SUMMARY

1. β_1 -Lactoglobulin has been examined for heterogeneity by the variable-solvent and constant-solvent solubility tests.

2. Both tests indicate that β_1 -lactoglobulin contains two proteins, the minor accounting for about 10 % of the total.

3. The theoretical basis of solubility tests and their application to salt fractionation are discussed.

4. Fractionation of β_1 -lactoglobulin has produced the major component in a more nearly pure state.

5. The behaviour of the major component on U-tube electrophoresis is discussed.

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The Isolation of Inositol Monophosphate from Liver

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Phospholipids containing inositol (phosphoinositides) are known to occur in a great variety of animal tissues such as ox brain (Folch, 1949), ox heart (Faure & Morelec-Coulon, 1954), liver (Macpherson & Lucas, 1947; McKibbin, 1956; Hawthorne & Hawthorne, 1955) and egg yolk (Malangeau, 1955; Dils & Hawthorne, 1956). Several recent observations emphasize the high turnover rate of the phosphoric acid component in the phosphoinositides of brain and pancreas (Dawson, 1954; Hokin & Hokin, 1955*a, b*, 1956) as compared with the other phospholipids present. Also, certain drugs seem to influence the turnover of phospholipids at pharmacologically significant concentrations (Magee, Berry & Rossiter, 1956; Ansell & Dohmen, 1956, 1957).

It seems therefore that phosphoinositides may have biochemical functions of dynamic rather than structural importance. A study of the biosynthesis of phosphoinositides might contribute to a better understanding of their metabolic significance.

The evidence is presented in this paper for the occurrence of free inositol monophosphate in liver; this has already been reported as a preliminary communication (Hawthorne & Hübscher, 1956).

MATERIALS AND METHODS

Paper chromatography. The solvent system propan-2-ol-acetic acid-water (3:1:1, by vol.) was used for the separation of ethanolamine, serine and amino acids (descending 18 hr.), of choline (descending 6 hr.) and of inositol and glycerol (descending 11 hr.). For the separation of inositol and glycerol, *n*-butanol-ethanol-water (4:1.9:9, by vol.; ascending 16 hr.; Hough, 1950) was also used.

The chromatograms were either sprayed with the ninhydrin reagent for the detection of ethanolamine, serine and amino acids, or according to the method of Chargaff, Levine & Green (1948) for the detection of choline. Inositol, glycerol and sugars were detected by dipping the chromatograms according to the method of Trevelyan, Procter & Harrison (1950). The solutions for dipping the chromatograms were prepared as follows. Solution 1: 0.1 ml. of saturated aq. AgNO₃ soln. was added to 20 ml. of acetone. Water was added with constant stirring until all the precipitate had just redissolved. Solution 2: saturated aq. NaOH soln. was diluted with 50 vol. of ethanol. The papers were dipped in solution 1, allowed to dry, and then dipped in solution 2. Inositol, glycerol and sugars showed up as dark-brown spots on a yellow background.

The phosphoric acid esters were separated by paper chromatography in the above propan-2-ol-acetic acid solvent and also in propan-1-ol-aq. NH₃ soln.-water

(6:3:1, by vol.). The papers were sprayed by the method of Wade & Morgan (1953).

Determination of inositol. Inositol was determined according to the method of Böhm & Richarz (1954) slightly modified as follows. Inositol, separated chromatographically on paper, was eluted and oxidized with an excess of HIO_4 . The unused HIO_4 was determined in phosphate buffer, pH 7.4, with $\text{Na}_2\text{S}_2\text{O}_3$. The theoretical uptake is 6 moles of HIO_4 /mole of inositol. Under the experimental conditions described below the uptake of HIO_4 was 96–98% of theory.

A volume (2 ml.) of 0.01M-periodic acid was added to 15 ml. of water containing up to 300 μg . of inositol. The mixture was kept in a glass-stoppered vessel for 2.5 hr. at 50°. After incubation, 2 ml. of phosphate buffer [12% (w/v) Na_2HPO_4 in 0.2N- H_2SO_4] and 2 ml. of the iodide reagent [5% (w/v) KI in a half-saturated aq. NaCl soln.] were added. The liberated iodine was titrated with 0.005N- $\text{Na}_2\text{S}_2\text{O}_3$. The accuracy of this method was $\pm 3\%$.

For the determination of esterified inositol alone or in impure fractions the sample was hydrolysed in 5.8N-HCl in a sealed tube at 110° for 20 hr. The phosphate esters of inositol are very stable and these conditions were necessary to ensure complete hydrolysis. A sample of the hydrolysate was analysed for orthophosphate. Other samples containing about 1 μmole of P were applied to paper chromatograms together with an inositol marker. After development of the chromatogram, the areas corresponding to the marker were cut into small pieces and eluted four times with 4 ml. portions of water. Then HIO_4 was added and the determination was performed as described above. For comparison, a known amount of inositol and pure inositol monophosphate were taken through the same procedure. The latter was prepared from phytate and purified by means of an ion-exchange resin (unpublished observations).

Extraction of the acid-soluble phosphates from liver and other tissues. The liver or other tissues were removed from the rats and guinea pigs as soon as possible after death and homogenized immediately. The ox tissues were obtained immediately after slaughtering and kept in ice during transport. They were treated as described below within 1 hr. from death. The organs were freed from large vessels and connective tissue, cut into small pieces and homogenized in 10 vol. of 5% (w/v) trichloroacetic acid. The protein-free filtrate was treated with 0.1 vol. of 20% (w/v) lead acetate and this mixture was made just alkaline to phenolphthalein with 5N-NaOH. The heavy white precipitate was collected by centrifuging and decomposed with H_2S . To 1 l. of the clear, slightly yellow filtrate, freed from excess of H_2S by aeration, 25 ml. of 2M-barium acetate was added and the pH adjusted to 8 with 5N-NaOH. The mixture was kept at 0° for 2 hr. The precipitate, which contained most of the inorganic phosphate and some of the organic phosphates, was filtered off and the filtrate was again treated with lead acetate, followed by decomposition of the precipitated lead salts with H_2S . The solution obtained after filtering off the lead sulphide was aerated free from excess of H_2S , neutralized (final pH, 7.5) and kept at 0°. It was used as starting material for further purification on ion-exchange resins.

It was later found that the starting material was contaminated with peptides. For this reason the solution was stirred with charcoal (2 g./l. of solution) for 10 min. and filtered before applying to ion-exchange columns. The charcoal was previously boiled in 6N-HCl and washed with

water until free from phosphate. Inositol monophosphate is not adsorbed on charcoal.

Ion-exchange resins. The ion-exchange resins used in this investigation were Deacidite FF (–16 + 50 mesh, formate form; column size 1 cm. $\times$ 10 cm.; rate of flow 5 ml./min.), Zeo-Karb 225 (–16 + 50 mesh, formate form; 2 cm. $\times$ 30 cm. rate of flow 5 ml./min.) and Nalcite SAR (–80 + 200 mesh, formate form; column size 1 cm. $\times$ 12 cm.; the rate of flow did not exceed 1 ml./min.). In large-scale preparative work, columns of the Nalcite SAR resin with dimensions of 2.5 cm. $\times$ 23 cm. were used. The anion-exchange resins were washed with 3N-HCl until free from material with an absorption at 260 m μ and then with 3N-ammonium formate until free from chloride. The excess of ammonium formate was removed by washing with water. All water used was freed from ions by passage down a mixed-bed resin column (Biodeminrolit, The Permutit Co. Ltd., London, W. 4).

After loading, the columns were washed with 200–500 ml. of water. For elution, two different systems were used. The first consisted of mixtures of formic acid and ammonium formate and the second of mixtures of sodium borate and ammonium formate (Khym & Cohn, 1954). Fractions (25 ml.) were collected and samples were analysed for phosphate.

Phosphate determinations. These were done according to the method of King (1932).

Borate. This was detected by the mannitol–bromothymol-blue test (Vogel, 1942). The sample was almost neutralized by adding acid or alkali until the indicator turned green. One drop of a 50% (w/v) solution of mannitol was added. If borate was present a yellow coloration was obtained, i.e. the pH decreased owing to the complexing of the very weak boric acid with mannitol to give a relatively strong acid. Under these conditions 0.001 μg . of boron can be detected in 0.03 ml. of solution.

Microanalytical determinations. These were performed by the Mikroanalytische Laboratorium, Bonn, Germany.

Inositol monophosphate. This was prepared from phytate as described by McCormick & Carter (1952).

Acid phosphomonoesterase. For the purification of this enzyme from prostate gland the procedure of Markham & Smith (1952) was followed. The assay system consisted of 0.78 ml. of 0.1N-acetate buffer, pH 5.2, 0.02 ml. of enzyme and 0.2 ml. of a solution containing 1.5–3.0 μmoles of phosphoric acid esters; it was incubated at 37°. Samples were withdrawn at intervals of 10 min. and analysed for inorganic phosphate.

RESULTS

Separation on ion-exchange resins

From several anion-exchange resins tested Nalcite SAR was chosen because it gave the best separation and reproducible results. The separation of inositol mono- and di-phosphate, glycerophosphate, inorganic phosphate, glycerylphosphorylinositol and glycerylphosphorylserine was investigated previously (Hawthorne & Hübscher, unpublished work).

In Fig. 1 a diagram of a typical separation of the acid-soluble phosphates is shown. For elution, the formic acid–ammonium formate system was used.

Peak *B* represents inorganic phosphate. Of all other fractions analysed only peaks *A* and *D* contained inositol. The amount of inositol present in these two fractions was appreciable. The presence of free inositol can be excluded since 100 mg. of inositol was quantitatively removed from the column by washing it with 200 ml. of water. Free inositol would therefore pass right through the column when it was loaded and washed. The positions of these peaks, which were eluted with 0.01 *N*-formic acid–0.03 *N*-ammonium formate and with 0.1 *N*-formic acid–0.5 *N*-ammonium formate respectively, correspond to those of inositol mono- and di-phosphates prepared from phytate. A determination of the molar ratio of inositol to phosphoric acid in the pooled fractions of peak *A*, however, gave low values. The inositol:phosphoric acid ratio usually varied between 0.1:1.0 and 0.2:1.0. Small amounts of serine, ethanolamine and glycerol were present in this fraction. Amino acids also occurred if the charcoal treatment of the starting material was omitted. The amino acids probably originated from a contaminating small polypeptide which could be removed from fraction *A* by stirring it with activated charcoal. The total phosphate content of fraction *A* was not altered by this procedure.

All attempts to separate the different compounds constituting peak *A* by re-application to the same resin, either ammonium formate or formic acid being used for elution, failed. The sodium borate–ammonium formate system (Khym & Cohn, 1954) was, however, very effective in this respect.

Fig. 2 gives an example of the separation of the compounds constituting peak *A*. In this diagram peaks 1–3 consist of pure inositol monophosphate, glycerophosphate and inorganic phosphate respectively. The position of these peaks corresponds

exactly with those of authentic inositol monophosphate, β -glycerophosphate and of inorganic phosphate, as can be seen from Fig. 3. Peak 2 was shown to contain only glycerophosphate. Since the naturally occurring compound has the α configuration, it must be assumed that the α and β isomers do not separate under the conditions used. Peaks 4–6

Fig. 2. Separation of fraction *A*. Conditions were as in Fig. 1. Eluted with the sodium borate–ammonium formate system. Fractions (25 ml.) were collected. Peak 1 represents inositol monophosphate, peak 2 glycerophosphate, peak 3 inorganic phosphate, peaks 4 and 5 unknown phosphates. Peak 6 contains small amounts of serine and ethanolamine. Actual values for the broken portion of peak 6, which is not to scale, are (left to right) 1375, 9850 and 1875 μ g. of P/50 ml. of fraction.

Fig. 1. Separation of the acid-soluble phosphates of liver. The ion-exchange resin used was Nalcite SAR (–80 + 200 mesh, formate form); size of column was 1 cm. $\times$ 12 cm.; rate of flow approx. 1 ml./min. Before elution the column was washed with 250 ml. of water. Fractions (25 ml.) were collected. Peak *B* represents inorganic P. Esterified inositol was present in peaks *A* and *E* only.

Fig. 3. Separation of inositol monophosphate, glycerophosphate and inorganic phosphate. Conditions were as in Fig. 1 except for the size of the column, which was 8 cm. $\times$ 1 cm. Inositol monophosphoric acid (46 μ moles), 88 μ moles of β -glycerophosphoric acid and 100 μ moles of phosphoric acid were applied and eluted with the sodium borate–ammonium formate system. Fractions (20 ml.) were collected. Peaks *a*, *b* and *c* consist of inositol monophosphate, glycerophosphate and inorganic phosphate respectively.

did not contain any inositol or glycerol. In peak 6 small amounts of serine and ethanolamine were found; each of them corresponds, on a molar basis, to about 10 % of the total phosphate present in this fraction. No further investigations were made to elucidate the nature of the compounds in peaks 4-6, but peak 6 still represents a mixture of several compounds.

Identification and characterization of inositol monophosphate

The pooled fractions of peak 1 were applied to a Deacidite FF column, borate ions were removed with 200 ml. of 0.5N-formic acid and inositol monophosphate (or the other phosphate esters present in peak 2 and peaks 4-6) with 200 ml. of 0.5N-formic acid-0.2N-ammonium formate. Finally the ammonium ions were removed by passing the solution through a Zeo-Karb 225 column (H^+ form) and the eluate was taken to dryness under low pressure at room temperature, the remaining formic acid thus being removed. The dry, hygroscopic material was taken up in about 2 ml. of water and the pH of this solution was adjusted to 7.5 with freshly prepared 0.1N-Ba(OH)₂. The barium salt of inositol monophosphate was precipitated by addition of an equal volume of 95 % (v/v) ethanol. The barium salt was washed with 95 % ethanol and ether, dried and reprecipitated several times from water. The recoveries were about 75 % of theory.

The barium salt of inositol monophosphate was hydrolysed in 5.8N-HCl at 110° for 20 hr. and analysed for its content of phosphate and inositol. In Table 1 the results of this and other analyses are compared with those obtained on pure inositol monophosphate prepared from phytate.

Distribution in tissues

Inositol monophosphate was found to be present in the liver of the rat, guinea pig and cow, in ox heart and ox brain. These were the only tissues examined. The inositol monophosphate was isolated from each by means of ion-exchange resins as

described above. Quantitative determinations were performed on ox liver only, the concentration of inositol monophosphate actually isolated being 4-5 μ moles/100 g. of fresh tissue. This value is smaller than those obtained for phosphorylcholine in various tissues (Dawson, 1955), but the true concentration of the inositol compound in liver will be somewhat higher than the value quoted.

Properties of inositol monophosphate

As would be expected from the complete saturation of the carbon ring in inositol monophosphate, no absorption was observed over a range 400-220 m μ except for slight end-absorption between 220 and 230 m μ .

Inositol monophosphate was hydrolysed very slowly by the acid phosphatase from prostate gland. Under identical experimental conditions, phosphoric acid was liberated from inositol monophosphate at only about 1 % of the rate of hydrolysis of glycerophosphate. This is surprising since phenyl phosphate and glycerophosphate are both suitable substrates for this not very specific enzyme.

Pure preparations of inositol monophosphate from liver or phytate applied to paper chromatograms gave R_f values which were in good agreement. This was found for the two solvent systems described. Though Desjobert & Petek (1956) described several solvent systems for the separation of phosphoric esters of inositol, it seems from our experiments that the identification of inositol monophosphate in mixtures of other phosphate esters by means of paper chromatography may easily lead to erroneous results owing to the presence of other compounds with similar low R_f values.

DISCUSSION

The phosphoinositides of liver (McKibbin, 1956) and also of heart are known to have a structure very similar to that of phosphatidyl-choline, -ethanolamine and -serine, the molar ratios of fatty acid:glycerol:P:inositol being 2:1:1:1. It seems

Table 1. *Analyses of barium inositol monophosphate from various sources*

Compound	Inositol (%)	P (%)	C (%)	H (%)	Molar ratio P:inositol
Inositol monophosphate from liver	41.1	7.4	17.0	3.2	1:0.95
Inositol monophosphate from phosphoinositides*	41.2	7.4	—	—	1:1
Inositol monophosphate from phytate	42.0	7.4	17.0	3.0	1:0.98
Theoretical for $C_6H_{11}O_9PBa, 1.5H_2O$	42.7	7.35	17.06	3.29	1:1

* These values are taken from McKibbin (1956). The inositol monophosphate was prepared from a highly purified phosphoinositide.

possible therefore that inositol monophosphate in liver and heart tissues may be either a breakdown product of this phospholipid or a precursor in its biosynthesis. Brain diphosphoinositide, on the other hand, has a somewhat more complex structure, based on inositol diphosphate (Folch, 1949). Sloane-Stanley (1953) and Rodnight (1956) claimed that inositol monophosphate was liberated from diphosphoinositide by the action of an enzyme present in brain and other tissues, though the monophosphate was not actually isolated in their experiments.

The work of Dawson (1954) and of Hokin & Hokin (1955a, b, 1956) regarding the high turnover of the phosphoric acid component in phosphoinositides, and the fact that phosphorylcholine is known to be a precursor in the biosynthesis of lecithin (Kennedy & Weiss, 1956), suggest that inositol monophosphate may play a similar role in the biosynthesis of phosphoinositides. Evidence was recently obtained in this laboratory that an enzyme catalysing the formation of inositol monophosphate from inositol and adenosine triphosphate is present in extracts of rat liver (unpublished observations).

From the behaviour of inositol monophosphate from liver or phytate on ion-exchange resins, no conclusion can be drawn as to which position of the inositol ring is esterified with phosphoric acid. Theoretically it should be possible to separate the isomers of inositol monophosphate on a resin column in the presence of borate, since the ease of formation of borate complexes will vary with the position of the phosphate group. Attempts to resolve the inositol monophosphate from phytate by this method have not been successful. This suggests that the phosphatase of brain used in its preparation produces only one isomer of inositol monophosphate. Earlier observations (Hawthorne, 1955) suggest that it is the 2-phosphate. The inositol monophosphate from liver behaves identically on borate-resin columns and so could be the 2-phosphate also. Without further evidence, particularly on the column behaviour of inositol phosphates other than the 2-compound, this must remain an open question.

The analytical values for the barium salt do not correspond with those of the anhydrous compound usually quoted. A barium salt with 1.5 moles of water of crystallization agrees best with the results. These also correspond closely with the inositol and P figures quoted by McKibbin (1956) for the barium salt of inositol monophosphate liberated on alkaline hydrolysis of liver monophosphoinositide.

SUMMARY

1. Free inositol monophosphate has been isolated from liver. The pure compound has been identified

by analysis and by comparison with inositol monophosphate prepared from phytate.

2. Inositol monophosphate is present in ox heart, ox brain and in the liver of various animals.

3. In ox liver the concentration of inositol monophosphate is at least 4–5 μ moles/100 g. of wet tissue.

4. The possible biochemical significance of inositol monophosphate is discussed.

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