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Phosphorylation of Carbohydrates in Retinal Extracts

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Warburg, Posener & Negelein (1924) first observed that the isolated rat retina together with tumour cells, the grey matter of brain and embryonic tissue possessed comparatively high glycolytic activity. Subsequently, numerous investigations were carried out on the metabolic activity of the isolated retina (e.g. Dickens & Greville, 1933; Laser, 1935). Kerly & Bourne (1939, 1940) and Süllman & Vos (1939), independently, were the first to study glycolysis in retinal extracts. Such extracts were found to convert added glucose, fructose, hexose diphosphate and glycogen into lactic acid; the latter workers also observed the formation of lactic acid from mannose and glucose 1-phosphate. Studies of the effects of dialysis and of activators and inhibitors supported the view that the pathway of lactic acid formation involved the phosphorylating glycolysis which operates in muscle extracts. However, no intermediary phosphate esters were isolated, nor were any of the individual enzymes studied. Kerly (1948) has reported preliminary experiments on a hexokinase in retinal extracts, but again the products of the reaction were not isolated nor identified. Lenti (1939) also studied lactic acid formation in retinal extracts and concluded that phosphorylation was not involved; he failed in all attempts to isolate phosphorylated intermediates (Lenti, 1943).

The present paper provides direct evidence for the occurrence in retinal extracts of the three glycolytic enzymes, hexokinase, phosphohexose isomerase and 6-phosphofructokinase. Some of the characteristics of the hexokinase have been examined and compared with those of yeast hexokinase; certain intermediate phosphoric esters have also been isolated and identified.

METHODS

Materials

AnalaR grade sugars were used without further purification. D-Glucosamine hydrochloride was purified by two recrystallizations from ethanol-water and had a purity of 98–99% on the basis of its nitrogen content. Glucose 1-phosphate (G 1-P) was prepared by the method of Hanes (1940) and was recrystallized as the di-potassium salt. We are indebted to Dr J. Ashby for samples of synthetic D-glucose 6-phosphate (G 6-P) and D-fructose 1:6-diphosphate (Fru 1:6-P) and to Dr C. W. Parr for D-fructose 6-phosphate (Fru 6-P). Adenosine triphosphate (ATP) was made from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948), and inosine triphosphate (ITP) was made from this by the method of Kleinzeller (1942). All the phosphate esters except Fru 1:6-P were prepared as the barium salts and were converted to the soluble sodium salts before use; Fru 1:6-P was prepared and used as the lithium salt, experiments having shown that lithium ions at the concentration employed did not inhibit the phosphorylating enzymes. All solutions were adjusted to pH 7.5 before use.

Preparation of extracts

(a) *From retina.* Ox eyes, in batches of one dozen, were fetched on ice from the slaughter house. The retinas were dissected out as soon as possible on arrival in the laboratory and were immediately suspended in 10 ml. ice-cold glass-distilled water. The suspension was ground in a chilled mortar with 2 g. acid-washed sand for 1 min. and was then centrifuged in thoroughly chilled tubes for 5 min. at 1500–2000 g. The top, slightly turbid, pink layer was decanted off (or removed with a pasteur pipette) and the residue (a small dark brown layer together with cell debris and sand) was re-extracted with a further 10 ml. glass-distilled water, again centrifuged and the top layer added to the first extract. The combined extracts were dialysed in tightly filled cellophan sacs against running ice-cold glass-distilled

water for 4 hr. The final volume of the extract was 16–18 ml., the dry weight 20–25 mg./ml. and the nitrogen content 2–3 mg./ml.

Attempts to stabilize and purify the hexokinase in retinal extracts were unsuccessful. The addition of 1% (w/v) glucose (cf. van Heyningen, 1942) or of 0.01M cysteine hydrochloride or sodium sulphide (Bailey & Webb, 1948) to the extracts did not increase their stability. Dialysed or undialysed retinal extracts could not be freeze-dried without large losses of enzyme activity: in many cases complete inactivation resulted. Fractionation with ammonium sulphate gave inconsistent results: an active fraction was precipitated, with little loss of activity, by complete saturation; removal of the precipitate formed by 20–30% saturation increased the activity of the supernatant but higher concentrations of salt caused a distribution of the hexokinase between the supernatant and residue, the activities of both being less than that of the original extract. Low temperature fractions with organic solvents, including butanol (Morton, 1950), resulted in complete loss of activity. Acetone powders, made by pulverizing retinas frozen in a mixture of solid CO₂ and acetone and washing the powder with cold acetone on a Büchner funnel, were stable up to 2 months. The hexokinase activity of aqueous extracts of such acetone powders was always less than that of the crude aqueous retinal extracts (about 60% on a comparable weight basis).

Freshly prepared extracts were used for the experiments reported here, although they were not very stable (hexokinase activity lasted only a few days if the extracts were frozen solid).

(b) *From baker's yeast.* In some experiments a partially purified yeast hexokinase preparation was used for comparison with the hexokinase in retinal extracts. The method of Bailey & Webb (1948) was followed as far as their 'stage 4', i.e. autolysis of baker's yeast, extraction in the presence of sulphide, ammonium sulphate precipitation, adsorption on to and elution from calcium phosphate gel and ammonium sulphate fractionation. The final ammonium sulphate fraction was dialysed against several changes of 1% (w/v) glucose solution and was freeze-dried. The preparation was stable for several months when stored *in vacuo* at about 0°. 2–5 µg. dry wt. of this preparation utilized a comparable amount of glucose to 0.1 ml. of dialysed retinal extract.

Tests for enzyme activity

Hexokinase. In most experiments activity was measured by the rate of disappearance of the sugar substrate. In a few cases the reaction was followed by measuring the rate of disappearance of the labile phosphate of ATP; this method is not satisfactory for use with crude extracts as the initial phosphorylated product may undergo further phosphorylation at the expense of more ATP, yielding phosphoric esters which are susceptible to the acid hydrolysis used in the estimation of ATP, so that results are difficult to interpret. A manometric technique, essentially that of Colowick & Kalckar (1941) was also used, particularly for some comparative experiments, but the limitations in the method for crude extracts are similar to those which apply to measurements by changes in labile phosphate.

Except in the manometric method, tests were carried out in open test tubes at 30°. The standard reaction mixture contained: sugar substrate, 0.002M; MgCl₂, 0.01M; NaF,

0.05M; ATP, 0.005M; extract, 0.1 ml. (=200–300 µg. N); veronal buffer pH 7.9, 0.071M.

The veronal buffer (0.5 ml.) (0.142M, Michaelis, 1931), together with all the reactants except ATP (total volume 0.9 ml., NaF added last to diminish the risk of precipitation of MgF₂), was equilibrated for 5 min. at 30°, after which the reaction was started by addition of 0.1 ml. 0.05M ATP. Incubation was usually continued for 10 min., then the reaction was stopped by addition of 1 ml. each of 0.3M-Ba(OH)₂ and 0.3M-ZnSO₄ if sugars were to be estimated, or of 1 ml. 10% trichloroacetic acid if phosphates were to be determined. Control tubes without ATP were included for each sugar substrate; control tubes for phosphate determinations contained ATP but no sugar. In some experiments the total volume of the reaction mixture was increased, but the concentration of all reactants was kept to that stated above.

In the manometric tests the volume of the reaction mixture was increased to 2 ml., the concentrations of the reactants being the same but the relative amount of extract being reduced to 100–200 µg. N per test. Veronal buffer was replaced by 0.02M-NaHCO₃ which was used to buffer the mixture in the main compartment and in the side bulb containing ATP. The flasks were gassed at 30° for 5 min. with a mixture of 95% N₂ and 5% CO₂ and equilibrated until the manometer readings were steady (about 10 min.). Control flasks were always included to correct for gas evolution arising from ATPase action. Rates of gas evolution were uniform over the first 20 min. although there was usually an initial 'jump' in gas evolution when the reaction was initiated, in spite of adequate buffering of the ATP.

Phosphohexose isomerase. Activity was measured by following the disappearance of Fru 6-P. The reaction mixture contained 0.1 ml. of retinal extract, 0.1 ml. 0.02M Fru 6-P and 0.8 ml. 0.142M veronal buffer pH 7.5. After incubation for 10 min. at 30°, the mixture was deproteinized by the addition of 1 ml. of 10% trichloroacetic acid and, after centrifuging, the supernatant was examined for residual fructose esters. Control tubes containing an equal amount of a boiled retinal extract were incubated simultaneously.

Phosphofructokinase. Activity was measured by following the changes in acid-hydrolysable phosphate and also manometrically. Conditions were the same as for estimation of hexokinase activity except that Fru 6-P was used as substrate.

Chemical methods

Reducing sugars were estimated colorimetrically by the Somogyi (1945) modification of Nelson's method; standard curves were constructed for fructose, mannose and glucosamine. Fructose both free and combined as Fru 6-P was also estimated, without interference by other reducing sugars, by the method of Bacon & Bell (1948); this method estimates ketose sugars, but as fructose is the only ketose likely to be present it has been assumed that it was in fact fructose that was estimated. Phosphate esters were determined from the increase in inorganic phosphate following hydrolysis for 10 and 30 min. in N-HCl in a boiling-water bath. It was assumed that all the labile phosphate of ATP was liberated in the first 10 min. hydrolysis. Fru 1:6-P is also partially hydrolysed during this period, but as an equal amount (30%) is liberated in the following 20 min. (Lohmann, 1928) a correction can be made, if this ester is present, in calculating changes in the labile phosphate from

ATP. If P_0 , P_{10} and P_{30} represent the amount of inorganic phosphate found after hydrolysis for 0, 10 and 30 min. respectively, then after incubation the decrease in labile phosphate from ATP is given by the decrease in

$$(P_{10} - P_0) - (P_{30} - P_{10})$$

(it being assumed further that the amount of phosphate liberated from other esters, e.g. adenylic acid, G 6-P, does not change appreciably during incubation), and any formation of Fru 1:6-P phosphate by the increase in $10/3(P_{30} - P_{10})$. Inorganic phosphate was estimated by the Fiske & Subbarow method (1929).

RESULTS

Hexokinase

D-Glucose, D-mannose, D-fructose and D-glucosamine, but not D-galactose, D-arabinose, D-ribose, D-xylose, nor L-sorbose were utilized by the retinal extracts. Meyerhof & Geliazkova (1947) and Hele (1950, 1953) have found that extraction of brain and intestine with a modified Ringer solution was necessary in order to demonstrate hexokinase activity towards galactose, but extracts of retina prepared with this solution were still unable to utilize D-galactose, L-sorbose or the above-listed pentose sugars. The pH optimum for the reaction, 7.4–8.6, was similar for all the sugars tested (Table 1). At pH values below 4.0 retinal extracts formed a precipitate and did not utilize any sugars.

The reaction rate for all the sugars utilized by the extracts, was, for any one extract, approximately proportional to the amount of extract added to the incubation mixture over the range 100–500 μ g. N (Fig. 1). Rates for the different sugars were similar except that glucosamine was utilized more rapidly (see Table 1). The reaction rate did not vary with sugar substrate concentration over the range 0.001–0.05 M, indicating that retinal hexokinase has a high affinity for the sugars. Competitive utilization was investigated by studying the effect of the addition of other sugars on the utilization of fructose. Glucose, mannose, and glucosamine, but not galactose, inhibited the utilization of fructose. Table 2 illustrates this finding in the case of the binary mixtures of fructose with glucose, and fructose with glucosamine. Weil-Malherbe & Bone (1951) have shown that G 6-P inhibits hexokinase in brain preparations, but in these crude extracts there is no question of inhibition of hexokinase by G 6-P which is rapidly converted into Fru 6-P (see below). G 1-P was not phosphorylated by the extracts and its presence did not interfere with the utilization of glucose.

Kleinzeller (1942) found that ITP could replace ATP as a phosphate donor for yeast hexokinase; this was tested with both retinal and partly purified yeast extracts, but glucose was not phosphorylated in the presence of either extract by ITP. The reason for the difference from Kleinzeller's results is not apparent.

Earlier work with retinal extracts (Kerly & Bourne, 1940; Süllman & Vos, 1939) showed that Mg^{2+} was necessary for glycolytic activity, but the effect of other divalent ions was not investigated. The effect of a number of divalent ions was studied in 12–18 hr. dialysed retinal extracts, and for comparison, in partially purified yeast extracts. The incubation period was increased to 30 min. as the retinal extracts had lost 50% of their activity during the extra period of dialysis. Since heavy metals form chelation complexes with veronal, the buffer was replaced by 0.1 M N-ethyl morpholine

Table 1. *Effect of pH on utilization of sugars by retinal extract*

0.1 ml. extract incubated 10 min. at 30°.

pH	Sugar used (μ mole/mg. N) when substrate was			
	Glucose	Fructose	Mannose	Glucosamine
4.6	1.7	2.3	1.5	0.3
5.8	2.4	2.5	2.1	3.4
7.4	3.6	4.8	3.5	7.3
8.6	3.7	3.7	4.7	7.2

Fig. 1. Effect of concentration of extract (measured as μ g. N) on hexokinase activity of retinal extract; μ l. CO_2 liberated in 5 min. from extract incubated at 30° with 0.002 M glucose.

Table 2. *The effect of glucose and glucosamine on the utilization of fructose by retinal extracts*

0.1 ml. extract incubated 10 min. at 30°, pH 7.9. Fructose concn. 0.002 M.

Concn. of glucose or glucosamine (M)	Glucose added		Glucosamine added	
	Fructose used (μ g.)	Inhibition (%)	Fructose used (μ g.)	Inhibition (%)
0	150	—	142	—
0.002	0	100	0	100
0.001	0	100	42	71
0.0005	50	67	65	51
0.0002	95	37	105	26
0.0001	145	0	115	20

adjusted to the required pH with 0.1 N-HCl. Hexokinase activity was not influenced by the change in buffer. Fluoride was omitted to avoid any effects which might have arisen from varying solubilities of the metal fluorides. Manganese, and to a much less extent cobalt, but not calcium, beryllium, copper, zinc, mercury, or ferrous iron, could replace magnesium (see Table 3). All the metal ions were added as the chlorides, except ferrous iron which was in the form of ferrous ammonium sulphate; they were all tested at a concentration of 0.01 M, that which was found to be optimum for Mg and Mn (Table 4). Bailey & Webb (1948), using yeast extract, found that calcium did not antagonize magnesium; this effect was tested with retinal extracts and a similar result was obtained, i.e. Ca^{2+} does not antagonize Mg^{2+} in retinal extracts.

An attempt was made to identify the products of the action of retinal hexokinase on the different sugar substrates. Test mixtures incubated with glucose, mannose or fructose were deproteinized by trichloroacetic acid and by barium hydroxide-zinc sulphate. Filtrates obtained by the former method of deproteinization gave a colour when tested with the Bacon & Bell ketose reagent, but those deproteinized by the latter method showed no colour, indicating that a fructose phosphate had been formed. When glucosamine was used as substrate no ketose colour appeared in either filtrate. The experi-

ments were repeated using a larger volume of reaction mixture and the trichloroacetic acid filtrates were fractionated with barium (LePage, 1949).

When glucose or fructose was used as substrate, the ketose reaction was found in the barium-insoluble fraction, but only weakly in the barium-soluble fraction, indicating that the predominant ester formed was Fru 1:6-P. Studies of the rates of hydrolysis of the phosphate esters formed after incubation of the extract with glucose further confirmed the formation of Fru 1:6-P, whereas simultaneous experiments with glucosamine as substrate gave no indication of formation of a readily hydrolysable ester such as Fru 1:6-P (Table 5). Calculation showed that more than one labile phosphate group from ATP was utilized per mole of glucose disappearing, but approximately one mole per mole of glucosamine. After incubation of the extract with glucosamine a phosphate ester was found in the barium-soluble-alcohol-insoluble fraction, indicating a hexose monophosphate as the product of

Table 3. *The effect of divalent cations on glucose utilization by yeast and retinal hexokinase*

0.1 ml. 18 hr. dialysed extract incubated, without addition of NaF, 30 min. at 30°, pH 7.9. Metal-ion concn. 0.01 M.

Cation added	$\mu\text{g.}$ Glucose used with enzyme from	
	Yeast	Retina
Mg	230	160
Mn	220	140
Co	50	30
Ratio of activity		
Mn/Mg	0.96	0.88
Co/Mg	0.22	0.19

Table 4. *The effect of concentration of Mg and Mn on glucose utilization by retinal extracts*

0.1 ml. 18 hr. dialysed extract, incubated without addition of NaF, 30 min. at 30°, pH 7.9.

Concn. of added ion (M)	$\mu\text{g.}$ Glucose used in presence of	
	Mg	Mn
0.02	130	—
0.01	140	130
0.005	120	90
0.001	90	50
0.0005	60	30

Table 5. *Utilization of glucose and ATP and formation of Fru 1:6-P in retinal extracts incubated with glucose and glucosamine*

0.1 ml. extract incubated for 30 min. at 30°, pH 7.9. P_0 , P_{10} and P_{30} represent $\mu\text{g.}$ inorganic phosphate (as phosphorus) found after hydrolysis for 0, 10 and 30 min. respectively. See text for method of calculating decrease in labile phosphate from ATP and formation of Fru 1:6-P.

	Extract incubated		
	Without sugar (control)	With glucose	With glucosamine
$P_{10} - P_0$	126	92	79
$P_{30} - P_{10}$	10	21	8
$(P_{10} - P_0) - (P_{30} - P_{10})$	116	71	71
Decrease in labile phosphate from ATP	—	45	45
Formation of Fru 1:6-P phosphate	—	37	—
$\mu\text{g.}$ Sugar utilized (as glucose)	—	160	300
Molar ratio: Labile phosphate/sugar	—	1.64	0.87

Table 6. *Acid hydrolysis of glucosamine phosphate*

1.6×10^{-3} M ester hydrolysed at 100° in N-HCl.

Time (min.)	...	30	60	120	
Phosphate liberated (% total)		5.4	9.4	20.0	
$10^5 K^*$		3.2	2.6	3.1	Mean, 3.0

* Dissociation constant calculated from the equation for a first-order reaction: $K = 1/t \log_e a/(a-x)$, where t = time in sec., a = initial concn. and $(a-x)$ concn. at time t .

hexokinase action on this sugar. Evidence that this compound was a phosphate ester and not a glycoside came firstly from the finding that it was a reducing sugar (the reducing value being increased after acid hydrolysis), and secondly from its stability to hydrolysis in acid solution (Table 6). By analogy with the work of Brown (1951) on brain extracts it is likely to be glucosamine 6-phosphate.

Phosphohexose isomerase

The results reported in the previous section indicate that phosphorylation continues to the Fru 1:6-P stage. By analogy with other glycolytic systems, this requires the presence of phosphohexose isomerase to convert the initial product of the reaction, presumed to be G 6-P, into Fru 6-P which is then phosphorylated to Fru 1:6-P. Direct evidence for the presence of this enzyme (phosphohexose isomerase) in crude retinal extracts was obtained in two ways, first by following the change in fructose colours when Fru 6-P was incubated with extract but no ATP (Table 7), and secondly by showing that G 6-P reacts in a similar manner to Fru 6-P when added to the test system for phosphofructokinase (Fig. 2). The enzyme must be more active than hexokinase since Fru 6-P disappeared rapidly even in extracts diluted 100 times, a dilution at which no hexokinase activity was apparent.

Phosphofructokinase

The identification of Fru 1:6-P as a product of the incubation of glucose with crude retinal extracts, and the direct demonstration of a hexokinase and a phosphohexose isomerase in the extracts indicate that Fru 1:6-P is formed by the consecutive action of these two enzymes and a phosphofructokinase. Direct evidence for the presence of this last enzyme was obtained by following the changes in hydrolysable phosphate when either G 6-P or Fru 6-P was added to the reaction mixture. Approximately 1 mole of Fru 1:6-P was formed for each mole of ATP utilized (Table 8). Utilization of G 6-P and Fru 6-P was also followed manometrically. There was no significant gas evolution without addition of ATP, indicating the absence of an active phosphatase hydrolysing either of these esters. The rates of gas evolution were always greater than that observed with glucose (Fig. 2) implying that the hexokinase reaction is the rate limiting step in the formation of Fru 1:6-P from glucose. With aged extracts, phosphofructokinase activity was lost more rapidly than that of hexokinase. In acetone-powder extracts also, phosphofructokinase activity was reduced relative to that of hexokinase (Fig. 3), indicating that phosphofructokinase is more labile than hexokinase.

Table 7. *Disappearance of Fru 6-P in retinal extracts incubated without ATP*

Extract containing 0.002M Fru 6-P incubated 10 min. at 30°, pH 7.5; control = boiled extract.

Concn. of enzyme (μ g. N/ml.)	Fructose remaining after incubation	
	Colorimeter reading	% Control
Control: 250	20	—
250	6	30
25	6	30
2.5	8	40

Fig. 2. Comparison of rates of activity of phosphofructokinase and hexokinase in retinal extract: μ l. CO₂ produced by 0.1 ml. extract incubated at 30°. x — x, ATP only, no substrate; ▲—▲, 0.002M glucose; ●—●, 0.002M G 6-P; ○—○, 0.002M Fru 6-P.

Table 8. *Utilization of ATP and formation of Fru 1:6-P in retinal extracts incubated with Fru 6-P*

0.1 ml. extract incubated 10 min. at 30°, pH 7.9. P₀, P₁₀ and P₃₀ represent μ g. inorganic phosphate (as phosphorus) found after hydrolysis for 0, 10 and 30 min. respectively. See text for method of calculating decrease in labile phosphate from ATP and formation of Fru 1:6-P.

	Extract incubated	
	Without Fru 6-P (control)	With Fru 6-P
P ₁₀ - P ₀	155	136
P ₃₀ - P ₁₀	22	45
(P ₁₀ - P ₀) - (P ₃₀ - P ₁₀)	133	91
Decrease in labile phosphate from ATP	—	42
Formation of Fru 1:6-P phosphate	—	77
Molar ratio: labile P/Fru 1:6-P	—	1.09

Fig. 3. Comparison of rates of activity of phosphofructokinase and hexokinase in an aqueous extract of acetone-dried retinas. Extract (0.1 ml.) containing 400 μ g. N, incubated at 30°. ▲—▲, 0.002M Glucose; ○—○, 0.002M Fru 6-P.

DISCUSSION

The hexokinase in retinal extracts is analogous to the hexokinase from yeast in its metal ion requirements and substrate specificity. The inhibition of fructose utilization by glucose and glucosamine suggests that the three sugars are substrates of a common hexokinase, as in yeast, in contrast to the separate gluco- and fructo-kinases which exist in muscle (Slein, Cori & Cori, 1950) and in liver (Vestling, Mylroie, Irish & Grant, 1950). It is of interest that all the sugars utilized, including glucosamine, contain the partial structure shown to

be essential for all substrates of yeast hexokinase (Gottschalk, 1950), whereas glucose 1-phosphate, which also contains this structure, is not attacked. In muscle a specific enzyme has been found which phosphorylates G1-P (Paladini, Caputto, Leloir, Trucco & Cardini, 1949). It may be noted that 2-deoxyglucose, another sugar containing the above structure, has recently been shown to be phosphorylated by yeast (Cramer & Woodward, 1952) and by brain hexokinase (Sols & Crane, 1953). The hexokinase from intestinal mucosa may be fundamentally different from that of yeast and retina since it phosphorylates sugars which do not have this structure, e.g. 3-methylglucose (Csaky, 1953), galactose and xylose (Hele, 1953) none of which are phosphorylated by yeast or retinal hexokinase.

It is significant that in retinal extracts glucosamine cannot be metabolized beyond the stage of the monophosphate, although fructose 6-phosphate and glucose 6-phosphate are rapidly metabolized. Very little is known of the metabolism of glucosamine. Harpur & Quastel (1949) first reported its phosphorylation in brain extracts and further showed that *N*-acetyl glucosamine, which was not phosphorylated, inhibited the phosphorylation of glucosamine, fructose and glucose by the brain preparation, and Brown (1953) has shown that glucosamine 6-phosphate is slowly converted into glucosamine 1-phosphate by rabbit muscle extracts and by purified phosphoglucumutase. It is of interest that liver and brain extracts and the lactose yeast *Saccharomyces fragilis*, which are capable of phosphorylating galactose, also phosphorylate the corresponding amino sugar galactosamine (Cardini & Leloir, 1953).

SUMMARY

1. The presence of hexokinase in retinal extracts has been demonstrated. In the presence of ATP it phosphorylates glucose, fructose, mannose and glucosamine. Magnesium, manganese and to a smaller extent cobalt activate the enzyme in dialysed extracts.
2. Both glucose and glucosamine inhibit the utilization of fructose, implying that all three sugars are substrates of a common hexokinase.
3. Glucosamine is converted into a monophosphate derivative which is not further metabolized by the extracts; the other sugars yield fructose 1:6-phosphate.
4. Direct evidence for the presence of phosphohexose isomerase and of phosphofructokinase is also presented.

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Monoamino Monocarboxylic Acid Content of Preparations of Herbage Protein

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The similarity in amino acid composition of numerous 'representative' leaf proteins and of the constituent cytoplasmic and chloroplastic fractions is fairly well established but some doubt remains as to the degree of similarity, variations with age and species having been reported (review by Lugg, 1949).

Earlier analyses were rendered difficult by the dearth of suitable techniques and have, in general, been confined to those amino acids which are readily determined by means of some characteristic group in the molecule. Unfortunately, it is often these substances that are most labile under the conditions of hydrolysis.

Before investigating the fate of amino acids liberated by proteolysis in herbages under conditions of starvation (e.g. wilting and ensilage) it was essential to know whether the residual-protein fractions were of constant composition or whether it would be necessary to undertake analysis in every case. The method of Kemble & Macpherson (1954), which was developed primarily for the amino acid analysis of plant extracts and which was to be used for the proteolytic investigations, was therefore applied to the analysis of protein-rich fractions extracted by several methods from a variety of herbages.

EXPERIMENTAL

Source and extraction of proteins

Protein A. The protein was extracted from perennial rye grass (S. 24 hay strain) by the method of Crook (1946), which entails repeated grinding with sodium borate buffer at

pH 8.0. The grass was senescent, being cut in December. 62.5% of its total protein-N was extracted, as material having N 10.8% of dry matter.

Protein B. Mixed grass from permanent pasture was macerated in 0.05M borax, filtered through paper pulp and precipitated at pH 4.5. The protein, after heat coagulation, was washed with ethanol and ether. 41.2% of the grass protein-N of the grass was obtained as material having N 14.5% of dry matter.

Protein C. Young lawn grass (height 4–6 in.) was macerated with a solution of borax (2%, w/v). After filtration through muslin the solution was brought to pH 4.5 and the protein coagulated by heating to 90°. 38.2% of the total protein-N was obtained; N, 8.9% of dry matter.

Protein D. Young perennial rye grass (S. 24 hay strain) was cut in May (height 4–6 in.). It was finely minced and the sap filtered by suction through muslin and then through paper. The protein was coagulated by heating to 90° at the pH of the sap (6.3). It was washed with hot water, ethanol and ether and represented 32.8% of the fresh grass protein-N; N, 13.4% of dry matter.

Protein E. A sample of alsike clover was cut before flowering and extracted as described for protein A. 39% of the clover protein-N was obtained as material having N 14.3% of dry matter.

Protein F. A similar grass sample to that used for protein C was allowed to wilt in the laboratory for 48 hr. In this time 10% of the protein had been degraded. The remainder was extracted as described for protein C. 37% of the total protein-N was extracted; N, 9.0% of dry matter.

Protein G. An identical sample of sap to that from which protein D was obtained was bottled, covered with a thick layer of liquid paraffin and placed in an incubator at 30°. These conditions simulated the ensilage process (Macpherson, 1952) and the pH dropped rapidly to 3.9. After