Biol. Chem.*, **231**, 813 (1958). M. Faure and M. J. Maurelec-Coulon, *Compt. rend. Acad.*, **236**, 1104 (1953); **238**, 411 (1954).

(12) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

(13) The lability required for this presumed intermediate precludes a 6-membered cyclic phosphate. See reference 15.

(14) C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

(15) H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, *ibid.*, **79**, 430 (1957).

(16) S. J. Angyal, P. T. Gilham and C. G. MacDonald, *J. Chem. Soc.*, 1417 (1957).

(17) G. L. Kilgour and C. E. Ballou, THIS JOURNAL, **80**, 3956 (1958).

phate, as well as in (−)-inositol 3-phosphate[17] and pinitol 4-phosphate.[17] When 0.1 molar solutions of the inositol phosphates, as their free acids, were heated at 90°, *myo*-inositol 2-phosphate and the soybean phosphate were readily interconverted. None of the other substances gave any indication of phosphate migration. This indicates that acid-catalyzed migration across *trans*-hydroxyl groups in the ring is particularly difficult. Since *myo*-inositol 4-phosphate would have the phosphate group flanked by *trans*-hydroxyl groups, as in the 5-phosphate, it probably would show the same resistance to acid migration. This ready interconversion of *myo*-inositol 2-phosphate and the soybean phosphate by acid migration is further evidence that the latter is the 1-phosphate.

As evidence that the optical activity of the soybean phosphate is associated with the inositol phosphate and not a contaminant, a study was made relating the change in rotation to phosphate group migration. Since migration from the 1-position to the 2-position will produce a compound with a plane of symmetry, the rotation should disappear, and an equilibrium mixture of the 2-phosphate and the (±)-1-phosphate should result. In the first experiment, the soybean phosphate, 0.02 molar concentration of the free acid, was heated at 100° until the rotation had changed to a small value (Fig. 1). However, a chromatogram indicated that very

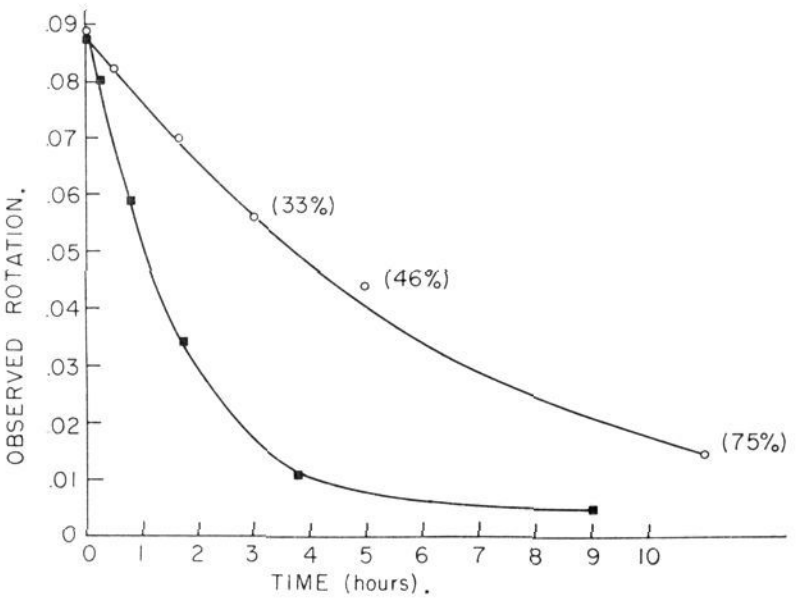


Fig. 1.—The change in rotation of a solution of the soybean inositol phosphate when heated at *p*H 2 and 100° (circles), and in 1 *N* acid at 80° (squares). The numbers in parenthesis indicate per cent. hydrolysis as measured by inorganic phosphate determination. No hydrolysis occurred in 1 *N* acid at 100°.

little migration had occurred (Fig. 2), free inositol and inorganic phosphate were produced and the change in rotation could be accounted for on the basis of phosphate hydrolysis. Other workers[18] have shown that phosphate monoesters can be hydrolyzed at *p*H 4 without appreciable phosphate migration, and it appears that the above conditions used (although certainly more acidic than *p*H 4) gave the same result. When a 0.02 molar solution of the soybean phosphate in 1 *N* hydrochloric acid was heated at 80°, a different result was obtained. The optical rotation decreased rapidly (Fig. 1), no

(18) J. Baddiley, J. G. Buchanan and R. Letters, *J. Chem. Soc.*, 1000 (1958).

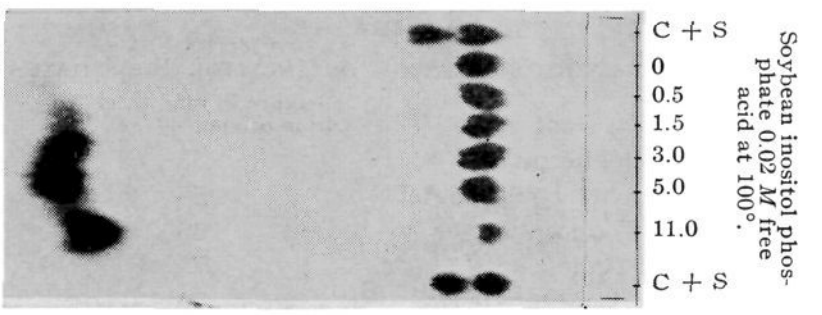


Fig. 2.—Chromatogram of the products obtained by heating the soybean inositol phosphate at 100° at about *p*H 2. C + S is the standard containing *myo*-inositol 1- and 2-phosphates. The fastest component is free *myo*-inositol. Time is in hours.

inorganic phosphate was produced, and an equilibrium mixture of the 1-phosphate and the 2-phosphate resulted (see Fig. 3). Under identical conditions, the 2-phosphate was rapidly converted to an equilibrium mixture of 1- and 2-phosphates (Fig. 3), while the 5-phosphate was not obviously affected (Fig. 3).

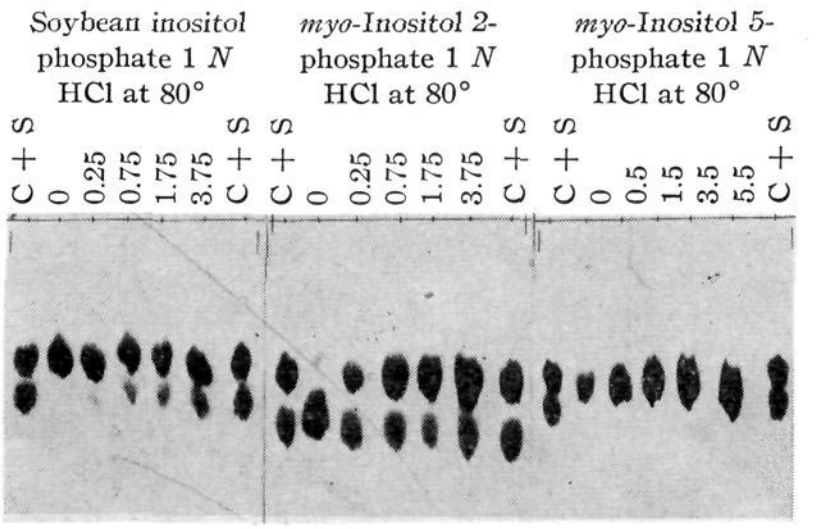


Fig. 3.—Chromatograms of the products obtained at various time intervals by heating inositol phosphates at 80° in 1 *N* hydrochloric acid. C + S is the standard containing *myo*-inositol 1- and 2-phosphates. Time is in hours.

As others have shown,[15,19] the phosphate group does readily cyclize across *trans*-hydroxyl groups in a 6-membered ring under the action of dicyclohexylcarbodiimide (DCC). We have found that *myo*-inositol 5-phosphate does react with DCC to give an unstable cyclic phosphate (presumably the 4,5-phosphate), which is converted to at least one other cyclic phosphate, before it accumulates as the stable 1,2-cyclic phosphate. The intermediates, which were only observed chromatographically, apparently hydrolyze and recyclize repeatedly until the stable product is formed. (See Experimental section and Table I.)

Additional evidence to confirm that the soybean phosphate is the 1-phosphate and not the 4-phosphate was obtained from a study of periodate oxidation rates. Angyal and McHugh[20] have compared the rates of periodate oxidation of the different *myo*-inositol monomethyl ethers. The 1- and 2-methyl ethers oxidize at similar slow rates compared with the 4- and 5-methyl ethers which oxidize much faster. The rate apparently depends on whether one of the three *cis*-hydroxyl groups is sub-

(19) D. M. Brown and H. M. Higson, *ibid.*, 2034 (1957).
(20) S. J. Angyal and D. J. McHugh, *ibid.*, 1423 (1957).

TABLE I

CHROMATOGRAPHIC SEPARATION OF INOSITOL PHOSPHATES

Compound[a]	Distance in mm. travelled from origin—30°, 36 hr.
Soybean monophosphate	55
(±)-*myo*-Inositol 1-phosphate[b]	55
myo-Inositol 5-phosphate	65
myo-Inositol 2-phosphate[c]	75
(−)-Inositol 3-phosphate	95
DL-Glycerol 1-phosphate	100
Pinitol 4-phosphate	110
Glycerol 2-phosphate	115
I-5-P—cyclic 1	180
I-5-P—cyclic 2	205
I-5-P—cyclic 3	228
myo-Inositol 1,2-phosphate	230
Inorganic phosphate	30–50 (streak)

[a] Applied as free acid or as the cyclohexylamine salt. [b] From DCC reaction or acid migration of the 2-phosphate, or from phosphorylation of *myo*-inositol tetraacetate. [c] Iselin, Carter or Desjobert preparation.

stituted or not. We find that the optically active phosphate from soybean as well as the (±)-1-phosphate oxidize at rates indistinguishable from the 2-phosphate but distinctly slower than the 5-phosphate (Fig. 4). Although the 4-phosphate is not

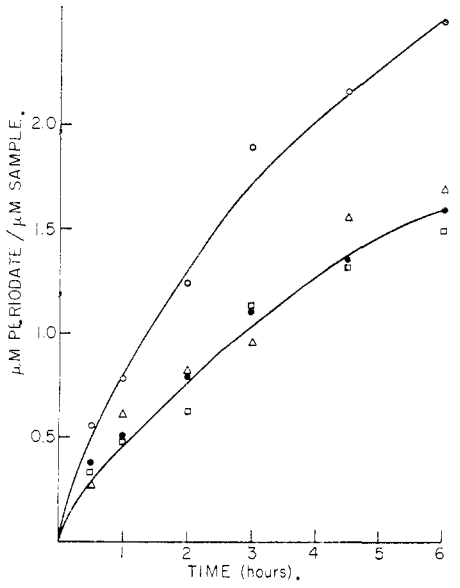


Fig. 4.—Periodate consumption of various *myo*-inositol phosphates as a function of time under standard conditions. *Myo*-inositol 5-phosphate (open circles), (±)-1-phosphate (solid circles), 2-phosphate (open squares) and soybean phosphate (open triangles).

available for comparison, it would be expected to parallel the 5-phosphate as they both have the three adjacent *cis*-hydroxyl groups unsubstituted.

Although our detailed study was carried out on the phosphoinositide from soybean, we have examined chromatographically the alkaline hydrolysis products of phosphoinositides from beef heart,[21] wheat germ and liver. They all showed the same two inositol monophosphates that were found in the soybean preparation. It is probable, therefore, that the inositol is attached in the same manner in the phosphoinositide from these different sources.

(21) We are indebted to Mlle. M. Faure for samples of the crystalline sodium salts of phosphoinositides from beef heart and wheat germ.

Experimental

Chromatography of Inositol Phosphates.—The modification of a solvent system described by Markham and Smith[15,22] was the only one found, of many tried, that would separate the isomeric *myo*-inositol monophosphates. However, it was only effective when the chromatograms were run at 30° or higher for at least 36 hr., and the best results were obtained at 40° with a run of 48 hr. Under these conditions, care had to be taken that drafts did not play on the jars, and this was prevented by covering each chromatography jar in the heated room with a card-board box. At least 1 inch of solvent was kept in the bottom of the jar, although this was done simply by pouring the used solvent from the tray into the bottom of the jar after each run. All chromatography described in this work, therefore, employed as solvent isopropyl alcohol:ammonia:water (70:10:20, v./v.) with Whatman No. 1 paper developed in a descending fashion. A 36 hr. chromatogram at 30° of the various inositol phosphates gave the results shown in Table I. These are expressed as actual distance run from the origin, and when related to the distance travelled by α-glycerol phosphate, these distances may be given a fractional designation with the prefix R_{gp}.

Mixtures of the 1- and 2-phosphate of *myo*-inositol can be separated cleanly by this method. However, the 5-phosphate runs between these two, and although it separates from the 2-phosphate, it forms an elongated spot with the 1-phosphate. When each of the three is run as a pure reference material, there is sufficient difference to tell them apart.

The movement of the inositol phosphates is very sensitive to small amounts of metal ions in the preparations; thus it is best to treat the solution with clean sulfonic acid resin, in the hydrogen form, before spotting the compounds on the paper. The metal ions in the paper did not seem to interfere, since the use of acid washed paper did not affect the separations. The separation is also sensitive to concentration, one or two applications from a capillary of a 0.1 molar solution being a satisfactory amount.

For detection of the compounds on the paper, we used either the Axelrod and Bandurski[23] modification of the Hanes–Isherwood phosphate spray or the Anet and Reynolds[24] modification of the alkaline silver nitrate reagent for glycols. The latter is by far the more sensitive of the two.

Melting Point Determinations.—Attempts were made to distinguish between the different isomeric *myo*-inositol monophosphates on the basis of melting points of the cyclohexylamine salts. Samples in open capillaries were placed in an oil-bath at 170–180°, and the temperature was raised at such a rate that the compound melted within about three minutes at around 200°. The melting point for a particular compound was variable, and melting points of mixtures of different isomers were depressed only slightly, if at all. The compounds usually melted over a range of 2–6°. Representative melting points on different isomers taken at different times are listed below.

Desjobert preparation:	194–203, 197–203 (hydrate).
	201–206, 197–203 (anhydrous).
Carter preparation:	198–210, 204–210 (hydrate).
	198–210, 206–212 (anhydrous).
Soybean preparation:	197–206, 200–205, 206–210, 203–209.

Determination of the Infrared Spectra.—The spectra were determined on potassium bromide pellets containing about 1% of the cyclohexylamine salt of the compound. The instrument used was the Baird-Atomic Model 4-55. The sample was ground carefully in a small agate mortar with powdered potassium bromide (Harshaw Chemical Co.). The spectra of all of the different inositol phosphates studied were distinctly different, thus we have assurance for the first time that these spectra can be used to distinguish the isomers. Although the fine spectra of the compounds differ throughout the range from 625–5000 cm.$^{-1}$,

(22) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952); *Nature*, **168**, 406 (1951); D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

(23) B. Axelrod and R. S. Bandurski, *J. Biol. Chem.*, **193**, 405 (1951).

(24) E. F. L. J. Anet and T. M. Reynolds, *Nature*, **174**, 930 1954.

the most obvious and useful differences occur from 625–1000.

Preparation of *myo*-Inositol 2-Phosphate. (a) **Desjobert Preparation.**—The method of Desjobert[1] was followed for the alkaline hydrolysis of phytic acid. Calcium phytate (Nutritional Biochemicals Corp.), 250 g., was converted to the sodium salt, and the hydrolysis was carried out as described.[1] The barium salt of the resulting inositol monophosphate weighed 14.3 g. This was converted to the free acid by use of Amberlite IR-120(H) resin, and inorganic phosphate was removed by precipitation as magnesium ammonium phosphate. The cations were removed from the filtrate, it was made basic with cyclohexylamine and was then concentrated *in vacuo* to dryness. The residue was crystallized from water by the addition of acetone. The yield was 7.2 g. This product was recrystallized several times and when air-dried, it analyzed for a diamine salt with 4 molecules of water. It was optically inactive.

Anal. Calcd. for $C_{18}H_{39}O_9PN_2 \cdot 4H_2O$ (530): N, 5.28; P, 5.85. Found: N, 5.02; P, 5.74.

The infrared spectrum for the region 625–1000 cm.$^{-1}$ had the following peaks: 964 (medium), 947 (strong), 923 (weak), 891 (weak), 832 (strong), 730 (medium), 681 (medium).

When dried in a high vacuum overnight, the hydrate lost the water of crystallization and analyzed as follows.

Anal. Calcd. for $C_{18}H_{39}O_9PN_2$ (458): N, 6.11; P, 6.77. Found: N, 6.25; P, 6.68.

Some preparations of the 2-phosphate by this method showed by chromatography a minor contaminant that corresponded to the 1-phosphate. Data on the chromatographic properties, melting point and periodate oxidation are given in the figures and tables.

(b) **Carter Preparation.**—The method of McCormick and Carter[3] was followed for the enzymic preparation of inositol phosphate from phytic acid. From 120 g. of calcium phytate, that was converted first to the sodium salt before hydrolysis with the phosphatase from wheat bran, was obtained 44.8 g. of the lead salt. This was converted to the free acid by use of a sulfonic acid resin, cyclohexylamine was added to the acid solution until it was basic, and the mixture was concentrated to a small volume. On addition of acetone, impure crystals formed. These were collected, dissolved in water and the inorganic phosphate precipitated as magnesium ammonium phosphate. The cations were removed from the filtrate, and cyclohexylamine was again added until the *p*H was about 9. Addition of acetone to turbidity caused the product to crystallize. The yield was 3.7 g. When air-dried, the material analyzed for a diamine salt tetrahydrate and was optically inactive.

Anal. Calcd. for $C_{18}H_{39}O_9PN_2 \cdot 4H_2O$ (530): N, 5.28; P, 5.85; H_2O, 13.6. Found: N, 5.26; P, 6.02; H_2O, 14.8.

The infrared spectrum was as follows: 968 (medium), 946 (strong), 922 (weak), 889 (weak), 825 (strong), 732 (medium), 680 (medium broad).

(c) **Iselin Preparation.**[5,25]—This preparation of *myo*-inositol 2-phosphate was carried out as described by Iselin except that the product never was isolated as the free acid. After hydrogenolysis of the phenyl groups and saponification of the acetyl groups, the product was converted to the cyclohexylamine salt and crystallized from water by the addition of acetone. The infrared spectrum and chromatographic property of this product were identical with those of the substances obtained by procedures (a) and (b) above.

Anal. Calcd. for $C_{18}H_{39}O_9PN_2 \cdot 4H_2O$ (530): N, 5.28; P, 5.85. Found: N, 5.10; P, 5.71.

The infrared spectrum for the region 625–1000 cm.$^{-1}$ was: 968 (medium), 946 (strong), 922 (weak), 889 (weak), 825 (strong), 732 (medium), 682 (medium broad).

Preparation of the Soybean Phospholipid Inositol Phosphate.—Commercial alcohol-insoluble soybean phospholipid (a gift from the American Lecithin Company, Inc., which is gratefully acknowledged) was fractionated by the method of Hawthorne and Chargaff.[4] From 300 g. of the starting material, we obtained 60–70 g. of the purified phosphoinositide. This was dissolved in 2 liters of 2 *N* potassium hydroxide, and the solution was then refluxed for 1 hr. After it had cooled, the solution was placed in an ice-bath and the potassium was precipitated by the addition of 60% perchloric acid. The filtrate from the potassium perchlorate was extracted with ether, and was then brought to *p*H 6.5 with potassium hydroxide, when 20% lead acetate was added to precipitate the phosphates. The lead salts were collected and decomposed with hydrogen sulfide, and the lead sulfide was removed by filtration. Sufficient magnesium acetate was added to the filtrate to precipitate the inorganic phosphate, and the solution was made basic with concentrated ammonia. After removal of the precipitate, the solution was concentrated to remove excess ammonia and was then treated with Amberlite IR-120(H) to remove all cations. The acid filtrate was brought to *p*H 9 with cyclohexylamine, and the solution was concentrated to dryness. The sirup weighed 28.6 g.

A chromatogram of the product at this stage showed about five components that contained organic phosphate, including two inositol monophosphates, two glycerol phosphates and one very minor component that might be an inositol diphosphate. The product at this stage could be worked up in two ways. It could be dissolved in a small amount of water, and the inositol phosphate precipitated by the addition of acetone. A second, and preferable, method was to triturate the residue with 300 ml. of boiling ethanol, and then filter off the solid by suction. This gave 7.0 g. of material that was a mixture of inositol phosphate and glycerol phosphate. A second extraction of the solid with the same amount of ethanol left 5.3 g., while a third extraction left 4.3 g. This purified inositol phosphate was dissolved in 10 ml. of water, the solution was filtered by suction, and the funnel was washed with 5 ml. of water. Acetone was added to the combined filtrate to turbidity. Crystallization occurred immediately. The product was collected by suction filtration and dried in air. It weighed 2.2 g. Addition of more acetone to the mother liquor gave a second crop of 0.7 g.

The crystalline inositol phosphate was recrystallized twice from water by the addition of acetone to remove all traces of glycerol phosphates and of *myo*-inositol 2-phosphate that are minor contaminants. The pure substance crystallized as an anhydrous diamine salt.

Anal. Calcd. for $C_{18}H_{39}O_9N_2P$ (458): N, 6.11; P, 6.77. Found: N, 5.84, 6.32; P, 6.61, 6.68.

The substance was optically active, and the same value for optical activity was found on three different preparations of the compound as well as on a sample that had been further purified by chromatography on a column of powdered cellulose. The value was $[\alpha]^{25}_{589}$ +3.4° (*c* 3, brought to about *p*H 9 with cyclohexylamine); which changed, when the solution was made acidic, to −9.8° (*c* 3, brought to *p*H 2, by adding Dowex-50(H) to remove the amine).

The infrared spectrum showed the following bands: 970 (strong), 958 (shoulder), 948 (strong), 925 (weak), 902 (medium), 893 (weak), 882 (weak), 842 (strong), 745 (strong), 680 (medium broad).

Phosphorylation of *myo*-Inositol 1,4,5,6-Tetra-O-acetate.—One gram of the tetraacetate, m.p. 139–141°,[16] was dissolved in 5 ml. of dry pyridine and 1.0 g. of diphenylphosphorochloridate was added. The container was stoppered and left at 40° for four days. A few drops of water were added, and after 10 minutes the reaction mixture was diluted with 50 ml. of benzene and 50 ml. of water. The mixture was shaken, and the two layers were then separated in a separatory funnel. The benzene layer was washed with 1 *N* hydrochloric acid, 1 *M* sodium bicarbonate and with water. It was dried over sodium sulfate and concentrated to dryness *in vacuo* but yielded no benzene soluble material.

The water layer from above was made basic by addition of 7.0 ml. of 3.9 *N* potassium hydroxide. The basic solution was refluxed for 30 minutes, then it was cooled and the pyridine was distilled off. More water was added, and the solution was refluxed another 15 minutes, after which it was concentrated to dryness *in vacuo*. The residue was extracted with 50 ml. of hot absolute ethanol, and the ethanol solution was decanted from the insoluble gum. The gum was dissolved in water, the solution was treated with Dowex 50(H) to remove cations, cyclohexylamine was added to the eluate to bring the *p*H to about 9, and this solution was concentrated to dryness. The residue was taken up in a few ml. of water and acetone was added to the solution to cause turbidity. After 18 hr. at room temperature, crystals

(25) Prepared by Dr. S. J. Angyal, to whom we are indebted for a sample of the material.

had formed. These were collected on a büchner funnel, washed with acetone and dried in air. The yield was 0.5 g. (38%), of the cyclohexylamine salt of an inositol phosphate. Recrystallization from water by the addition of acetone gave 0.35 g., which was dried in a high vacuum at room temperature for 1 hr.

Anal. Calcd. for $C_{18}H_{39}O_9N_2P$ (458): N, 6.11; P, 6.77. Found: N, 5.98; P, 6.87.

The infrared spectrum in potassium bromide pellet was identical with the spectrum of the product obtained by the action of DCC on *myo*-inositol 2-phosphate (Desjobert preparation) and was very similar to that of the soybean phosphate. The infrared spectrum showed peaks at: 963 (strong), 958 (shoulder), 942 (shoulder), 921 (weak), 900 (medium), 890 (weak), 833 (strong), 745 (strong), 680 (medium broad). It had chromatographic properties identical with the soybean phosphate, and on treatment with 1 *N* acid at 80°, it was converted partially to the 2-phosphate. Thus, although the synthesis cannot be considered to be a definitive one, we know that the product is not the 2- or 5-phosphate. Since only positions 1 and 2 were available for phosphorylation and since the product is readily converted to the 2-phosphate by acid migration, it follows that the most likely structure is that of *myo*-inositol (±)-1-phosphate.

Reactions with Dicyclohexylcarbodiimide. (a) **For Chromatographic Study.**[15]—About 10 mg. of the cylcohexylamine salt of the inositol phosphate was dissolved in 1.0 ml. of water, the solution was mixed for a short time with 0.5 ml. of Amberlite IR-120 (pyridinium form) to remove the cyclohexylamine, the resin was removed by filtration and the filtrate was concentrated to dryness. Ten drops of water were added to dissolve the sample, followed by 1.5 ml. of pyridine. To start the reaction, 20 mg. of DCC was added, and the mixture was shaken while the reaction proceeded. At intervals, samples of the reaction mixture were spotted directly onto Whatman No. 1 filter paper strips for chromatography. Dicyclohexylurea usually began to precipitate within an hour. The chromatograms were developed in the isopropyl alcohol–ammonia solvent, and the spots were detected with the phosphate spray or the silver nitrate dip. The R_f's of the compounds were approximately as described[15]: monophosphates 0.05–0.1, cyclic phosphates 0.4–0.5 and (presumed) N-phosphorylureas 0.8.

It was noticed that, during the first few hours of the reaction of *myo*-inositol 2-phosphate with DCC, some of the 1-phosphate appeared to be formed; this was before all of the 2-phosphate had reacted. The reverse occurred when the starting compound was the pure 1-phosphate. Apparently the cyclic phosphate was opening up during the reaction to form a mixture of isomers. The starting material was eventually all converted to the cyclic phosphate which was stable enough to be isolated (see below) and which gave only 1- and 2-phosphate on hydrolysis.

myo-Inositol 5-phosphate gave after 1.75 hr. a new product with R_{gp} 1.80, after 4.75 hours two products with R_{gp} 2.05 and 2.28 and after 10 hours a main product with R_{gp} 2.30.

(b) **For Preparative Work.**[26]—About 250 mg. of *myo*-inositol 2-phosphate cyclohexylamine salt (Desjobert preparation) was dissolved in water and converted to the pyridine salt by passage through a column containing 10 ml. of Amberlite IR-120 (pyridinium form). The eluate was concentrated to 5 ml., and 50 ml. of pyridine was added followed by 1.0 g. of DCC. The solution was left for 18 hr. at room temperature. (A 3 hr. sample showed, on chromatography, some inositol monophosphate remaining and a trace of the urea adduct.[15] The 18 hr. sample showed no inositol monophosphate but a fair amount of urea adduct.) The solution was diluted with 100 ml. of water, and the mixture was extracted three times with 50-ml. portions of ether. The water layer was then concentrated *in vacuo* to 25 ml., and one equivalent of cyclohexylamine per mole of inositol phosphate was added. The solution was concentrated further to about 5 ml. at 20° bath temperature, about 100 ml. of absolute ethanol was added, the mixture was concentrated to a thin sirup. This was taken up in a mixture of ethanol and acetone (amounts uncertain) and left to crystallize. Crystals formed after several hours. After 18 hr. at 5°, these were filtered off and dried in air. The weight was 85 mg. and the compound melted at 130–140° with foaming. The crystals were recrystallized from a small amount of water by addition of acetone. The product was dried in a desiccator over calcium chloride, then in a high vacuum at room temperature. It picked up water when exposed to air, but the dried sample analyzed correctly for the anhydrous monoamine salt.

Anal. Calcd. for $C_{12}H_{24}O_8NP$ (341): N, 4.11; P, 9.09. Found: N, 3.98; P, 9.12.

The cyclic phosphate was completely hydrolyzed when heated at 100° for 15 minutes in 0.1 *N* acid or base, and the product was a mixture of *myo*-inositol 1-phosphate (about 80%) and 2-phosphate (about 20%). The infrared spectrum of the reisolated DL-1-phosphate had the following peaks: 980 (strong), 960 (strong), 940 (shoulder), 923 (weak), 905 (medium), 890 (weak), 835 (strong), 736 (medium).

Acid Migration of Inositol Phosphates.—No evidence for migration was found when 0.01 molar aqueous solutions of the free acids of the inositol phosphates were heated at 50° for as long as 60 hr. However, a 0.1 molar solution of the free acid, obtained by treating a solution of the cyclohexylamine salt with Dowex-50(H), when heated at 90° did give evidence, on chromatography, of the formation of the 2-phosphate from the 1-phosphate and of 1-phosphate from the 2-phosphate. *myo*-Inositol 5-phosphate, (−)-inositol 3-phosphate and pinitol 4-phosphate did not show the formation of another isomer.

A 0.9% solution of the soybean phosphate, cyclohexylamine salt, in water was treated with Dowex-50(H) to remove the amine and give a 0.02 molar solution of the free acid in water. This solution was heated in a boiling waterbath, and the rotation was observed at various time intervals. The change in rotation with time is shown in Fig. 1. At each time that the rotation was observed, the solution was spotted on paper for chromatography. The composition of the mixture at each time is shown in the accompanying Fig. 2. Another solution of the same concentration, but in 1 *N* hydrochloric acid was heated at 80°. The change in rotation is shown in Fig. 1, and a chromatogram of the products are shown in Fig. 3. The 2-phosphate and 5-phosphate were also heated in 1 *N* acid, with the results shown in Fig. 3.

Comparison of Inositol Phosphates by Periodate Oxidation.—The sample of inositol phosphate cyclohexylamine salt, 7.5–10 micromoles, was dissolved in water in a 10-ml. volumetric flask. At zero time, 2.00 ml. of 0.024 *M* sodium periodate was added followed by water to 10 ml. The reaction flask was incubated in the dark, and at each time interval a 1.00-ml. aliquot was pipetted into a quenching flask which contained 5.00 ml. of 0.0126 *M* sodium arsenite, 5 ml. of phosphate buffer *p*H 7, 0.5 ml. of 4% sodium iodide and 2 ml. of starch solution. After at least 15 minutes at room temperature, the aliquots were titrated with iodine solution having approximately the same normality as the arsenite. The results of such titrations are shown in Fig. 4. Variations on the method were to run the oxidation at 0° instead of at room temperature and in the presence of 0.25 ml. of 0.1 *N* hydrochloric acid instead of as described above.

The results in Fig. 4, as well as in other determinations, showed that the Carter, Desjobert and soybean inositol phosphates as well as the synthetic 1-phosphate, could not be distinguished. The *myo*-inositol 5-phosphate, however, was oxidized at a distinctly faster rate. Although the figure shows data collected to 6 hr., the oxidations were followed for 24–48 hr. with the same general results.

Alkaline Hydrolysis of Phosphoinositides for Chromatographic Analysis.—Small samples of phosphoinositide, 0.1–1.0 g., were dissolved in 2 *N* potassium hydroxide, 50 ml. per g., and the solution was refluxed for 1 hr. Octanol was usually added to minimize foaming. The cooled solution was treated with excess Dowex-50(H) to remove the cations, and the acid filtrate was extracted with ether. The water layer was made basic with cyclohexylamine and concentrated to dryness. The residue was extracted with hot absolute ethanol, in which case most of the inositol phosphate was insoluble and most of the glycerol phosphate and other contaminants were dissolved. The insoluble cyclohexylamine salt of inositol phosphate was dissolved in a little water, and Dowex-50(H) was again added to ensure removal of all metal ions which otherwise interfered with the chromatography. This acid solution was used directly or

(26) A similar study has been reported recently by Th. Posternak, *Helv. Chim. Acta*, **41**, 1891 (1958).

was brought to *p*H 8 with cyclohexylamine before chromatography.

By the above analysis, hydrolysates of crystalline phosphoinositides from beef heart, wheat germ (supplied by Dr. M. Faure) and a crude phosphoinositide from liver (isolated from Viobin liver fat by solvent fractionation), all showed the same two inositol monophosphates in the same proportions. The two compounds are tentatively identified as *myo*-inositol 1-phosphate (major component) and *myo*-inositol 2-phosphate (minor component).

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The Inhibition of β-Amylase by Ascorbic Acid[1]

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The effect of ascorbic acid on β-amylase has been shown to be the result of the formation of an inactive cuprous enzyme. The extent of this inactivation at a fixed ascorbic acid concentration has been shown to be proportional to the copper concentration. The same type of inactivation has been encountered with bisulfite-copper ion solutions demonstrating that this effect is general rather than specific for ascorbic acid. Ascorbic acid actually decreases the extent of inhibition caused by mercuric ions. In this case the potent inactivator, mercuric ion, is reduced to the less active mercurous ion. This effect again demonstrates that the ascorbic acid acts through the reduction of cupric ion to cuprous followed by the formation of the inactive cuprous enzyme compound.

Introduction

The inhibition of β-amylase by ascorbic acid was reported some years ago by Purr[3] and Hanes.[4] The problem was later studied by Seshagirirao and Giri[5] who divided the action in two parts. They reported a reversible inhibition in the presence of ascorbic acid alone and an irreversible inactivation in the presence of ascorbic acid plus cupric ion. Ito and Abe[6] further divided the inactivation into a reversible and an irreversible phase.

Hanes suggested a reaction between the dienol of the ascorbic acid and the sulfhydryl of the enzyme as the cause of inhibition. Seshagirirao and Giri showed that any substance which prevented the oxidation of ascorbic acid also prevented the inhibition. Most of these compounds presumably complexed with the cupric ion. However, no explanation for the inhibition phase was offered. They again suggested the same possible reaction put forth by Hanes.

More recently Rowe and Weill[7] investigated the nature of the inhibition. They found that the inhibition was non-competitive with respect to ascorbic acid. This was contrasted with typical sulfhydryl reagents such as *p*-chloromercuribenzoate which show a competitive type of inhibition. These results suggest that the inhibition is not the result of any direct reaction between the dienol of the vitamin and the sulfhydryl of the enzyme.

The results of the current study demonstrate that the difference between the inhibition and the so-called irreversible inactivation is merely one of degree. All of the data suggest that the function of the ascorbic acid is to reduce cupric ion to cuprous. This then reacts with the enzyme to inactivate it. In this respect the results are similar to those reported by Mapson[8] for urease in that inactivation is due to the formation of a cuprous mercaptide complex.

Results and Discussion

In previous work[6] it has been shown that ascorbic acid resulted in a reversibly non-competitive inhibition with the enzyme in the absence of added cupric ions. The inhibition is dependent upon the oxidation of ascorbic acid. Raising the *p*H will increase the rate of oxidation of ascorbic acid and subsequently the amount of inhibition. Figure 1 shows the increased inhibition by ascorbic acid as the *p*H is increased.

Any substance which decreases the rate of oxidation of ascorbic acid should also reduce the extent of the inhibition. Mapson[9] has shown that KCl will decrease the rate of oxidation of ascorbic acid, presumably through the formation of a $CuCl_2^-$ ion with cuprous ion.

The effect of KCl in retarding the oxidation of ascorbic acid at *p*H 4.5 has been verified. This same concentration of KCl also removes a considerable amount of the inhibition of enzyme activity caused by the vitamin. If an antioxidant does not retard the oxidation of ascorbic acid, it does not remove the inhibiting effect of the ascorbic acid. Dihydrocaffeic acid is an example of this type.

No cupric ions have been added to the inhibition studies previously reported; however, the decrease in inhibition caused by KCl suggested that cupric ions might be present in very small quantities. Large volumes of the reaction mixture were concentrated, and it was demonstrated that there was about 1 part of copper per 20 million parts of solution. While this amount of copper is not sufficient to cause an "irreversible" inactivation, it is sufficient to induce a reversible inactivation or inhibition.

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(2) Submitted in partial fulfillment of the requirements for the Doctor of Philosophy Degree.

(3) A. Purr, *Biochem. J.*, **28**, 1141 (1934).

(4) C. S. Hanes, *ibid.*, **29**, 2588 (1935).

(5) P. Seshagirirao and K. V. Giri, *Proc. Ind. Acad. Sci.*, **16B**, 190 (1942).

(6) M. Ito and M. Abe, *J. Agr. Chem. Soc. Japan*, **27**, 486, 762 (1953); **28**, 15, 368, 751 (1954).

(7) A. W. Rowe and C. E. Weill, *Cereal Chem.*, **35**, 289 (1958).

(8) L. W. Mapson, *Biochem. J.*, **40**, 240 (1946).

(9) L. W. Mapson, *ibid.*, **39**, 228 (1945).