Acid-Soluble Phosphates in the Lens

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(Received 9 May 1957)

Pirie, van Heyningen & Flanders (1955) showed that the concentrations of certain acid-soluble phosphates in the lens, when estimated by the enzymic method of Slater (1953), were much lower than the concentrations reported by Nordmann & Mandel (1952), who used the barium-fractionation method of LePage (1948, 1949). This method has, until recently, been the accepted and standard one for estimation of acid-soluble phosphates. Using the enzymic method we found large deficits in creatine phosphate, hexose phosphates and phosphoglyceric acid. It seemed doubtful whether creatine phosphate was present in measurable quantity and the sum of the hexose phosphates and phosphoglyceric acid amounted to only 12–40 μ moles of P/100 g. of lens when estimated enzymically, whereas the chemical estimation had given about 400 μ moles of P/100 g. of lens in this form. Similar discrepancies were found in the analyses of the acid-soluble phosphates of rat kidney and liver, but not of those of rat muscle, where enzymic and chemical analyses agreed. LePage's analysis of the rat muscle has also been confirmed recently by Threlfall (1957), using a method based on chromatography.

The enzymic method can claim greater specificity than the chemical one and it seemed likely that it gave truer values. This implied that the nature of a considerable part of the organic acid-soluble phosphates of lens and of other tissues was still unknown. We have therefore analysed the acid-soluble phosphates of lens by chromatographic and other methods in an attempt to identify further substances and narrow the gap between total phosphorus and the phosphorus in known compounds.

The analysis which we report has not uncovered any compound not already known in other tissues but it has radically altered our conception of acid-soluble phosphates in the lens, and this may make necessary a reorientation of views of lens metabolism. We have examined the lens of both rabbit and ox.

METHODS

Animals. Cattle (calves of 1–7 days old and older beasts) and rabbits of known age were used. Cattle eyes were removed as soon as possible after death and brought from the slaughterhouse in ice; the lenses were removed and either used immediately or kept frozen. Hereafter the calves and

older beasts will be referred to as calves and cattle respectively. Rabbits were killed with intravenous Nembutal and the lenses were chilled and used at once.

Lens extracts. A calf lens weighs about 1 g., a cattle lens about 2–2.5 g., and the lens of a rabbit about 0.3 g. at 3 months of age and about 0.6 g. when 2 years old or more. About 20 g. of calf or cattle lenses was used to make an extract and about 5 g. of rabbit lenses. Three types of extract were used:

(i) Trichloroacetic acid (TCA) followed by barium precipitation. It is essential that trichloroacetic acid extracts of lens be fractionated and deionized for chromatographic separation of the phosphate compounds. The following procedure was found satisfactory. The lenses were weighed, ground in three or five times their weight of ice-cold TCA (8%, w/v), stored at 0° for 30 min. and centrifuged at 12 000 g for 15 min. at 0°. Most of the TCA was removed by three extractions each with 3 vol. of ether. The extract was then made just pink to phenolphthalein (pH 8.2) with dil. aq. NH_3 soln., and 1 ml. of saturated barium acetate was added for each 60 ml. of TCA extract. After 15 min. at 4° the barium precipitate was centrifuged down; it was washed according to the method of LePage (1949) and the extract and washing were combined. The water-insoluble barium precipitate was kept frozen.

The combined extract and washing were passed through a column of Amberlite IR(120)H (16–50 mesh), 20 cm. long and 1 cm. diameter, at the rate of about 0.5 ml./min., and washed through with a volume of water equal to about four times the volume of the combined extract and washings. The effluent was collected and, after removal of a small volume for the estimation of total P, passed through a column of Amberlite IR(4B)OH (20–50 mesh) (Ansell & Norman, 1956). The effluent from the second column was concentrated at 40–50° *in vacuo*, evaporated to dryness in a desiccator and made up to a suitable volume with water. This was called the 'basic fraction'. The more acidic compounds which were retained by the Amberlite IR(4B)OH column were eluted from it with aq. 3N- NH_3 soln. This eluate was taken to dryness, made up to a known volume and called the 'acidic fraction'.

The barium precipitate was suspended in a little water in a centrifuge tube and shaken with sufficient Amberlite IR(120)H to remove all the barium and thus bring the phosphorus-containing compounds into solution. The Amberlite was centrifuged down and washed twice with water. The combined extract and washings were neutralized with dil. aq. NH_3 soln., concentrated *in vacuo* and dissolved in a known volume of water. The treatment with Amberlite was done quickly and at 0° to prevent the breakdown of adenosine triphosphate (ATP) and other phosphorus-containing compounds.

Thus the TCA extract of lens was divided into three fractions, a 'basic fraction' and an 'acidic fraction', both of

Table 1. *Fractionation of trichloroacetic acid extract of calf lens*

which contain compounds having water-soluble barium salts, and a fraction containing compounds having water-insoluble barium salts. Table 1 gives the results of an experiment with calf lens. About half the total phosphate compounds have water-insoluble barium salts. Ninety per cent of the P in the extract containing the soluble barium salts was recovered in the effluent from the Amberlite IR(120)H column. On one occasion the small amount of organic P remaining on this column was eluted with aq. 3N-NH₃ soln. and examined chromatographically. It appeared to contain traces of a number of the compounds subsequently detected in the three analysed fractions and it was therefore assumed that, had the column been more exhaustively washed, all the P would have been recovered in the effluent.

(ii) Boiling dilute acetic acid. This method is a modification of that used by Campbell & Work (1952). Frozen or ice-cold lenses were weighed and dropped into five times their weight of boiling 0.07% (w/v) acetic acid. They were stirred and broken up with a glass rod and after 3 min. the boiling suspension was poured into a mortar and the lenses were thoroughly ground. The suspension was then boiled for a further 2 min., cooled and its volume measured, and centrifuged at 0° at 12 000 *g* for 20 min. The clear colourless extract was poured off and its volume measured. The pH was about 6.3.

The possible advantage of this type of extraction is that acid-labile substances may be preserved, and that phosphate esters will not be liberated from phospholipids (Dawson, 1955*a, b*). There is evidence of breakdown of phospholipid during trichloroacetic acid extraction of tissues (Ansell & Norman, 1953). A disadvantage of this method of extraction, when applied to the lens, is that the extract still contains a considerable amount of protein which has to be removed during the subsequent fractionation.

(iii) Ethanol. Extraction of lenses in 80% ethanol at room temperature (Shaw, 1955) was used occasionally, as an

alternative to trichloroacetic acid extraction, in the estimation of phosphorylethanolamine in lens.

Paper chromatography. Whatman no. 1 paper was washed with 2N-acetic acid followed by six successive lots of water. Both descending and ascending chromatography were used, usually the former.

Solvents. Phenol-aq. NH₃ soln. (sp.gr. 0.890 diluted with water, v/v to 0.3%); 10:28 (w/v). Phenol-water, 100:28 (w/v). Butanol-acetic acid-water, 40:9:20 (Waley, 1956). Propan-1-ol-aq. NH₃ soln. (sp.gr. 0.890)-water, 60:30:10 (Hanes & Isherwood, 1949). *iso*Propyl ether-formic acid (90%), 90:60 (w/v) (Hanes & Isherwood, 1949). *iso*Butyric acid-aq. N-NH₃ soln., 100:60 (Krebs & Hems, 1953). Propan-2-ol (680 ml.)-HCl (176 ml.)-water (to 1 l.) (Wyatt, 1951). 2-Methylpropan-2-ol (700 ml. at 26°)-constant-boiling HCl (132 ml.)-water (to 1 l. at 20°) (Smith & Markham, 1950). Butanol-water-formic acid (98-100%), 77:13:10 (Markham & Smith, 1949). Butanol-diethylene glycol-water, 4:1:1 (Levine & Chargaff, 1951).

Detection and determination of substances after chromatography. Compounds containing P were detected by dipping the papers in the molybdate reagent of Burrows, Grylls & Harrison (1952), and exposing the papers when thoroughly dry to direct sunlight or, when this was impossible, to light from an Osira ultraviolet lamp. Phosphate was estimated quantitatively by ashing the paper according to the method of Hanes & Isherwood (1949), and determining the resulting orthophosphate (P_i) by the method of Berenblum & Chain (1938). Choline was located by the method of Levine & Chargaff (1951) or Munier (1951), glycerol by the silver nitrate method of Trevelyan, Proctor & Harrison (1950) and amino compounds with the ninhydrin reagent of Wiggins & Williams (1952). Nucleotides were located on the chromatograms by their ultraviolet absorption with a Hanovia 'Chromatolite' (Hanovia Ltd., London) and their positions ringed in pencil. On some occasions the absorbing areas were extracted in 0.01N-HCl, and the absorption was measured in a Beckman spectrophotometer.

Paper electrophoresis. Horizontal strips of Whatman no. 1 or 3MM paper between sheets of polythene and glass were used (Kunkel & Tiselius, 1951). The potential difference along the paper was about 10 v/cm. Borate buffer, pH 9 (7.63 g. of sodium borate, 0.62 g. of boric acid in 1 l. of water), pyridine-acetic acid buffer, pH 4 (50 ml. of acetic acid, 15 ml. of pyridine and 2.5 l. of water; Grassmann, Hannig & Plöckl, 1955) and ammonium formate (0.05M, pH 3.2) were used.

Other estimations. Phosphate was estimated by the method of Fiske & Subbarow (1925), choline by the reineckate method of Engel, Salmon & Ackerman (1954) and pentose by the adaptation of Ceriotti (1955) of the method of Barrenschein & Perham (1941). Propane-1:2-diol phosphate was estimated by the method of Huggins & Miller (1956).

Materials

Chromatographic markers. Glycerylphosphorylethanolamine (GPE), glycerylphosphorylcholine (GPC) and phosphorylcholine (PC) were gifts from Dr R. M. C. Dawson. Phosphorylethanolamine (PE) was made by the method of Outhouse (1937). Inosine mono-, di- and tri-phosphates (IMP, IDP, ITP) were gifts from Mr R. Hems. Other substances used as markers were obtained commercially. Acid phosphatase was made from potato by the method of A. Kornberg (unpublished work), the details being supplied by Dr B. L. Horecker.

RESULTS

The three fractions into which the lens extract was separated by method (i) were examined separately.

'Basic fraction'

Spots (5 μ l.) of this fraction were superimposed on paper until about 35 μ g. of P in all had been applied, and this was submitted to two-way chromatography (phenol-aq. NH_3 soln., propanol-aq. NH_3 soln.-water). The paper was treated first with ninhydrin and then with the phosphate-detecting reagents. The spots were cut out and their P content was measured, and it was shown that most of the P was accounted for in the five areas which stained blue with the molybdate reagent (Table 2).

Three ninhydrin-positive spots were found; of the

three, two contained P and from their R_f values were presumed to be PE and GPE; the third, a very large spot, contained no P and had R_f values corresponding to taurine (Campbell & Work, 1952). Glutathione, other peptides and amino acids were retained on the Amberlite IR (120)H column (Campbell & Work, 1952).

The two areas which contained P but which were ninhydrin-negative were presumed to be PC and GPC, again from their R_f values in these solvents (Dawson, 1955a).

The streak of phosphorus-containing compounds from the origin was not further characterized.

The whole of the basic fraction which remained was applied as narrow bands across the width of several chromatographic papers and run in phenol-aq. NH_3 soln. overnight. About 300 μ g. of P was loaded on to each paper. Narrow strips from the long edges of the paper were developed first with ninhydrin and then with molybdate, to locate the position of the various compounds. Three areas of paper were removed for elution, the first around R_f 0.26 containing the presumed PE, the second around R_f 0.67 containing the presumed GPE and the third around R_f 0.90 containing the presumed PC and GPC. The three eluates were all taken to dryness *in vacuo* and dissolved in a known volume of water.

Identification of phosphorylcholine and glycerylphosphorylcholine

(i) Identification of P and choline. The presence of P in the compounds had already been shown. To show the presence of choline (Dawson, 1955a) spots of the eluate were chromatographed in propanol-aq. NH_3 soln.-water, and the two phosphorus-containing areas were cut out and extracted with boiling water. Ammonium reineckate in methanol was added and the tubes were left in the refrigerator. After 1 hr. shining crystals of choline reineckate could be detected in both. No crystals appeared when pieces of paper adjacent to the spots were treated in the same way.

Table 2. Analysis of two-way chromatogram of 'basic fraction' from calf lens

See text for details. Solvents, phenol-water (A), followed by propanol-aq. NH_3 soln.-water (B). Approx. 38.5 μ g. of P was applied to paper.

Nature of compound	Approximate R_f		Spot tests on chromatogram		P in spot (μ g.)
	(A)	(B)	Ninhydrin	Molybdate (for P)	
Phosphorylethanolamine	0.26	0.17	+	+	2.8
Taurine	0.37	0.40	+	-	None
Glycerylphosphorylethanolamine	0.66	0.42	+	+	2.0
Phosphorylcholine	0.89	0.25	-	+	6.3
Glycerylphosphorylcholine	0.90	0.41	-	+	16.9
Unknown	Streak from origin		-	+	6.2
				Total	34.2

(ii) Cochromatography with synthetic markers. When spots of PC and of GPC were superimposed on spots of the eluted fraction from lens extract and these were chromatographed in phenol-aq. NH_3 soln., propanol-aq. NH_3 soln.-water or isopropyl ether-formic acid, no separation of the markers occurred and the R_F values of the phosphorus-containing spots agreed with expectation.

(iii) Paper electrophoresis with synthetic markers. Paper electrophoresis of the eluted lens fractions at pH 9 showed three phosphorus-containing spots. The first had not travelled and corresponded in position with the GPC marker. The second had moved 3.7 cm. to the positive pole. It was a very weak spot and was not identified. The third spot had moved 4.3 cm. to the positive pole and corresponded to the PC marker spot. Inorganic P moved about 13 cm. under these conditions.

(iv) Hydrolysis and identification of hydrolysis products. GPC is hydrolysed to choline and α -glycerophosphate by hydrolysis in N-HCl for 30 min. at 100° , whereas PC requires 24 hr. hydrolysis in 2N-HCl at 123° (Dawson, 1955a).

Part of the eluate was hydrolysed in N-HCl for 30 min. It was then evaporated to dryness *in vacuo*, dissolved in water, run on a two-dimensional chromatogram (phenol-aq. NH_3 soln., propanol-aq. NH_3 soln.-water) and sprayed for P. The spot corresponding to PC was unchanged but the spot corresponding to GPC had disappeared and a spot corresponding to α -glycerophosphate was now present.

A chromatogram of the unhydrolysed and hydro-

lysed lens fraction and of choline hydrochloride was developed in phenol-aq. NH_3 soln. and the paper sprayed to detect choline. There was a spot corresponding to choline after hydrolysis of the lens fraction, but not before.

Since the reineckate precipitation may not be specific for choline, the formation of choline on mild and severe hydrolysis of lens extracts was confirmed chromatographically. Butanol-acetic acid-water and butanol-diethylene glycol-water were used as solvents. In both solvents a spot having the same R_F value as choline was obtained and cochromatography with authentic choline gave a single spot.

Estimation of phosphorylcholine and glycerylphosphorylcholine in the lens

After two-dimensional chromatography of the 'basic fraction' of calf, cattle and rabbit lens (phenol-water, propanol-aq. NH_3 soln.-water) the spots were made visible with the molybdate spray, and the P in the spots was estimated. Table 3 shows that the calf lens contains 230–400 μmoles of P/100 g., as choline compounds, with rather more GPC than PC. Cattle lens contains 100–200 μmoles of P/100 g. as these compounds. The lens of the rabbit contains less. Similar values were obtained with the three types of extract. The level in the calf lens is comparable with levels found by Dawson (1955a, b) in other tissues.

Choline was estimated in lens extracts after short and prolonged periods of hydrolysis. Unhydrolysed extracts contained none. Table 3 gives

Table 3. *Phosphorylcholine (PC) and glycerylphosphorylcholine (GPC) in lens*

P was measured in chromatographic spots and choline was measured as the reineckate in hydrolysed extracts.

Type of extract	How measured	P ($\mu\text{moles}/100 \text{ g. of lens}$)		
		As PC	As GPC	As PC + GPC
Calf (ii)	P	87	—	—
(ii)	P	168	216	384
(ii)	Choline	194	210	404
(ii)	Choline	—	181	—
(ii)	{ P	100	129	229
	{ Choline	—	—	236
(i)	P	116	216	332
(i)	P	113	219	332
(i)	P	79	232	311
Cattle (ii)	{ P	64	132	196
	{ Choline	45	106	151
(ii)	{ P	32	76	108
	{ Choline	—	—	144
(i)	Choline	64	93	157
(i)	Choline	—	93	—
Rabbit (i)	P	55	77	132
(ii) Age about 3 months	P	45	—	—
(i) Age 5 months	P	45	—	—
(i) Age 7 months	P	45	32	77
(i) Age 7–9 months	P	55	—	—
(i) Age 9 months	P	87	10	97
(i) Age 2 years or more	P	45	23	68

the values for choline liberated by mild and strong hydrolysis, expressed as μ moles of P/100 g. of lens, on the assumption that all lens choline is in the form of PC and GPC. There is reasonable agreement between the values for P as GPC and PC expressed in this way and the values calculated from the P content of the spots which correspond to PC and GPC on a chromatogram of a lens extract.

Identification of glycerylphosphorylethanolamine

(i) Cochromatography with synthetic marker. Spots of the concentrated eluate containing the substance presumed to be GPE were superimposed on a spot of synthetic GPE, and chromatographed in phenol-water followed by propanol-aq. NH_3 soln.-water. The papers were treated with ninhydrin and it was found that no separation of the two substances had occurred.

(ii) Paper electrophoresis before and after hydrolysis. GPE is hydrolysed to glycerophosphate (GP) and ethanolamine by 60 min. hydrolysis at 100° in N-HCl . Part of the eluate was hydrolysed under these conditions, evaporated to dryness *in vacuo* over NaOH and dissolved in water.

Paper electrophoresis at pH 4 of the eluate before and after hydrolysis, with GP, ethanolamine and P_i as markers, gave the following results. Before hydrolysis the eluate gave one spot, containing P and ninhydrin-positive, which did not migrate. After hydrolysis this spot had almost disappeared and instead there was a ninhydrin-positive spot migrating to the cathode in the position of ethanolamine, and a phosphorus-containing spot migrating to the anode in the position of GP.

Identification of phosphorylethanolamine

(i) Cochromatography with synthetic markers. In the basic fraction of the lens extract, the compound presumed to be PE travelled in phenol-water at a rate considerably lower than that of taurine and this made possible the separation of these two compounds. A spot of eluted compound, superimposed on a spot of synthetic PE, did not separate from it when run in a two-way chromatogram (phenol-water, propanol-aq. NH_3 soln.-water).

(ii) Paper electrophoresis before and after hydrolysis. A sample of the hydrolysate was incubated for 18 hr. at 120° in 6N-HCl in a sealed tube, conditions known to hydrolyse PE. Paper electrophoresis at pH 4 of the eluate before and after hydrolysis gave the following results. Before hydrolysis the substance did not migrate, and gave positive reactions with the ninhydrin and phosphate reagents. After hydrolysis, spots of inorganic phosphate and ethanolamine appeared and the PE spot was greatly reduced in intensity.

Estimation of phosphorylethanolamine and glycerylphosphorylethanolamine in lens

These compounds were estimated in the same two-way chromatograms as those prepared for the estimation of PC and GPC. PE was also estimated in a similar way in extracts made by method (iii) (Table 4).

'Acidic fraction'

This fraction accounts for about 413μ moles of P/100 g. of calf lens and 608μ moles of P/100 g. of young-rabbit lens. A $5\mu\text{l.}$ spot, containing about $10\mu\text{g.}$ of P, was run in phenol-water, and the paper was treated with the molybdate reagent to locate phosphorus-containing areas. There was a considerable amount of P_i together with some organic phosphate, running between R_f 0 and 0.18, and a small fast-running phosphorus-containing spot (R_f 0.9) which subsequently was shown to be due to PC, which had been retained by the Amberlite IR (4B) OH column. The rest of the phosphorus-containing compounds were found on incineration in the part of the paper between R_f 0.18 and 0.5, but their presence was not always shown at this stage by the appearance of a blue colour with the molybdate reagent. In the calf lens the P in this area accounted for about 230μ moles of P/100 g. of lens, the P_i for about 100μ moles of P/100 g. of lens, and the PC for 30μ moles of P/100 g. of lens.

The whole fraction was loaded in narrow bands along the width of chromatographic papers and chromatographed in phenol-water overnight. About $250\mu\text{g.}$ of P was applied to each paper. The papers

Table 4. *Glycerylphosphorylethanolamine (GPE) and phosphorylethanolamine (PE) in the lens*

P was measured in chromatographic spots.

Type of extract	P (μ moles/100 g. of lens)	
	As GPE	As PE
Calf (i)	32	35
(ii)	40	34
(iii)	—	23
Rabbit (i) Age about 3 months	29	13
(iii) Age about 6 months	—	None detected
(i) Age 7 months	24	None detected
(i) Age over 2 years	None detected	None detected

were thoroughly dried and strips from each long edge cut off and stained for P. The area of the papers from the boundary of the area containing P_i (about R_F 0.18) up to R_F 0.5 was cut out, eluted with water, taken to dryness *in vacuo* and dissolved in water. A spot of this eluate containing about 10 μ g. of P was run in propanol-aq. NH₃ soln.-water overnight. There were now found to be five distinct phosphorus-containing compounds in the fraction which reacted with molybdate giving a blue colour: a trace of P_i, R_F 0; three compounds absorbing strongly in ultraviolet light, R_F 0.11, 0.15 and 0.26; and a compound running at R_F 0.38 and giving a strong blue colour with the phosphate reagents.

The rest of the phenol eluate was run in a narrow band in propanol-aq. NH₃ soln.-water and, after drying the paper, the three ultraviolet-absorbing components were located. As separate elution of these three components resulted in three fractions each of which appeared still to contain all three components, further attempts to separate them were abandoned and they were eluted together, concentrated *in vacuo* and dissolved in water. The compounds were found to contain pentose and are presumably nucleotides.

The phosphate-containing compound running faster than the nucleotides in propanol-aq. NH₃ soln.-water was located by staining the edges of the paper as before, and was collected by elution and concentration. It was subsequently shown to be α -glycerophosphate (α GP).

The nucleotides accounted for about 113 μ moles of P/100 g. of calf lens and 146 μ moles of P/100 g. of rabbit lens, and the α GP for 97 μ moles of P/100 g. of calf lens and 146 μ moles of P/100 g. of young-rabbit lens.

It must be pointed out that the concentration of these nucleotides and of α GP are calculated from their relative proportions in the phenol eluate (R_F 0.18–0.50), since they did not show up with the molybdate reagent at an earlier stage. We were not able to elute all the phosphate from the paper, and it is therefore possible that either the α GP or the nucleotides were preferentially eluted, and concentrations given are not accurate. All the other compounds found in the lens were estimated before elution from paper.

A check on the concentrations of the glycerophosphate compounds in the lens is given by the following experiment. The alkali-stable, periodate-labile phosphate (Olley & Blewett, 1950) was measured in three batches of calf lens and found to be 381, 355 and 365 μ moles of P/100 g. of lens. The only alkali-stable, periodate-labile compounds we have found in the lens are α GP, GPC and GPE, and the figures agree well with the sum of the values found by chromatographic analysis for these three

compounds ($97 + 219 + 32 = 348$ μ moles of P/100 g. of calf lens).

Nucleotides in acidic fraction from calf lens

This ultraviolet-absorbing fraction was chromatographed before and after mild or severe acid hydrolysis in solvents designed to separate nucleotides and purines and pyrimidine bases. Before hydrolysis, adenylic acid (AMP) and uridylic acid (UMP), both presumably in the 5' form, seemed to be present, judged from the R_F in 2-methylpropan-2-ol-HCl-water and in propan-2-ol-HCl-water. There were no absorbing compounds without P. Eluted spots which corresponded in position to AMP or to UMP showed maximal absorption at 258 and 261 m μ in acid solution. Occasional traces of a compound with absorption maximum 250 m μ were found, suggesting the presence of a hypoxanthine nucleotide. After hydrolysis with N-HCl at 100° for 1 hr. adenine and UMP were obtained, judged by R_F values in 2-methylpropan-2-ol-HCl-water, P detection and absorption maxima of the eluted spots. After hydrolysis in 9N-HClO₄ at 100° for 1 hr., chromatography in the same solvent showed uracil and adenine. No ultraviolet-absorbing spot contained P after this treatment.

On one occasion the nucleotides prepared in this way liberated on hydrolysis not adenine but hypoxanthine.

Identification of α -glycerophosphate

The eluate contained a periodate-labile substance (Baddiley, Buchanan, Handschumacher & Prescott, 1956; Ansell & Norman, 1956), which was found to migrate at pH 4 in paper electrophoresis at the same rate as synthetic α -glycerophosphate. On treatment with acid phosphatase it was shown by paper electrophoresis that P_i and a substance giving a positive reaction with AgNO₃-NaOH (Trevelyan *et al.* 1950) was liberated. This substance had the same R_F value as glycerol, run simultaneously, in three solvents (propanol-aq. NH₃ soln.-water, phenol-water and butanol-acetic acid-water).

Spots of the eluate from lens and synthetic sodium α -glycerophosphate, chromatographed side by side in phenol-water, had different R_F values (0.35 and 0.19), whereas superimposed spots of the two compounds ran as one spot with an R_F value of 0.35. It was found that α -glycerophosphoric acid, neutralized with NaOH to pH 8, ran with R_F 0.19, whereas the same acid unneutralized, or neutralized with aq. NH₃ soln. to pH 8, had R_F 0.35. This accords with the fact that the lens extract, having been eluted from the Amberlite column IR (4B) OH with aq. NH₃ soln., would contain the ammonium salt of α -glycerophosphate, which has the higher R_F value.

It is possible that a similar explanation may be applied to the great variability in R_F values of certain phosphate compounds, including β -glycerophosphoric acid, reported by Olley & Dawson (1956), and also to the difference in R_F values between synthetic and naturally occurring phosphoglyceric acid (Threlfall, 1957).

Fraction precipitable by barium

This fraction was analysed chromatographically with isobutyric acid-aq. $N-NH_3$ soln. as solvent. This solvent separates the six nucleotides adenosine mono-, di- and tri-phosphate (AMP, ADP, ATP) and inosine mono-, di- and tri-phosphate (IMP, IDP, ITP) (Krebs & Hems, 1953). Four phosphorus-containing areas, all of which also absorbed in ultraviolet light, were found in both calf and rabbit lens, and these, together with P_i , accounted for nearly all the P in the fraction. The P_i ran together with ATP, and is easily located by means of the yellow colour it gives with the molybdate reagent. Since its concentration in the extract could be determined by a separate analysis, the value for P in ATP could be calculated by subtracting P_i from the total P found in the area. All the chromatographic analyses were done in duplicate and the agreement was good.

Two of the four phosphorus-containing areas corresponded in position on the chromatograms to ATP and ADP and were assumed to be these compounds without further identification. The presence of easily hydrolysable P in the lens has for a long time been taken as an indication of the presence of ATP and ADP. The fact that extracts of lens will convert glucose into lactic acid without the addition of ATP is proof of presence of this substance in the lens. Further proof comes from the measurement of the high-energy phosphate groups

of ATP and ADP by enzymic methods (Pirie *et al.* 1955), and Dr Gustav Born, using an enzymic method specific for ATP (Strehler & Totter, 1954), has found ATP in calf and rabbit lens in amounts comparable with those found in the present work.

The two other components corresponded in position to IDP and ITP, but on the evidence given below it was concluded that they were uridine diphosphate (UDP) and uridine triphosphate (UTP) respectively. Table 5 gives an analysis of the fractions containing insoluble barium salts, obtained from the calf and rabbit lens. In the analysis reported in Table 5 the ultraviolet absorption of the UDP spot was weak in relation to the amount of P it contained and it seems probable that the UDP was mixed with compounds not absorbing in ultraviolet light. Hydrolysis of the extract containing insoluble barium salts in 9N-HClO₄ for 1 hr. at 100° resulted in the formation of only two spots which absorbed in ultraviolet light, and which, when chromatographed, corresponded in position to uracil and adenine.

Characteristics of the nucleotides, probably uridine tri- and di-phosphate

The compounds were both shown to contain pentose. With the molybdate reagent they gave a blue-green colour, characteristic of easily hydrolysable phosphate compounds. The colour was not removed by subjecting the paper to an atmosphere of ammonia, again a characteristic of easily hydrolysable substances (Fleckenstein, Gerlach & Janke, 1953).

Spots of the chromatographed nucleotides, extracted in 0.01N-HCl, both had a maximum absorption at 260 m μ . Incineration of similar spots showed that the 'UTP' had 3 atoms of P for each molecule of base. The actual values obtained for the number of atoms of P/molecule of uracil was 2.7, 2.7, 3.0 and 4.0. Incineration of the 'UDP' spot gave a value of 2.1 atoms of P/molecule of uracil and therefore, in this preparation, there did not appear to be appreciable amounts of other substances running with the 'UDP'.

The solutions of 'UTP' and 'UDP' were hydrolysed for 1 hr. in N-HCl at 100°. Measurement of the total P and inorganic P showed that approximately two-thirds and one-half respectively of the P in these compounds was acid-labile. Chromatography of the hydrolysates showed the liberation of P_i and the formation of a new substance containing P, absorbing in ultraviolet light and running in the position of uridylic acid (UMP) in propan-2-ol-HCl-water (Wyatt, 1951) and 2-methylpropan-2-ol-constant-boiling HCl-water (Smith & Markham, 1950).

Hydrolysis of the nucleotides in 9N-HClO₄ for 1 hr. at 100° resulted in the formation of P_i and a

Table 5. *Analysis of fraction containing compounds having water-insoluble barium salts, in calf lens and young-rabbit lens*

Rabbits were about 3 months old. For preparation of the fraction see Methods section. The analysis was made chromatographically with isobutyric acid-aq. $N-NH_3$ soln. as solvent; for further details see text.

	P (μ moles/100 g. of lens)	
	Calf	Young rabbit
Trichloroacetic acid extract	1757	1660
Compounds having insoluble barium salts	870	844
ATP	347	448
ADP	60	46
UTP	128	88
UDP plus unknown	68	52
P_i	267	210

Table 6. *Trichloroacetic acid-insoluble phosphate in lens*

See text for preparation of extracts.

	P (μ moles/100 g. of lens)		
	TCA extract	Water suspension	TCA-insoluble
Calf	1620	2040	420
Rabbit	1650	2170	520

spot which absorbed in ultraviolet light but contained no P. Chromatography showed the substance to be the pyrimidine uracil. The evidence therefore supports the view that the nucleotides are UTP and UDP. In the absence of samples of the authentic compounds for comparison, however, it is not possible to establish this identity unequivocally.

Trichloroacetic acid-insoluble phosphate in lens

A lens was ground with five times its weight of water and a little sand in a pestle and mortar. To a known volume of the suspension an equal weight of 10% TCA (w/v) was added and the mixture was centrifuged. Total P was estimated in the lens suspension and the TCA extract. The results are shown in Table 6.

DISCUSSION

Tables 7 and 8 give the results of the analyses of the acid-soluble fractions of calf lens and young-rabbit lens respectively. In both about 85% of the acid-soluble phosphate is accounted for. Compounds whose presence in the lens extract is certain are PC, GPC, PE, GPE, α GP, ATP and ADP. Adenylic acid, uridylic acid and uridine di- and tri-phosphates are very probably present also, together with other nucleotides. Compounds which have been reported previously to be present at concentrations greater than 30 μ moles of P/100 g. of lens, but which have not been found by us, are creatine phosphate, hexose diphosphate, glucose 6-phosphate, glucose 1-phosphate, propanediolphosphate (Nordmann & Mandel, 1952) and fructose 6-phosphate (Frohman & Kinsey, 1952). Enzymic experiments had already shown the relative absence of all these compounds except propanediolphosphate (Pirie *et al.* 1955), and this compound has now been looked for by the method of Huggins & Miller (1956) but was not found. A comparison of the results of our analyses and those obtained by the method of LePage (1949) is given in Table 9.

The 15% of acid-soluble phosphate compounds not accounted for (220–260 μ moles of P/100 g. of lens) probably includes traces of the sugar phosphates mentioned above, phosphate compounds involved in the hexose monophosphate pathway, as

well as other phosphate components of metabolic processes.

We do not know whether all the substances we have found are present in the living lens, or whether they are breakdown products formed in the fractionation processes. PC, GPC, PE and GPE have been found in many tissues and are not believed to be formed during the extraction process. The same applies to ATP, ADP and UTP. The other nucleotides, however, and the α -glycerophosphate, may or may not be formed from more complex molecules. We do not know of any report of the occurrence of α GP in tissues, determined by methods which differentiate between the free form and the choline and ethanolamine esters.

The fractionation process used here resulted in an appreciably higher proportion of orthophosphate (350 μ moles of P/100 g. of lens) than had been found in a trichloroacetic acid extract of lens, prepared quickly and in the cold. Nordmann & Mandel (1952) and Müller (1937) found 260–290 μ moles of orthophosphate/100 g. of calf lens and for the young-rabbit lens values of 171 μ moles of orthophosphate/100 g. of lens (Nordmann & Mandel, 1952) and 278 μ moles of orthophosphate/100 g. of lens (Palm, 1948) have been recorded. We do not know the source of the extra orthophosphate found in our experiments, but some of it probably comes from the breakdown of the pyridine nucleotides during the elution from the Amberlite IR (4B) OH column with aq. 3N-ammonia solution.

The method of fractionation of lens extracts used was worked out for the analysis of the non-nucleotide portion of the acid-soluble phosphates. A more detailed analysis of the nucleotides in the fractions prepared in this way would not be profitable, since better methods are available for the analysis of nucleotides of tissue extracts (Hurlbert, Schmitz, Brumm & Potter, 1954).

The role of the five non-nucleotide phosphate compounds found in lens (α GP, PC, PE, GPC, GPE) is not known, and reactions in which they take part in other tissues have not been investigated in the lens. α GP has recently been shown to be involved in the formation of phosphatidic acids by tissue extracts (Kornberg & Pricer, 1953) and is an intermediary in the biosynthesis of phospholipids (Kennedy, 1953). It is formed in tissue extracts by the phosphorylation of glycerol with ATP, UTP or cytidine triphosphate (Bubitz & Kennedy, 1954). It is also formed as a side reaction of glycolysis by the reduction of dihydroxyacetone phosphate by the enzyme glycerophosphate dehydrogenase and reduced DPN.

PE and PC have been shown to be precursors of phosphatidylethanolamine and lecithin respectively (Dawson, 1954; Kennedy, 1956). They are probably both synthesized in tissues by phosphorylation of

Table 7. *Balance sheet of acid-soluble phosphorus compounds in the calf lens*

Analysis of the compounds with water-insoluble barium salts is given in Table 5.

		P (μ moles/100 g. of lens)			
Total	1757				
Insoluble barium salts	870				
		ATP, ADP, UTP	P _t	267	
				535	
Soluble barium salts	887				
		'Basic'	371		
		'Acidic'	413		
		Basic fraction	371		
			PC	81	
			GPC	219	
			PE	35	
			GPE	32	
		Acidic fraction	413		
			PC	32	
			P _t	126	
			α GP	97	
		Nucleotides including AMP and UMP		113	
					Unaccounted for
					45*
		Total accounted for	1537	Total unaccounted for	220

87% of the acid-soluble P compounds accounted for. Sum of P_t from the various fractions is 393 μ moles of P/100 g.* R_f below 0.18 in phenol-water.Table 8. *Balance sheet of acid-soluble phosphorus compounds in the young-rabbit lens*

Rabbits were 3 months old; average lens weight, 324 mg. Analysis of the compounds with water-insoluble barium salts is given in Table 5.

		P (μ moles/100 g. of lens)			
Total	1660				
Insoluble barium salts	844				
		ATP, ADP, UTP	P _t	209	
				582	
Soluble barium salts	816				
		'Basic'	208		
		'Acidic'	608		
		Basic fraction	208		
			PC	55	
			GPC	77	
			PE	13	
			GPE	29	
		Acidic fraction	608		
			PC	—	
			P _t	143	
			α GP	146	
		Nucleotides including AMP and UMP		146	
					Unaccounted for
					121*
					52†
		Total accounted for	1400	Total unaccounted for	260

84% of the acid-soluble P compounds accounted for. Sum of P_t from the various fractions is 355 μ moles of P/100 g.* R_f below 0.2 in phenol-water.† R_f above 0.2 in phenol-water and about 0.26 in propanol-aq. NH₃ soln.

Table 9. *Acid-soluble phosphates of lens. Comparison of the present findings with those obtained by the method of LePage (1949)**

	P (μ moles/100 g. of lens)			
	Calf		Young rabbit	
	Present results	Method of LePage	Present results	Method of LePage
Total	1757	1765	1660	1534
P _i	393	231	355	171
Creatine phosphate	0†	127	0†	86
ATP + ADP	407	571	494	663
AMP	—	88	—	115
Nucleotides including AMP, UMP, UTP	241	—	234	—
Glucose 1-phosphate, glucose 6-phosphate, hexose diphosphate and phosphorylglyceric acid	12§	393	30†	402
Propanediol phosphate	0	135	0	Not done
Glycerophosphate	97	284†	146	377†
Glycerylphosphorylcholine	219	0	77	0
Glycerylphosphorylethanolamine	32	0	29	0
Phosphorylcholine	113	0	55	0
Phosphorylethanolamine	35	0	13	0

* Nordmann & Mandel (1952).

† Nordmann & Mandel (1952), by method of Fleury & Courtois (1941).

§ A. Pirie & R. van Heyningen (unpublished work).

† Pirie *et al.* (1955).

the bases ethanolamine and choline in the presence of ATP (Ansell & Dawson, 1951; Wittenberg & Kornberg, 1953). GPE and GPC are not believed to be precursors of phospholipids, but may be breakdown products (Dawson, 1955*b*). Phospholipids in the cattle lens are found at a concentration of about 200 mg./100 g. of lens; most of these contain choline (Krause, 1935; D'Asaro, Young & Williams, 1954). This is equivalent to about 266 μ moles of P/100 g. of lens and is therefore the largest part of the TCA-insoluble phosphorus (Table 6).

Phosphorylcholine accounts for about one-tenth of the total acid-soluble phosphate of lens. It is present in the same proportion as it is in liver and the actual amount present in calf lens (av. 25 mg./100 g. of lens) is equal to that in rat spleen and intestine and only surpassed by the amounts in liver and testis (Dawson, 1955*a*). It is not possible to assess the importance of a substance in the metabolism of a tissue from a measure of its concentration, but it is possible that lipid metabolism in lens may be more important than we have thought hitherto and that glycerophosphate and phosphorylcholine may actively participate.

SUMMARY

1. About 85 % of the phosphorus in the acid-soluble phosphate compounds of the lens of the calf and young rabbit have been accounted for.

2. The compounds which have been identified and estimated and which have not previously been found in the lens are phosphorylcholine, glycerylphosphorylcholine, phosphorylethanolamine and

glycerylphosphorylethanolamine. Uridine 5'-phosphate, uridine diphosphate and uridine triphosphate are also probably present at fairly high concentrations.

3. In addition, the presence of α -glycerophosphate, adenosine 5'-phosphate, adenosine triphosphate and adenosine diphosphate has been confirmed.

4. The low concentration of hexose phosphate and phosphoglyceric acid in lens extracts is confirmed.

We wish to record our thanks to Dr S. G. Waley, Dr R. M. C. Dawson and Dr June Olley for helpful advice; to the National Council to Combat Blindness, New York, for a Fight for Sight Grant for the purchase of a Beckman spectrophotometer and chromatographic equipment; to Dr G. Born for estimating adenosine triphosphate enzymically in some extracts; to Miss Joyce Blackwell for the collection of calves' eyes and to the Staffs of the Oxford Co-operative and Eastwyck Farm Slaughterhouses for their help.

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The Reactivation of Cholinesterase Inhibited with Organophosphorus Compounds

1. REACTIVATION BY 2-OXOALDOXIMES

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(Received 13 May 1957)

The ability of many chemical compounds to restore the activity of cholinesterase (ChE) after inhibition by organophosphorus compounds is now well established (Childs, Davies, Green & Rutland, 1955; Wilson, 1955). It has also been shown that recovery of enzymic activity is due to chemical displacement of the phosphoryl group of the inhibitor from the group to which it is attached at or near the active centre of the enzyme (Davies & Green, 1956; Jandorf, Crowell & Levin, 1955). The most powerful reactivators, hydroxamic acids and oximes, will react rapidly with many organophosphorus inhibitors themselves (Hackley, Plapinger, Stolberg & Wagner-Jauregg, 1955; Green & Saville, 1956), but there is little correlation between these reactivities

and the ability of hydroxamic acids and oximes to reactivate the inhibited enzyme (Childs *et al.* 1955). It has been suggested (Davies & Green, 1956) that this lack of correlation may be due to the formation of a preliminary complex between the reactivator and the inhibited enzyme. In this and the subsequent paper, this possibility has been tested kinetically for the two most potent groups of reactivating agents, 2-oxoaldoximes (I) and quaternized pyridine aldoximes.

(I)

(R = methyl, ethyl, *n*-propyl, *isopropyl*, *tert.*-butyl, phenyl and *cyclohexyl*).