Sugar Accumulation by Sugar-Cane Storage Tissue: the Role of Sucrose Phosphate

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Sucrose is the first sugar detected when sucrose, glucose or fructose is actively accumulated by storage tissue of sugar-cane stems. Glucose and fructose are accumulated more rapidly than sucrose by immature and mature storage tissue (Glasziou, 1960; Sacher, Hatch & Glasziou, 1963). The first and generally the rate-limiting step for sucrose accumulation is hydrolysis by an invertase located outside the cytoplasmic membrane. To explain these observations Sacher *et al.* (1963) proposed that the compound that moved across the limiting membrane into the cellular storage compartment was a sucrose derivative that was only derivable from sucrose via hexoses. Sucrose phosphate (phosphorylated at the 6-position of fructose) appears to be the only known sucrose derivative found in plants that would fulfil these requirements. The present studies provide evidence consistent with the proposition that sucrose phosphate is an intermediate in the sugar accumulation process.

METHODS

Preparation and assay of uridine diphosphate glucose-fructose 6-phosphate glucosyltransferase. Rind-free storage tissue or leaf tissue was cut into small pieces and frozen, and then 50 g. homogenized with 100 ml. of cold 10 mM-potassium phosphate buffer, pH 8.0. The extract obtained by squeezing the homogenate through fine muslin was treated with solid $(\text{NH}_4)_2\text{SO}_4$ to obtain the protein that precipitated between 40% and 50% saturation. This treatment removed most of the UDP-glucose-fructose glucosyltransferase and alkaline phosphatase present in the original extracts. The protein was suspended in 10 ml. of 2 mM-potassium phosphate buffer, pH 7.4, and dialysed for 16 hr. against 3 l. of the same buffer at 3°. This preparation was used for enzyme studies. Reaction mixtures containing $[\text{U}^{14}\text{C}]\text{fructose 6-phosphate}$ as substrate are described in the Results section. After heating in a boiling-water bath for 3 min. to stop the reaction, 0.01 ml. was transferred to the base lines of paper chromatograms. The remainder of the reaction mixture was adjusted to pH 9.3 with 0.01 ml. of 1M-tris, and then 0.01 ml. of an alkaline phosphatase solution was added. This mixture was incubated for 1 hr. at 30° and then 0.01 ml. chromatographed together with the sample from the untreated reaction mixture, with ethyl acetate-pyridine-water (8:2:1, by vol.) as the developing solvent. With this solvent sugar phosphates remain at the origin and sucrose, glucose and fructose are separated.

Sugars were located by spraying with *p*-anisidine phosphate. The radioactivity on the chromatograms was measured with a Geiger-Müller tube. The counting efficiency was 5%, and samples containing approx. 4000 counts/min. were chromatographed. The increase in the proportion of radioactivity located in sucrose after phosphatase treatment was determined.

Preparation and assay of extracts for phosphatase activity. A 20 g. portion of tissue was cut into small pieces, cooled to 0° and then homogenized for 2 min. with 40 ml. of cold 50 mM-potassium phosphate buffer, pH 8.0, containing sucrose (0.4M). The temperature was kept below 5° during the following steps. Cell debris retained by fine muslin was discarded and the cell particulate fractions that were precipitated at 2000g for 3 min., 25000g for 10 min. and 150000g for 60 min. were each suspended in 3 ml. of water. The protein in the 150000g supernatant was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to give 80% saturation and then suspended in 3 ml. of water. All the fractions were dialysed against 3 l. of water for 16 hr. Part of the 150000g supernatant fraction was divided into six sub-fractions with $(\text{NH}_4)_2\text{SO}_4$. The various fractions were tested for phosphatase activity at pH 5.4 and 8.2 with sucrose phosphate and fructose 6-phosphate as substrates. Reaction mixtures contained: enzyme solution (0.03 ml.); either $[\text{fructosyl-}^{14}\text{C}]\text{sucrose phosphate}$ (5 μM -moles, 42000 disintegrations/min.) or $[\text{U}^{14}\text{C}]\text{fructose 6-phosphate}$ (15 μM -moles, 140000 disintegrations/min.); MgCl_2 (0.5 μM -mole); and either potassium acetate buffer, pH 5.4 (2 μM -moles), or tris-HCl buffer, pH 8.2 (2 μM -moles); the total volume was 0.1 ml. Appearance of non-phosphorylated sugars was measured after chromatography of samples of the reaction mixture by using the procedure described above.

Formation of sucrose phosphate in tissue slices. Tissue was cut into 1 mm. slices with a microtome and washed for 1 hr. in running tap water. Tissue slices were then incubated with $[\text{U}^{14}\text{C}]\text{glucose}$ as described in the Results section. After 10 min. the tissue was tipped into a strainer, washed for 10 sec. in running tap water, and then placed in 6 ml. of hot ethanol, macerated with a glass rod and heated in a boiling-water bath for 5 min. After shaking for 8 hr. the mixture was extracted with chloroform by the procedure of Bligh & Dyer (1958). The aqueous extract was placed in a band across Whatman no. 3MM paper and chromatographed, with ethyl acetate-pyridine-water (8:2:1, by vol.) as the developing solvent. The radioactivity remaining at the origin of the chromatograms was eluted from the paper with water. Portions of the eluate were chromatographed in the same solvent after treatment with alkaline phosphatase or alkaline phosphatase followed by invertase.

Accumulation of radioactive sugars by tissue slices. Tissue disks of mature (5 mm. $\times$ 1 mm. thick) or immature (5 mm. $\times$ 0.5 mm. thick) stem tissue were cut and washed for 1 hr. in running tap water. Equal numbers of disks were added to solutions of radioactive substrates and shaken in a water bath at 30° for 4 hr. Disks were then washed for 1 hr. in running tap water to remove radioactive compounds from the free space of the tissue (Sacher *et al.* 1963). The stored sugars were extracted by crushing the tissue in 2 vol. (w/v) of hot ethanol, heating at 80° for 5 min. and shaking for 16 hr. at room temperature. Samples of the bathing medium and the tissue ethanol extract were chromatographed on paper, as described above, to determine the radioactivity in sugars and sugar phosphates. To determine the distribution of radioactivity in the hexose moieties of accumulated sucrose the remainder of the ethanol extract was chromatographed and the sucrose eluted from the paper with water. The eluate was treated with invertase and rechromatographed to determine the radioactivity in glucose and fructose.

MATERIALS

Internodal stem tissue of a hybrid sugar-cane (var. Pindar) was used. Tissue taken from internodes in the process of expanding was classified as immature. The following compounds were obtained from commercial sources: ATP, UDP-glucose, yeast hexokinase, mucosa alkaline phosphatase, wheat-germ acid phosphatase (all from Sigma Chemical Co., St Louis, Mo., U.S.A.), [^{14}C]-fructose, [^{14}C]-glucose (both from The Radiochemical Centre, Amersham, Bucks.) and yeast invertase (from Difco Laboratories, Detroit, Mich., U.S.A.).

Sucrose labelled with ^{14}C in the fructose moiety was prepared enzymically from [^{14}C]-fructose and UDP-glucose as described by Sacher *et al.* (1963).

[^{14}C]-Fructose 6-phosphate was prepared from [^{14}C]-fructose by using crystalline yeast hexokinase. Unchanged fructose, ATP and ADP were separated from fructose 6-phosphate by chromatographing the reaction mixture as a band on Whatman no. 3MM paper, with propyl acetate-90% (v/v) formic acid-water (6:3:1, by vol.) as the developing solvent (Bielecki & Young, 1963). The fructose 6-phosphate band was located by radioautography and eluted from the paper with water. On treatment with wheat-germ acid phosphatase more than 99% of the radioactivity was located in fructose.

Sucrose phosphate, phosphorylated at the 6-position of fructose and containing radioactivity only in the fructose moiety, was prepared by incubating [^{14}C]-fructose 6-phosphate (8.8 μmoles , 70×10^6 disintegrations/min.), UDP-glucose (27 μmoles) and tris-HCl buffer, pH 7.1 (60 μmoles), with wheat-germ UDP-glucose-fructose 6-phosphate glucosyltransferase, prepared as described by Mendicino (1960). The reaction was stopped by heating at 100° for 5 min. and the precipitated protein removed. The reaction mixture was then treated according to the procedure of Leloir & Cardini (1955) up to and including the removal of borate by vacuum-distillation and reprocessing with Dowex 50. The eluate from Dowex 50 was neutralized with NaOH and a large part of the inorganic salt removed with an electrolytic desalting apparatus. The preparation was then chromatographed on Whatman no. 3MM paper, with propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1,

by vol.) (Hanes & Isherwood, 1949) as the developing solvent. The sucrose phosphate band was eluted with water and samples were chromatographed before and after treatment with alkaline phosphatase. Analyses of these samples showed that 99.5% of the radioactivity was located in sucrose phosphate and approx. 0.5% in fructose 6-phosphate. After treatment of a sample with phosphatase and then invertase all the radioactivity was located in fructose. Approx. 60% of the original radioactivity was recovered in sucrose phosphate.

RESULTS

Synthesis and breakdown of sucrose phosphate in sugar-cane tissue extracts. Hatch, Sacher & Glasziou (1963) briefly reported low sucrose phosphate-synthesizing activity by extracts of mature storage tissue of sugar-cane. Extracts of storage tissue and leaf have now been fractionated with ammonium sulphate to remove some interfering enzymes, and higher activities have been observed (Table 1). The sucrose released by treatment of reaction mixtures with phosphatase provided a measure of sucrose phosphate synthesis. The identity of the sucrose so formed was confirmed by co-chromatography with unlabelled sucrose and by treatment with yeast invertase. After invertase treatment the radioactivity was located in fructose. Efforts to identify a sucrose kinase in sugar-cane tissue extracts were not successful.

With sucrose phosphate as substrate most storage-tissue phosphatase activity at pH 5.4 and 8.2 was located in the 150000g supernatant. The combined activities of the particulate fractions that were precipitated at 2000g, 25000g and 150000g were less than 10% of the total. Sub-

Table 1. *Uridine diphosphate glucose-fructose 6-phosphate-glucosyltransferase activity of sugar-cane storage tissue and leaf*

Reaction mixtures contained: enzyme (0.1 ml. of the preparations described in the Methods section); [^{14}C]-fructose 6-phosphate (0.2 μmole ; 1.6×10^6 disintegrations/min.); UDP-glucose (0.7 μmole); tris-HCl buffer, pH 7.4 (5 μmoles); potassium fluoride (2.5 μmoles); the total volume was 0.2 ml. Sucrose released after phosphatase treatment provided a measure of sucrose phosphate formed (see the Methods section). No sucrose was formed when UDP-glucose was omitted.

	Time (hr.)	Sucrose formed ($\mu\text{m-moles}$ /reaction mixture)	
		Storage tissue	Leaf tissue
Before phosphatase treatment	1	1.0	1.8
After phosphatase treatment	1	22.2	25.0
Before phosphatase treatment	2	1.6	2.4
After phosphatase treatment	2	31.0	27.2

fractionation of the supernatant with ammonium sulphate revealed two major fractions of activity at pH 5.4 and pH 8.2. In the unfractionated supernatant, the activity at pH 8.2 was less than 20 % of the activity at pH 5.4. None of the fractions hydrolysed sucrose phosphate at a significantly greater rate than fructose 6-phosphate.

Synthesis of sucrose phosphate in tissue slices. When [^{14}C]glucose was supplied to mature and immature storage-tissue slices a compound with the properties of sucrose phosphate became labelled (Table 2). After treatment of the 'origin' compounds with alkaline phosphatase approx. 50 % of the radioactivity remained immobile in the chromatography solvent used, approx. 15 % chromatographed with sucrose and the remainder with glucose and fructose. If the 'origin compounds' were treated with phosphatase and invertase, radioactivity appeared in glucose and fructose but not sucrose.

Distribution of ^{14}C in sucrose accumulated from sucrose phosphate and other sugars. Sucrose and sucrose phosphate, containing radioactivity only in the fructose moiety, were supplied to tissue disks, and the total radioactivity and distribution of radioactivity in the sucrose accumulated by the tissue were determined (Table 3). For comparison the sucrose accumulated from radioactive fructose and fructose 6-phosphate was also examined. The more rapid formation of stored sucrose from free sugars, compared with formation from phosphorylated sugars, was probably due to restricted entry of the latter into the cytoplasm. Irrespective of the substrate supplied approx. 60 % of the ethanol-soluble radioactivity taken up by the tissue was located in sucrose. Incubation media were buffered to pH 6.3 or above because at lower pH values phosphorylated sugars were rapidly hydrolysed by a phosphatase that was apparently located outside the cytoplasmic membrane. Sodium

Table 2. *Formation of sucrose phosphate in storage-tissue slices*

Tissue slices (1.5 g.) were incubated for 10 min. in 3 ml. of a solution containing [^{14}C]glucose (3 μmoles , 24×10^6 disintegrations/min.) and then extracted as described in the Methods section. Chromatographed samples of 'origin compounds' treated with phosphatase or invertase contained at least 1000 counts/min. 'Origin compounds' refer to water-soluble compounds, including sugar phosphates, that remained at the origin of chromatograms during preparative chromatography. This procedure removes essentially all the free sugar present in the original tissue extract (see the Methods section). The total counts/min. in sucrose were determined from the radioactivity in the sucrose spot on chromatograms.

Type of storage tissue	Incorporation of ^{14}C into 'origin compounds'		Analysis of 'origin compounds' (total counts/min. in sucrose)		
	Percentage of total ^{14}C supplied	Total counts/min.	Before phosphatase treatment	After phosphatase treatment	After phosphatase and invertase treatment
Immature	0.58	6960	60	990	36
Mature	0.33	3950	76	650	30

Table 3. *Radioactivity in sucrose accumulated into tissue slices and in the medium after supplying different radioactive sugars and sugar phosphates*

Immature storage-tissue slices (0.3 g.) were incubated with 0.7 ml. of a solution containing: radioactive substrate (0.3 μmole , 2.5×10^6 disintegrations/min.); either potassium phosphate buffer, pH 7.2 (10 mM) (Expt. 1), or potassium phosphate buffer, pH 6.3 (10 mM), tris-HCl buffer, pH 6.3 (25 mM), and sodium molybdate (0.5 mM) (Expt. 2). After incubating for 4 hr. at 30° analysis was carried out as described in the Methods section. Chromatographed samples of the medium contained approx. 4000 counts/min. and samples of the tissue ethanol extracts and of sucrose treated with invertase contained at least 200 counts/min. 'Origin compounds' refer to those remaining at the origin of the chromatograms.

	Substrate supplied	Percentage distribution of ^{14}C in medium after 4 hr.			^{14}C accumulated as sucrose (counts/min./reaction mixture)	Ratio of ^{14}C in glucose and fructose moieties of sucrose
		'Origin compounds'	Sucrose	Glucose + fructose		
Expt. 1	[^{14}C]Fructose	4	1	95	10050	0.82
	[^{14}C]Fructose 6-phosphate	96	0.5	3.5	1400	1.02
	[fructosyl- ^{14}C]Sucrose	1	95	4	2100	0.32
	[fructosyl- ^{14}C]Sucrose phosphate	91	8.5	0.5	650	0.04
Expt. 2	[^{14}C]Fructose	4	2	96	12600	0.80
	[^{14}C]Fructose 6-phosphate	95	0.6	4.4	1250	0.95
	[fructosyl- ^{14}C]Sucrose	1	91	8	4600	0.36
	[fructosyl- ^{14}C]Sucrose phosphate	96.5	3.3	0.2	510	0.02

molybdate (Spencer, 1954) was used in the medium buffered at pH 6.3 to inhibit the phosphatase. In the same experiment tris was used to partially inhibit free-space invertase, which would be more than three times as active at pH 6.3 as at pH 7.2 (Sacher *et al.* 1963).

The distribution of radioactivity in the glucose and fructose moieties of sucrose accumulated from radioactive fructose and fructose 6-phosphate provided evidence for the rapid interconversion of glucose and fructose within the tissue (Table 3). When [*fructosyl*- ^{14}C]sucrose phosphate was supplied there was little randomization of radioactivity in accumulated sucrose. This contrasted with the results obtained when [*fructosyl*- ^{14}C]sucrose was supplied. A small proportion of the sucrose accumulated from sucrose phosphate would be derived from radioactive fructose, formed in the medium by the combined action of phosphatase and invertase. This proportion, calculated from the observed fructose concentrations in the medium and the uptake of supplied fructose, was approx. 4 % for Expt. 1 and 2 % for Expt. 2. If allowance is made for sucrose derived in this manner, the ratios of ^{14}C in the glucose and fructose moieties of the remaining accumulated sucrose would be close to zero. Results similar to those described in Table 3 were obtained for mature storage tissue.

The concentration of [*fructosyl*- ^{14}C]sucrose, as supplied, was 0.43 mM, whereas the final concentration of sucrose in the medium of experiments containing [*fructosyl*- ^{14}C]sucrose phosphate was less than 10 % of this value. A separate experiment showed that the distribution of radioactivity in accumulated sucrose did not alter significantly when [*fructosyl*- ^{14}C]sucrose was supplied at concentrations from 0.2 mM to 20 mM. However, with different batches of tissue, and with medium buffered to different pH values, ratios of radioactivity in the glucose and fructose moieties of accumulated sucrose have varied from 0.25 to 0.75. In one experiment (J. A. Sacher, M. D. Hatch & K. T. Glasziou, unpublished work) the ratio for sucrose accumulated from invert sugar containing [U - ^{14}C]fructose was 0.68 compared with 0.95 for

[U - ^{14}C]fructose and 0.42 for [*fructosyl*- ^{14}C]sucrose when all sugars were supplied at a concentration of 1 % (w/v). A more significant comparison would have been provided by experiments in which fructose and invert sugar were supplied at a concentration approximating the average concentration of glucose and fructose in reaction mixtures supplied with [*fructosyl*- ^{14}C]sucrose.

When sucrose is supplied to tissue disks a component of stored sucrose would be derived by passive diffusion. Hence estimates of the randomization of hexose moieties during the active accumulation process would be minimal. This component becomes significant when the active process is decreased by high pH or the addition of tris (Sacher *et al.* 1963). From estimates of diffusion, based on the diffusion of fructose into the storage compartment, the ratio of radioactivity in hexose moieties of sucrose accumulated by the active process can be calculated. For the results in Table 3 the values of 0.32 and 0.36 become 0.45 and 0.43 respectively.

DISCUSSION

Sugar-cane storage tissue accumulates sucrose against a concentration gradient, the process being dependent on respiratory energy (Bieleski, 1960). Reasons for considering that sucrose phosphate may be an intermediate in this process have already been presented. The synthesis and subsequent hydrolysis of sucrose phosphate could provide the expenditure of energy necessary from thermodynamic considerations of the process. The present concept of the sugar accumulation process is outlined in Scheme 1, which modifies one presented by Sacher *et al.* (1963). Evidence for the steps leading to the formation of hexose phosphates and UDP-glucose has been reported (Hatch *et al.* 1963; Sacher *et al.* 1963). The following evidence described in the present paper is consistent with sucrose phosphate being an intermediate in the sugar accumulation process: (a) Enzymes for the synthesis and hydrolysis of sucrose phosphate are present in stem tissue. (b) When [U - ^{14}C]glucose is supplied to slices of storage tissue labelled sucrose phosphate is formed. (c) Sucrose accumulated by

Scheme 1. Sugar accumulation process.

storage-tissue slices from [*fructosyl*- ^{14}C]sucrose contained label in both hexose moieties, whereas sucrose accumulated from [*fructosyl*- ^{14}C]sucrose phosphate retained its asymmetric labelling pattern.

The assay of UDP-glucose-fructose 6-phosphate glucosyltransferase is subject to interference by a number of commonly occurring enzymes. Frydman & Hassid (1963) were unable to detect sucrose phosphate synthesis by extracts of sugar-cane leaves. The present report describes the synthesis of sucrose phosphate by sugar-cane storage tissue and leaf extracts, but with all preparations the rate decreased rapidly with time. Subsaturation concentrations of substrate were used and the decline in rate was considered to be largely due to a decline in fructose 6-phosphate concentration. Glucose 6-phosphate-fructose 6-phosphate isomerase and phosphatases were probably the major contributors to this decline. Hydrolysis of sucrose phosphate to sucrose and inversion of sucrose were also contributing factors. Activity of all the enzymes mentioned was detectable under the conditions of assay.

A phosphatase specific for sucrose phosphate has not been described. The presence of such an enzyme, suitably located in storage-tissue cells, could explain the apparently exclusive role of sucrose as an initial product of the sugar storage process. Phosphatases that hydrolyse sucrose phosphate were identified in sugar-cane extracts but no evidence was obtained for the presence of a specific enzyme.

According to the above scheme, sucrose should accumulate at least as rapidly from sucrose phosphate as from sucrose, provided that entry of sucrose phosphate into the metabolic compartment is not restricted. In fact, less sucrose was accumulated into the tissue from sucrose phosphate than from sucrose. Restricted movement through the plant cytoplasmic membrane of anionic compounds, including metabolic inhibitors (James, 1953), organic acids (Hatch, Pearson, Millard & Robertson, 1959) and glucose 1-phosphate (Bialeski, 1960), has been demonstrated. The present studies also show that fructose enters the sugar-cane cells much more readily than does fructose 6-phosphate. The first step in the metabolic utilization of fructose would almost certainly be phosphorylation to fructose 6-phosphate.

The processes of sugar accumulation by sugar-cane stem tissue and *Canna* leaves (Putman & Hassid, 1954; Hassid, 1958) have many features in common. In other plant tissues (Hellebust & Forward, 1962; Robinson & Brown, 1952), yeast (Fuente & Sols, 1962) and mould (Metzenberg, 1962) there is evidence that hydrolysis of sucrose by an invertase located outside the cytoplasmic

membrane is a prerequisite for the metabolic utilization of sucrose. However, there are a number of tissues that store sucrose, including tobacco leaf (Porter & May, 1955), sugar-beet root (Bacon, 1961) and artichoke tuber (Edelman & Hall, 1963), but contain low or undetectable invertase activity. Porter & May (1955) provided evidence that sucrose moved into leaf-tissue cells without being hydrolysed but did not distinguish between sucrose stored by active accumulation and sucrose moving into the tissue free space or cells by passive diffusion. At least two possible mechanisms exist for the active accumulation of sucrose via sucrose phosphate without the operation of invertase. Direct phosphorylation of sucrose would give rise to accumulated sucrose without interconversion of its hexose moieties. However, sucrose kinase was not detected in sugar-cane and has not as yet been found in other plant tissues. A more likely pathway involves the cleavage of sucrose by UDP-glucose-fructose glucosyltransferase followed by phosphorylation of fructose and synthesis of sucrose phosphate. UDP-glucose-fructose glucosyltransferase is widely distributed in plant tissues and the equilibrium constant for the reaction it catalysed does not prohibit its operation as a sucrose-cleaving enzyme (Cardini, Leloir & Chiriboga, 1955). Sucrose could be accumulated by this pathway without interconversion of its hexose moieties if UDP-glucose is utilized for sucrose phosphate synthesis without prior equilibration with the hexose phosphate pool. Such a pathway apparently could contribute to only a minor extent to sucrose accumulation by sugar-cane storage parenchyma. In addition to the direct evidence implicating a free-space invertase (Sacher *et al.* 1963), M. D. Hatch & J. S. Hawker (unpublished work) have shown that UDP-glucose-fructose glucosyltransferase is located almost exclusively in the vascular tissue of the stem. A uridine diphosphatase present in the storage parenchyma (Hatch, 1963) would, if suitably located within the cell, prevent breakdown of sucrose by UDP-glucose-fructose glucosyltransferase.

SUMMARY

1. Enzymes that catalyse the synthesis (uridine diphosphate glucose-fructose 6-phosphate glucosyltransferase) and breakdown (phosphatases) of sucrose phosphate were isolated from stem and leaf tissue of sugar-cane.

2. A compound with the properties of sucrose phosphate was formed from [U - ^{14}C]glucose in slices of stem tissue.

3. Studies in which radioactive sugars and sugar phosphates, including [*fructosyl*- ^{14}C]sucrose and [*fructosyl*- ^{14}C]sucrose phosphate, were supplied to

storage-tissue slices provided evidence consistent with the proposition that sucrose phosphate is an intermediate in sugar accumulation.

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Amino Acids and Related Compounds in the Lens

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During the course of the fractionation of extracts of calf lens that led to the isolation of *S*-($\alpha\beta$ -dicarboxyethyl)glutathione (Calam & Waley, 1963) several unidentified compounds were detected. Two of these have now been isolated, by ion-exchange chromatography followed by paper electrophoresis or paper chromatography, and identified; one is probably the *O*-phosphate of hydroxylysine, and the other is the mixed disulphide derived from cysteine and glutathione.

An improved general method for the analysis of the ninhydrin-positive compounds in lens extracts was outlined by Calam (1962). The application of this method, which consists of paper electrophoresis at pH 1.6 in one direction followed by paper chromatography in the second direction, to bovine and human (cataractous) lenses is now described in detail. Specific reagents have revealed the presence of ergothioneine, and of several other compounds not previously known to be present in lens. A quantitative analysis of the more abundant amino acids has also been carried out.

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METHODS

Paper electrophoresis. This was carried out in a ridge-pole apparatus (Cliffe & Waley, 1958), usually at 8v/cm., on Whatman papers. The buffers used were: pyridine acetate, pH 4 (50 ml. of acetic acid, 15 ml. of pyridine and 2.5 l. of water; Grassmann, Hannig & Plöckl, 1955) (no. 52 paper); formic acid-acetic acid-water, pH about 1.6 (125 ml. of 90% w/v, formic acid and 375 ml. of acetic acid in 2.5 l.) (no. 3 paper); 10% (v/v) acetic acid, pH about 2.3; 0.05M-veronal, pH about 8.4 (10.3 g. of sodium diethylbarbiturate and 2.8 g. of diethylbarbituric acid/l.). Continuous electrophoresis was carried out at pH 4 in a Beckman model CP apparatus (Calam & Waley, 1963).

Paper chromatography. The solvents used were: butan-1-ol-acetic acid-water (40:9:20, by vol.); butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953); 72% (w/v) phenol-aq. 3% (w/v) NH_3 soln. Chromatography on cation-exchange papers (Amberlite SA-2) was carried out with 0.2M-acetate buffer, pH 5.2 (Tuckerman, 1958). Whatman no. 1 or no. 4 papers were usually used; for the two-dimensional 'maps', no. 3 paper was used. Amino compounds were detected with ninhydrin (0.4%) and CoCl_2 (0.2%) in propan-2-ol (Wiggins & Williams, 1952); this reagent gives red colours which do not fade. The Sakaguchi, Pauly and molybdate reagents were as described by Smith (1960), the Ehrlich reagent as de-