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An Amino Acid Sequence in the Active Centre of Phosphoglucomutase

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The presence of phosphate in phosphoglucomutase was first demonstrated by Jagannathan & Luck (1949), who also showed that it may readily be exchanged with phosphate in the substrates and that it is thus probably involved in the active centre of the enzyme. Anderson & Jollès (1957) observed that it was not released from the protein even under strong acid conditions and were able to isolate serine phosphate from the partially hydrolysed enzyme. This offered the possibility of studying the amino acid sequence around the point of attachment of the phosphate by isotopic techniques. In a preliminary study of the peptides produced from a partial hydrolysate of phosphoglucomutase labelled with ^{32}P, Koshland & Erwin (1957) and Koshland, Ray & Erwin (1958) suggested that the sequence around the serine phosphate (SerP) residue was Asp.SerP.Gly.Glu.Ala.Val. [For definitions of the abbreviations of amino acids used in this paper see *Biochem. J.* (1957), **66**, 6.] This was very similar to that found in trypsin and chymotrypsin around the serine residue that can react with di*iso*propyl phosphorofluoridate. The sequence Asp.Ser.Gly thus appeared to be a common feature of enzymes with rather dissimilar functions, and these conclusions have been discussed by several authors in connexion with the problem of the relationship between structure and function (Lumry, 1959; Koshland, 1959; Linderstrøm-Lang & Schellman, 1959; Dixon, Neurath & Pechère, 1958). In the work of Koshland & Erwin evidence for the purity of the peptides studied was lacking, and, by comparing partial hydrolysates of [^{32}P]phosphoglucomutase with similar hydrolysates of labelled chymotrypsin, we were able to show (Milstein & Sanger, 1960) that the sequence around the serine phosphate residue in phosphoglucomutase was not Asp.SerP.Gly. The present paper describes the determination of a pentapeptide sequence around the serine phosphate residue.

In most experiments with peptides of serine phosphate it is usually possible to separate them from other peptides by virtue of their strong acid group by ion-exchange methods (Flavin, 1954) or by ionophoresis at an acid pH. Since most of the labelled peptides in a partial hydrolysate of [^{32}P]-phosphoglucomutase contain a basic residue, they are neutral and purification is much more difficult. We have thus determined as much as possible of the sequence by isotopic methods, since it is relatively easy to separate the labelled peptides from one another but very difficult to separate them from the very complex mixture of non-labelled peptides that is derived from such a large protein. Some such methods have been described previously (Naughton, Sanger, Hartley & Shaw, 1960; Sanger & Shaw, 1960; Milstein & Sanger, 1960). The interrelationships of the various peptides (Tables 3 and 4) were first determined by studying the effect of partial acid hydrolysis and Edman degradation on them. The presence of a histidine residue after the serine phosphate and of an acidic residue next to the histidine was concluded from the ionophoretic rates of the peptides at different pH values.

Another method for the identification of histidine peptides was also developed by making use of the lability of the imidazole ring to photo-oxidation. Weil, Gordon & Buchert (1951) showed that some amino acids can be oxidized when they are exposed under a visible light in the presence of methylene

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blue and that peptide bonds do not participate in the photochemical action of the dye. Of the amino acids studied, the most reactive appeared to be histidine, tyrosine, tryptophan, methionine and cysteine. It was suggested that, during the reaction, the imidazole ring breaks, with formation of an aldehyde group. It would therefore be expected that the electrophoretic mobility of a peptide containing histidine will change after exposure to photo-oxidation. Since no other basic amino acid should react under those conditions, differentiation of histidine peptides from other basic amino acids containing peptides is possible.

Another specific test that proved useful was the use of periodate (Sanger & Shaw, 1960). If the ionophoretic rate of a peptide was altered after treatment with periodate it could be concluded that it contained serine or threonine in the *N*-terminal position.

Further information about the individual residues present in the peptides could be obtained by studying their R_F values on paper chromatography. Pardee (1951) showed that the chromatographic rate of a peptide in a given solvent system can be related to the chromatographic rate of the amino acid components by the following formula:

$$RT \ln (1/R_{F(p)} - 1) = (n-1)A + B + \Sigma RT \ln (1/R_{F(a)} - 1) \quad (1)$$

in which R is the molar gas constant, T the absolute temperature, n the number of amino acid residues in the peptide, and A and B are constants. $R_{F(p)}$ and $R_{F(a)}$ are the R_F values of the peptide and the amino acids respectively. This relationship has been shown to fit well with the experimental data obtained on different solvent systems with 46 peptides (Moore & Baker, 1958). These authors reported no significant differences for groups of peptides in which the sequence of amino acids was altered.

From this it follows that if, for a given solvent system, the values of the constants and the R_F values of a peptide with one unknown amino acid are known, the R_F of the unknown amino acid could be easily deduced. The R_F values of an unknown amino acid will not in most cases be enough to characterize it. However, from the combination of R_F data on more than one system and information obtained by mobility studies and specific reactions, it should be possible to obtain valuable information leading to the resolution of the sequence around one known amino acid. With these techniques it could be concluded that the most probable sequence was Thr.Ala.SerP.His.-(Glu or Asp). In order to confirm this and to identify the acidic amino acid it was necessary to purify certain of the peptides and identify the amino acids present by established techniques. A rather extensive purification was found to be necessary, but since the size and approximate composition of the peptides was known it was easy to ascertain when they were pure. From these results it was shown that the sequence was Thr.Ala.SerP.His.Asp.

METHODS

Phosphoglucomutase (PGM) was prepared by the method of Najjar (1948) and dialysed against distilled water before use. Trypsin and chymotrypsin were crystalline salt-free preparations from Worthington Biochemical Corp. Elastase was a gift from M. A. Naughton (Naughton & Sanger, 1960).

Preparation of [^{32}P]*phosphoglucomutase*

Two methods were used for the preparation of [^{32}P]PGM. In method 1 PGM was labelled by allowing it to react with glucose 6-[^{32}P]phosphate, which was prepared through the hexokinase reaction. A mitochondrial suspension was used to generate adenosine [^{32}P]triphosphate from [^{32}P]phosphate. In method 2 glucose 1-[^{32}P]phosphate was prepared by the action of potato phosphorylase on starch in the presence of [^{32}P]phosphate, and allowed to react with the PGM.

Method 1. Each 8·2 ml. of the incubation mixture contained (amounts in μmoles unless otherwise stated): $MgSO_4$, 60; glucose, 225; 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, 125; glutamate or proline, 75; KCl, 250; adenosine triphosphate (ATP), 2; $H_3{}^{32}PO_4$ (carrier-free), 0·2–2 mc; fresh mitochondria suspension, 0·75 ml.; and excess of hexokinase. The mitochondria were freshly prepared from sucrose homogenates of rat liver and tested before use. Yeast hexokinase was prepared according to Berger, Slein, Colowick & Cori (1946), by using the preparation of stage 5. The total volume of the incubation mixture was 8·2 ml. and the pH 7·3. After the addition of the hexokinase, the mixture was kept for 40 min. at room temperature. Histidine (75μmoles, pH 7·5) was then added together with a dialysis bag containing a solution of PGM in a small volume (0·5–5 ml.); the whole was allowed to equilibrate in the cold overnight. The dialysis bag was then transferred to a 5 l. beaker and dialysed against distilled water with stirring. After 12 hr. the water was changed, and the dialysis repeated five times, unless otherwise indicated. Even after additional changes of the water, considerable amounts of radioactive phosphate and glucose phosphate were released when the protein was subjected to tryptic digestion or partial acid hydrolysis and to ionophoresis at pH 6·5 or 3·5. This preparation was used for the structural studies, unless otherwise indicated. The specific activity of the protein ranged from about 0·05 to 0·5μc/mg.

As a control in one experiment this procedure was repeated exactly as above, except that PGM was denatured before use. The denatured PGM was prepared by precipitating 2 mg. of protein with 10% trichloroacetic acid and then dialysing it against 1·5 l. of distilled water. Native enzyme (5 mg.) was included in the same experiment. After the usual procedure (except that a smaller amount of ^{32}P was used) the native enzyme contained 7·5μmc/mg. of PGM, whereas the denatured one contained 0·3μmc/mg. of protein. As will be seen below, in the denatured preparation, only phosphate and glucose phosphate were detected

after partial acid hydrolysis or tryptic digestion, whereas the native preparation contained phosphopeptides in addition.

Method 2. A crude phosphorylase preparation was prepared by blending 20 g. of potatoes with 20 ml. of water. Kaolin (0·6 g.) was added and the mixture centrifuged. To 5 ml. of 2·5% starch solution were added 0·5 ml. of 0·2M-phosphate buffer, pH 7·0, 1·5 ml. of the above supernatant solution, a few drops of toluene and 1 mc of $H_3{}^{32}PO_4$ (carrier free). The mixture was kept at room temperature for 8 hr. A solution of 5 mg. of PGM in 1·0 ml. of water containing histidine (0·04M, pH 7·5) and $MgSO_4$ (2·5 mM) was dialysed overnight against the above mixture. The dialysis bag was then placed in a 5 l. beaker and dialysed against several changes of water.

Tryptic hydrolysis of [^{32}P]*phosphoglucomutase*

[^{32}P]PGM was twice dialysed against 40% urea and then against water until the urea was removed. Under these conditions the denatured PGM was precipitated. The precipitate was suspended in $(NH_4)_2CO_3$ (0·5%) to produce a 0·25% suspension, and crystalline trypsin (7·5μg./ml.) was added. Incubation was carried out at 37° with occasional stirring. Samples were removed at various times and subjected to ionophoresis after removal of the $(NH_4)_2CO_3$ *in vacuo*.

Partial acid hydrolysis. Two methods of partial acid hydrolysis were used:

(*a*) 5·7N-HCl at 100°. In general the reaction was carried out in small test tubes with approx. 0·2 ml. of acid. The time of hydrolysis was 30 min. unless otherwise indicated.

(*b*) 12 N-HCl at 37°. This method was used principally when larger amounts of protein were hydrolysed. Between 20 and 70 mg. of protein was incubated with 3–10 ml. of 12 N-HCl, for 30–40 hr. at 37° in tubes fitted with ground-glass stoppers.

Ionophoretic fractionation of peptides

The peptides were fractionated by high-voltage paper ionophoresis as previously described (Michl, 1951; Naughton *et al.* 1960). The buffer at pH 4·0 was prepared by adding pyridine to the buffer at pH 3·5. Unless otherwise stated, Whatman no. 52 paper was used with voltage gradients of 40 v/cm.

The pH–mobility curves (Fig. 9) were determined in the apparatus of Gross (1955) with Whatman no. 1 filter paper and 50 v/cm. (Naughton *et al.* 1960).

In order to standardize the positions of peptide spots and the conditions of each ionophoretic run, it was found convenient to use a mixture of coloured markers, which were usually applied at one side of the paper and run in parallel with the radioactive peptides. A suitable mixture for running at pH 3·5 or 6·5 towards the anode was made by using the following dyes: Pappenheim's panoptic stain; isamine blue; xylene cyanol FF; phenol red; methyl orange; safranine; Bordeaux; methyl green; light green SF.

For two-dimensional ionophoresis the following method, which was preferable to those previously described (Naughton *et al.* 1960), was used. Ionophoresis was carried normally in the first direction and the paper was radio-autographed. A strip containing the peptides was cut out and sewn with a sewing machine to a second strip of paper. The paper was then wetted with the buffer to be used in the second dimension in such a way that it flowed evenly towards the strip from each side, thus sharpening the bands. The paper was then subjected to ionophoresis in the second dimension.

Paper chromatography

Two systems were used for studying the R_F values of the peptides: butan-1-ol–acetic acid–water–pyridine (30:6:24:20, by vol.) (Waley & Watson, 1953); phenol–water: phenol saturated with water at 25°. Whatman no. 52 filter paper was used with both systems.

Photo-oxidation technique for the detection of histidine peptides

The method was tested first with pure amino acids. The following amino acids were applied on a sheet of Whatman no. 52 paper, in two series one next to the other: tryptophan, tyrosine, arginine, lysine, histidine and methionine. The paper was moistened with pyridine–acetate buffer (10% of pyridine, 0·4% of acetic acid, pH 6·5) and then with methylene blue (0·2% in absolute ethanol). The paper was then covered with a polythene sheet, and one of the series of amino acids covered with a black paper to use as control. The paper was pressed between glass plates and the part containing both series of amino acids put under a 150 w lamp at 30 cm. for 2 hr. It was then dried in the dark, the amino acids were subjected to ionophoresis at pH 2·1 for 20 min. and the paper was sprayed with ninhydrin. The methylene blue, which covers part of the sheet, does not seem to interfere with the ninhydrin reaction and the spots can easily be located. It was observed (Fig. 1) that tryptophan, present in the control, completely disappeared after photo-oxidation. Histidine, which ran together with the basic amino acids in the control, is slightly slower than tyrosine after the treatment. No difference was detected in the other amino acids tested.

The test for histidine peptides was made as follows: a partial acid hydrolysate of [^{32}P]PGM containing about 2·5μmc of ^{32}P was applied on Whatman no. 52 paper in a 1·5 cm. wide band and subjected to ionophoresis at pH 4·0

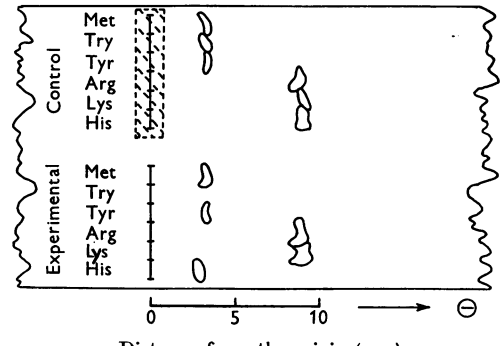

Fig. 1. Effect of photo-oxidation on the electrophoretic mobilities of amino acids at pH 2·1. Before electrophoresis both series of amino acids were photo-oxidized as indicated in the Methods section. During photo-oxidation the shaded area was covered with a black paper.

for 2 hr. It was then radioautographed, and a strip was cut to include peptides 7, 6, 5 and 4, and part of the glucose [^{32}P]phosphate to be used as a marker. The strip was moistened with buffer solution and methylene blue, and placed under the 150 w lamp in a dark room. Another strip of the same paper containing histidine, lysine and arginine, covered and uncovered with black paper, was subjected to the same treatment and used as further control. Both strips were placed together under the same light, and exposed for 80 min. After being dried in the dark, they were sewn with a sewing machine on a sheet of Whatman no. 52 paper at the same distance from the edge of the paper as the hydrolysate was applied in the first run. Ionophoresis was repeated under the same conditions (pH 4·0, 2 hr.).

Direct identification of amino acids in peptides

In order to obtain sufficient quantities of the peptides to identify the amino acids present in them directly, larger-scale experiments were carried out: [^{32}P]PGM (70 mg.) was hydrolysed for 2 days at 37° in 12N-HCl and passed through a column (20 cm. × 0·8 cm. diam.) of Dowex 50X4 (200–400 mesh). About 50% of the radioactivity passed straight through the column when washed with water and appeared in the first 20 ml. of eluate. This was collected, freeze-dried and labelled F_1. The column was then developed with ammonium acetate solution (2%, pH 3·7), which eluted about 30% of the radioactivity. This fraction, which contained most of the material in the partial hydrolysate, was freeze-dried and labelled F_3. No more radioactivity could be eluted from the column by increasing the pH to 7·0.

Samples of fractions F_1 and F_3 were subjected to ionophoresis at pH 4·0 and 6·5. The bands obtained were designated by numbers as shown in Fig. 4. F_1 contained the acidic components, namely bands 2 (serine phosphate), 4 and 5 as well as 1 (phosphate) and 3 (glucose phosphate). F_3 contained the 'neutral' components (bands 6 and 7). This fractionation on Dowex 50 was very useful for the purification of peptide 5 but unnecessary for the 'neutral' peptides.

All the peptides were purified further by paper ionophoresis and chromatography (Table 6), generally with the two-dimensional method. Whatman no. 52 paper was used where possible, but, when the amount of material was so large that it would have caused overloading on no. 52, it was applied to no. 3 MM paper.

The first run of fraction F_3, or when the Dowex 50 step was omitted, was made on Whatman no. 3 MM paper (peptides 6 and 7, Table 6) and the bands were eluted and dried. In other cases the samples were applied as 2 cm. bands and run in two dimensions. After location of the spots by radioautography they were cut out and were either eluted and hydrolysed for amino acid determination or sewn on another sheet of paper and again run in two directions.

The amino acids were identified by two-dimensional paper electrophoresis (pH 2·1; 30 min.; 80 v/cm.) and chromatography in butanol–acetic acid–water (3:1:1, by vol.) for 3 hr. (L. F. Smith, unpublished work), except for 7A and 7C (Table 6), in which case only electrophoresis was used.

RESULTS

Labelling of phosphoglucomutase

According to Kennedy & Koshland (1957) PGM is inhibited by di*iso*propyl phosphorofluoridate (DFP). An attempt was therefore made to label the enzyme by treatment with [^{32}P]DFP. The conditions used were similar to those used by the above authors for obtaining 100% inhibition of PGM in 15 min.: PGM was incubated with [^{32}P]-DFP (1·0 mM), cysteine (25 mM), $MgSO_4$ (1·5 mM) and glucose 1-phosphate (5 mM). The pH was adjusted to 8·2. After 3 hr. the mixture was dialysed extensively against water. A partial acid hydrolysate of the freeze-dried protein was examined by ionophoresis at pH 3·5 followed by radioautography. No 'phosphopeptides' or 'phospho-amino acids' were detected but only inorganic phosphate and a trace of mono*iso*propyl phosphate. A second experiment in which the concentration of the DFP was increased ten times, the incubation period increased to 12 hr. and the enzyme precipitated with HCl before dialysis to avoid the possibility of removal of the phosphate during dialysis, was also negative.

Labelling the PGM by exchange of phosphate with labelled substrate proved much more successful. According to Kennedy & Koshland (1957) their preparation contained about 1·5 μmc/mg. of protein, by using as starting material 1 mc of ^{32}P. This would have been insufficient for our purposes. With the techniques described above, it was possible to prepare a labelled protein containing as much as 0·5 μc/mg. This offered the possibility of using minute amounts of protein for sequence studies.

Stability of the bound ^{32}P in labelled phosphoglucomutase. The [^{32}P]PGM prepared by these methods still contained some diffusible ^{32}P. When dialysed five times against 100 ml. of water for 24 hr., some radioactivity still diffused out. The material after such treatment was dialysed under various conditions and the amount of radioactivity that diffused out was measured (Table 1). Three separate experi-

Table 1. *Removal of ^{32}P from [^{32}P]phosphoglucomutase by dialysis*

Expt.	Dialysis against	Amount diffused out (%)
A	Water	5·6
	Water	6·1
	Water	6·0
B	0·1 mM-Phosphate (pH 6·0)	1·5
	4·0 mM-Phosphate (pH 6·2)	3·7
	Water	1·0
C	40% Urea	7·1
	Water	1·3
	Water	1·2

ments were performed, in which samples containing about 50 μmc of [^{32}P]PGM were dialysed against 25 ml. of the indicated solution for 24 hr. The radioactivity of the dialysate was determined and the dialysis was repeated with a further 25 ml. of solution. In all cases some radioactivity diffused out, although in a small proportion compared with the total amount. When the urea-treated enzyme (Table 1, Expt. C) was digested with trypsin and the hydrolysate subjected to ionophoresis at pH 6·5, a spot corresponding to phosphate and amounting to about 30 % of the total remaining radioactivity was obtained. This suggests that there is still some labile phosphate remaining inside the sac even after very extensive dialyses, namely three times against 5000 vol. of water (12 hr. each with stirring), five times against 100 vol. of water (24 hr. each), once against 16 vol. of 40 % urea (24 hr.) and finally twice against 16 vol. of water for 24 hr.

Tryptic hydrolysate of [^{32}P]*phosphoglucomutase*

Fig. 2 indicates the time course for the hydrolysis of [^{32}P]PGM with trypsin. The results clearly suggest that band B (not necessarily a single radioactive peptide) is the primary breakdown product formed by the splitting of very susceptible bonds, the reaction being complete when all the denatured material has come into solution (usually less than 4 hr.). A second slower reaction takes place and a radioactive peptide is released from band B which moves quicker towards the anode at pH 6·5 (band A). This second reaction hydrolyses band B after incubation for 17 hr. A third product, band C, begins to be apparent at that time. Trypsin usually contains traces of other pancreatic enzymes, especially chymotrypsin, and it is possible that band C is due to the action of such a contaminant on band A or B. Partial acid

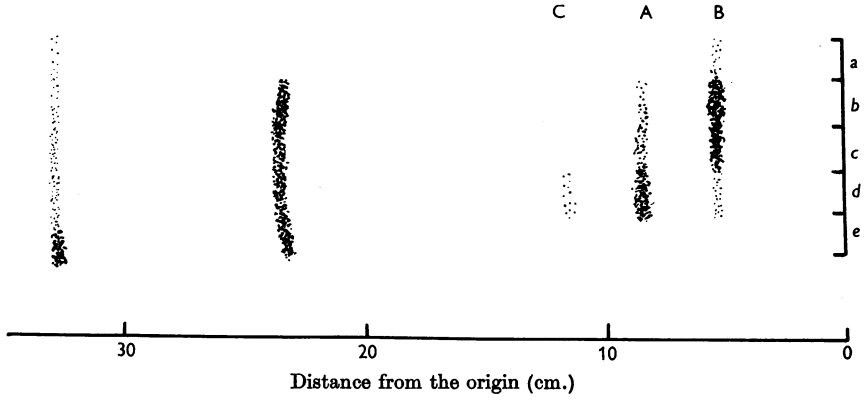

Fig. 2. Tracing of radioautograph obtained after ionophoresis (pH 6·5; 40 v/cm.; 2 hr.) of tryptic digest of [^{32}P]PGM. Conditions of the digestion were as described in text. Times of digestion: *a*, 1½ hr.; *b*, 4 hr.; *c*, 7 hr.; *d*, 17 hr.; *e*, markers of bands 1 and 3 (Fig. 4).

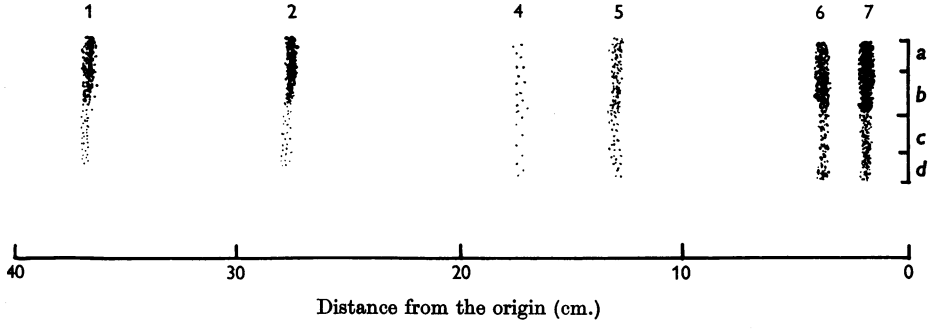

Fig. 3. Tracing of radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of partial acid hydrolysates of tryptic peptides (Fig. 2) derived from [^{32}P]PGM prepared by methods 1 and 2. *a*, Band B (method 1); *b*, band A (method 1); *c*, band A (method 2); *d*, band B (method 2).

hydrolysates of the three bands were made and since no difference of pattern was observed, either the pure bands or a mixture of them was used in further partial hydrolysis studies.

The [^{32}P]PGM prepared by method 2 was also subjected to hydrolysis by trypsin. Fig. 3 shows an ionogram of the partial acid hydrolysate of the tryptic bands, which was run together with a similar hydrolysate of [^{32}P]PGM prepared by method 1. The exact matching of the bands indicates that both methods of labelling are equivalent.

As a control a sample of PGM that had been denatured before the labelling procedure was also subjected to tryptic digestion. The small amount of radioactivity which remained associated with the protein after dialysis gave after digestion a spot moving with phosphate and a trace of a second spot moving with glucose phosphate on ionophoresis at pH 6·5.

Partial acid hydrolysis of [^{32}P]*phosphoglucomutase*

Fig. 4 shows the pattern obtained when [^{32}P]-PGM was subjected to partial acid hydrolysis (5·7 N-HCl; 100°; 30 min.) and ionophoresis at pH 3·5. Band A from the tryptic digest (Fig. 2) was similarly hydrolysed and run side by side. As a control, denatured PGM was also subjected to the same treatment. For comparison the pattern obtained from di*iso*propoxy[^{32}P]phosphinylchymotrypsin, which had been subjected to the same treatment, is shown. It is quite clear that the main features in the patterns of PGM and chymotrypsin are different, so that it may be concluded that PGM does not contain the sequence Gly.Asp.SerP.Gly which is in chymotrypsin.

Band 1 runs in the position of inorganic orthophosphate and band 2 was identified as serine phosphate by comparison with a known marker on ionophoresis at pH 3·5 and 2·1.

Band 3 appeared to be an impurity, which can be released during the isolation of the tryptic peptide. A sample eluted from band 3 was run in parallel with glucose 6-phosphate in ionophoresis at pH 3·5. The paper was treated with phosphate reagent (Burrows, Grylls & Harrison, 1952) and then radioautographed. There was a satisfactory match between the blue colour and the radioactive spot. It thus seems likely that it is a glucose phosphate, and the known stability of the phosphate residues would suggest that it is glucose 6-phosphate rather than glucose 1-phosphate.

Bands 6 and 7 are mixtures of a number of components which can be separated at pH 2·1 or 6·5. The ionophoresis of the peptide mixture at pH 6·5 is shown in Fig. 5. Fig. 6 shows a two-dimensional ionogram run at pH 3·5 and 6·5, and shows the relationships between the patterns obtained at the two pH values. At pH 6·5 bands 4 and 5 are contaminated by a 'tailing' of band 6A and can best be purified at pH 3·5, whereas the other peptides are present as mixtures (bands 6 and 7) at pH 3·5 and are best purified at pH 6·5. The proportions of the peptides in hydrolysates obtained by two different methods were determined by measuring the relative degree of darkness of the radioautographs of the ionograms run at pH 6·5. The measurements were carried out in a Chromatograph automatic recording reflective densitometer (Joyce, Loebel and Co. Ltd., A8, Princeway Team Valley, Gateshead, Co. Durham) and the results are shown in Table 2.

The time course of the hydrolysis was followed by ionophoresis at pH 3·5 and 6·5. A sample of the tryptic bands was hydrolysed with 5·7 N-HCl in a boiling-water bath and samples were taken out at different times and subjected to ionophoresis at pH 3·5. The relative amounts of each peptide were determined by densitometry of the radioautographs. Fig. 7 shows clearly that bands 7, 2 and 1 increased continuously. Bands 5 and 6, on

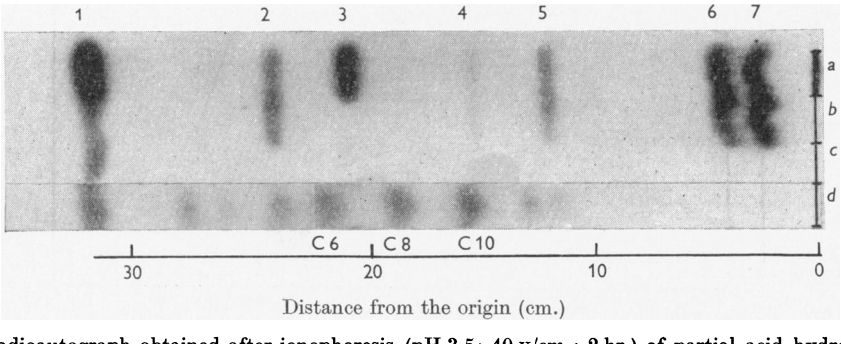

Fig. 4. Radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of partial acid hydrolysates of ^{32}P derivatives. *a*, [^{32}P]PGM; *b*, band A (Fig. 2); *c*, denatured PGM subjected to the same labelling and subsequent treatment as *a*; *d*, di*iso*propoxy[^{32}P]phosphinylchymotrypsin; the Asp.SerP (C6), Asp.SerP.Gly (C8), and SerP.Gly (C10) bands are indicated (Naughton, Sanger, Hartley & Shaw, 1960).

the other hand, pass through a maximum. Band 4 does not seem to change much after reaching a low level. The time course of the hydrolysis was also followed at pH 6·5. In this case two different hydrolysis conditions were used: 5·7 N-HCl and 12 N-HCl. The time course of the hydrolysis is more or less similar for both conditions, when 10 min. hydrolysis in the 5·7 N-HCl is compared with 24 hr. in 12 N-HCl as suggested by Naughton *et al.* (1960). Bands 6A and 7A are the main ones present after 30 min. in 5·7 N-HCl (or 72 hr. in 12 N). Band 7A is, however, still increasing whereas band 6A is beginning to diminish. Both seem to be quite stable peptides, especially the former.

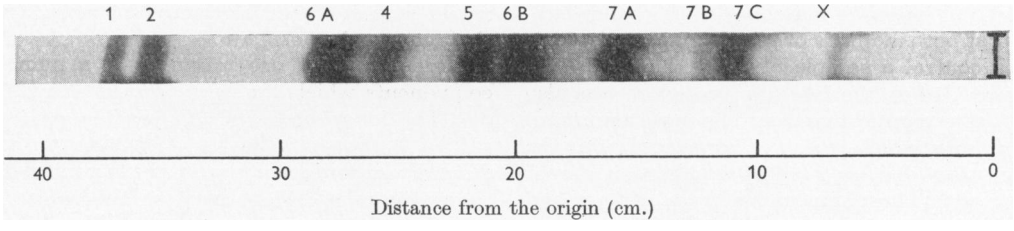

Fig. 5. Radioautograph obtained after ionophoresis (pH 6·5; 40 v/cm.; 2 hr.) of the partial acid hydrolysate of band B (Fig. 2).

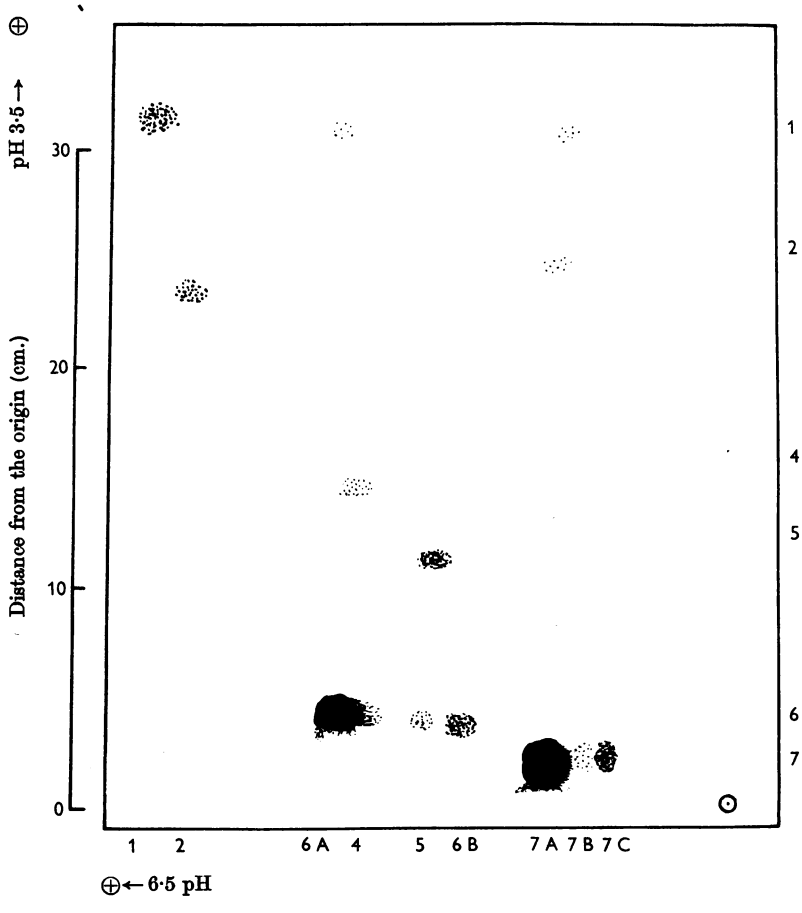

Fig. 6. Tracing of radioautograph of a two-dimensional ionogram (pH 3·5 and 6·5; 40 v/cm.; 2 hr. and 110 min. respectively) of the partial acid hydrolysate of a mixture of isolated bands from the tryptic digest of [^{32}P]PGM.

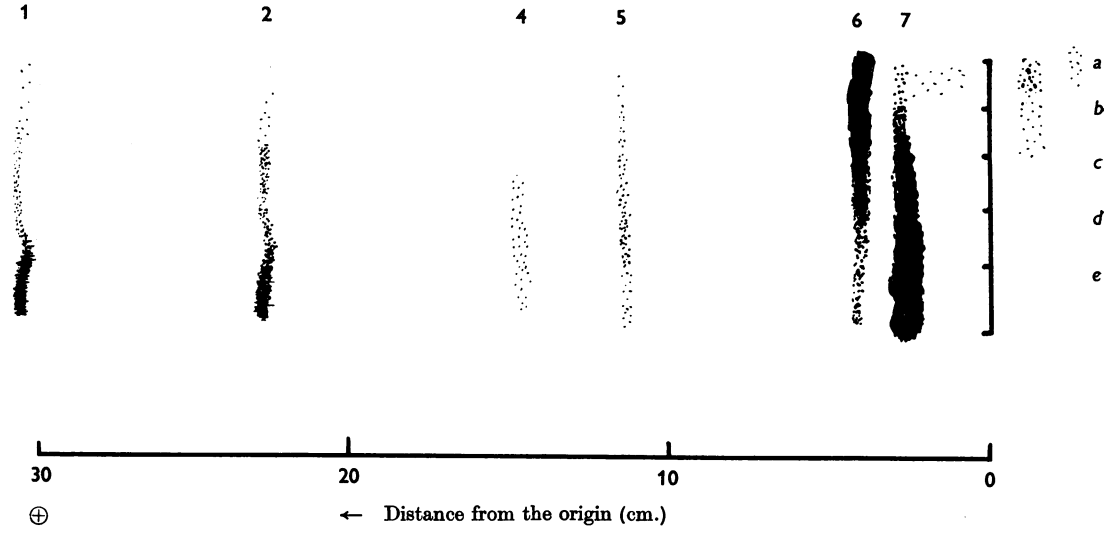

Fig. 7 (*a*). Tracing of radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of the partial hydrolysis of band A (Fig. 2) for different times: *a*, 5 min.; *b*, 10 min.; *c*, 20 min.; *d*, 30 min.; *e*, 45 min.

Table 2. *Yields of peptides* (%) *from partial acid hydrolysis of* [^{32}P]*phosphoglucomutase*

Band no.	5·7 N-HCl for 30 min. at 100°	12 N-HCl for 3 days at 37°
1	14·0	5·5
2	15·6	11·6
4*	2·5	2·6
5	8·2	10·8
6A	18·4	26·8
6B	3·9	4·6
7A	24·4	28·6
7B	1·7	1·2
7C	6·2	7·7
Others	4·7	1·2

* Contaminated with material running as band 6 at pH 3·5.

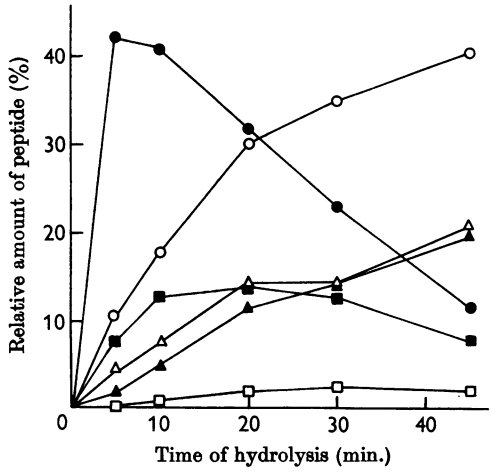

Fig. 7 (*b*). Relative amounts of each band during the hydrolysis, calculated by densitometry of the radioautograph [Fig. 7 (*a*)]. △, Band 1; ▲, band 2; □, band 4; ■, band 5; ●, band 6; ○, band 7.

Structure of the peptides

In order to determine the interrelations of the various bands they were subjected to a further partial acid hydrolysis (5·7 N-HCl; 100°; 45 min.) and the products identified by ionophoresis together with markers of the original partial acid hydrolysate. The results are shown in Table 3. Table 4 shows the products produced when the various bands were subjected to the Edman degradation (Naughton *et al.* 1960).

Peptide 7A gives only serine phosphate on partial hydrolysis, suggesting that it is a dipeptide, and, since phosphate and not serine phosphate was obtained as the main product after the Edman degradation, the serine phosphate is *N*-terminal. Peptide 6A also has *N*-terminal serine phosphate and gives 7A on partial hydrolysis, indicating that it is the tripeptide formed by addition of a residue to the *C*-terminal end of 7A. Peptide 5 gives 4 and serine phosphate on partial hydrolysis. It does not give 7A and therefore the serine phosphate must be the *C*-terminal residue. It is converted into 4 by the Edman degradation. It is thus most probably a tripeptide and peptide 4 the other dipeptide. The yield of band 4 is very low and it was not possible to carry out the re-hydrolysis and Edman degradations with it. The various relationships of the

Table 3. *Products of the rehydrolysis of radioactive bands*

Bands 1, 2, 4 and 5 were isolated by ionophoresis at pH 3·5, the other bands at pH 6·5, and hydrolysed in 5·7 N-HCl for 45 min. at 100°. They were again subjected to ionophoresis at the same pH used for their isolation and identified by their position with respect to markers of the original partial hydrolysate. The relative amount of each product is indicated with crosses; tr., trace.

Band rehydrolysed (Figs. 4 and 5) ...	1	2	4	5	6A	7A	7B	7C	X
1	×	.	.	.	.	.	.	.	.
2	×	× ×	.	.	.	.	.	.	.
4	×	× ×	× × ×	.	.	.	.	.	.
5	× ×	× × ×	×	×	.	.	.	.	.
6A	×	× ×	.	.	× ×	× × × ×	.	.	.
6B	×	× ×	×	× ×	× ×	× × × ×	tr.	tr.	.
7A	×	×	.	.	.	× × × ×	.	.	.
7B	×	× ×	× × ×	.	.	× × × ×	× × ×	.	.
7C	×	× × ×	×	×	.	× × × ×	×	×	.
X	×	×	.	.	.	× × × ×	.	.	× ×

Table 4. *Products of the Edman degradation of radioactive bands*

Band 5 was isolated at pH 3·5 and bands 6A and 7A at pH 6·5 as indicated in Figs. 4 and 5 respectively. The bands were eluted and subjected to the Edman degradation followed by ionophoresis at the same pH used for their isolation. Identification of the products was made by their position with respect to markers of the original partial hydrolysate. The relative amount of the products is indicated with crosses; tr., trace.

Band	Products
5	1 (×)+4 (× × ×)+5 (× ×)
7A	1 (× × × ×)+2 (tr.)+a* (× ×)+7A (× ×)
6A	1 (× × ×)+2 (tr.)+6A (× × × ×)

* Unknown spot, running between 5 and 7A.

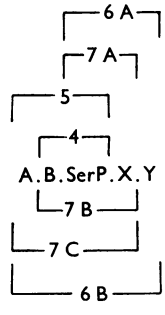

Fig. 8. Diagram showing the interrelationships of peptides of the partial acid hydrolysate.

peptides are shown in Fig. 8, where the unknown amino acid residues are indicated by A, B, X and Y. It can be seen that all possible partial hydrolysis products of the pentapeptide sequence have been detected with the exception of the tetrapeptide B.SerP.X.Y.

Fig. 9 shows the pH–mobility curves for peptides 6A and 7A and a SerP.Gly marker. The results were calculated relative to a serine phosphate marker in the following way: a titration curve

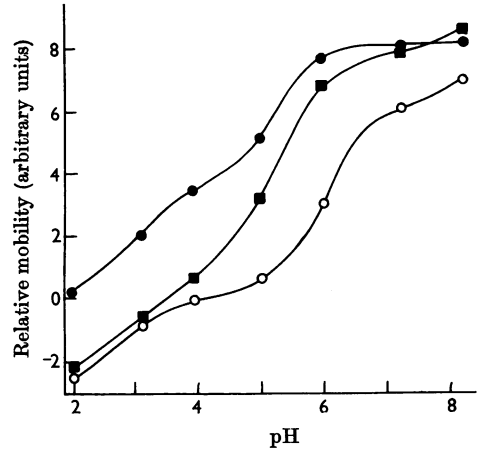

Fig. 9. Mobilities at different pH values of some of the peptides isolated as shown in Fig. 5. For comparison, the mobility of SerP.Gly (●) is also included. Mobilities are expressed relative to a standard serine phosphate marker. The points are experimental values and the curves are theoretically calculated from the following pK values: Peptide 7A (○); 1·1 (PO_4), 2·5 (CO_2H), 5·7 and 6·2 (PO_4 and basic), 8·8 (α-NH_2); Peptide 6A (■); 1·1 (PO_4), 2·5 (CO_2H), 4·5 (CO_2H), 5·7 and 5·7 (PO_4 and basic), 8·8 (α-NH_2).

of serine phosphate was drawn, and, assuming mobility to be proportional to net charge, an arbitrary mobility curve was obtained by substituting the alkali volume by an arbitrary centimetre scale. Glucose markers were used to correct for the displacement of the origin due to endosmosis. The relative mobility for a peptide at a given pH is equal to: (Distance of peptide from glucose marker)/(distance of SerP from glucose marker) × arbitrary mobility of SerP at same pH (Naughton *et al.* 1960). Values for band 3 (glucose phosphate) were also determined and showed good agreement with the theoretical curve calculated

from the known pK values. At pH 4·0, SerP.Gly has one negative charge, whereas the dipeptide 7A is neutral. This suggests that the unknown amino acid called X in Fig. 8 is a basic one. Comparing the increase in rates of SerP.Gly and peptide 7A between pH 4·0 and pH 7·2, it can be seen that although the molecular weight of peptide 7A is greater, its mobility has increased more than the increase in mobility of SerP.Gly, suggesting another group ionizing near the range of the second phosphate. Fig. 9 also shows the theoretical curve, based on ionizable groups in peptide 7A, including a basic group with a pK of 5·7 or 6·2. It can be seen that this curve fits the experimental data. The tripeptide 6A is more acidic than 7A at pH values higher than 3·5. At pH 4·5, when the second phosphate group is completely discharged, the tripeptide is clearly acidic. The inclusion of an acidic group with pK 4·5, and slight changes in the pK of the phosphate or basic, or both, groups of peptide 7A, fit in with the experimental data. The mobility data, strongly suggest then, the presence of a basic amino acid (pK between 5 and 7) and an acidic amino acid (pK about 4·5) on the C-terminal side of serine phosphate. Peptides 4 and 5 run at both pH values (3·5 and 6·5), as would be expected for a di- and tri-peptide having neutral amino acids and a C-terminal serine phosphate.

The above curves indicate that residue X (Fig. 8) is a basic amino acid having a pK about 6·5 and therefore histidine. This was further confirmed by photo-oxidation (see below) and by the following experiment: band 7 (3 μmc) isolated by ionophoresis at pH 3·5 was added to 3 mg. of a hydrolysate (5·7 N-HCl; 100°; 1 hr.) of unlabelled PGM and subjected to ionophoresis at pH 3·5 (2 hr.; Whatman no. 1 paper). The radioactive band was cut out, eluted and re-run at pH 6·5 for 90 min. and then at pH 2·1 for 50 min. The paper was then radioautographed and sprayed with Pauly reagent (Fraenkel-Conrat & Singer, 1956). A faint but distinct red spot fell in exactly the same position as the radioactivity. Since the possibility of a histidine peptide contamination having the same mobility as peptide 7A in the three different systems used seems unlikely, the assumption that SerP.His is one of the dipeptides of the partial hydrolysis of PGM seems reasonable.

Fig. 10 shows the results of the photo-oxidation experiment. The pattern obtained in the first run is shown at the origin. After photo-oxidation and re-running in the second dimension but with the same buffer, glucose phosphate and peptides 4 and 5 are mostly unchanged, whereas most of peptides 6 and 7 are in a new position, indicating an increase in negative charge. Some of the unchanged peptides are still present. Band 6 shows two unchanged spots, and two corresponding faster-moving spots (presumably 6A, 6B and their oxidized derivatives) and some free phosphate. Band 7 gave one spot in the position of the unchanged material and several other spots. The strongest one, running in a position between 4 and 5, is probably the oxidized 7A, and the slower-moving spots the other peptides present in 7 which can be separated at pH 6·5. There are also a phosphate spot and three very faint spots whose nature is unknown. It is quite clear from this experiment that the main components in 6 and 7 are histidine peptides. The presence of spots in the positions of the unchanged peptides from 6 and 7 suggests that in these conditions the reaction is not quantitative. In this respect it has to be considered that 10 % of unchanged histidine would hardly be detected in the ninhydrin control experiment, but would give a very clear spot in the radioactive experiment.

In order to identify the presence of N-terminal serine or threonine residues, peptides 4 and 5 were

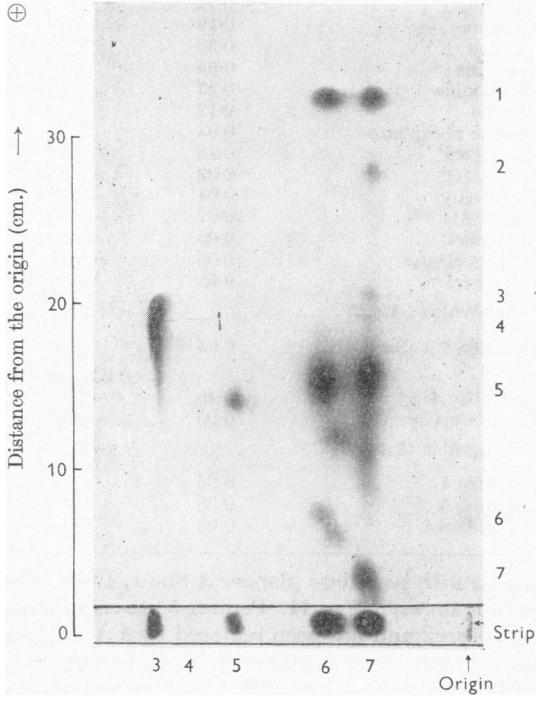

Fig. 10. Radioautograph obtained after ionophoresis of the photo-oxidized peptides. A paper strip obtained by ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) was subjected to photo-oxidation and then sewn to another sheet of Whatman no. 52 filter paper and subjected to ionophoresis in the second dimension with the same conditions. The photograph also shows the radioautograph of the photo-oxidized strip, and the position in which it was sewn before ionophoresis in the second dimension.

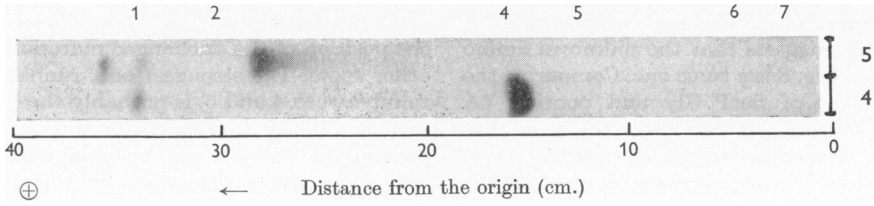

Fig. 11. Radioautograph obtained after ionophoresis (pH 3·5; 40 v/cm.; 2 hr.) of the periodate-treated peptides 4 and 5 from Fig. 3.

Table 5. *Chromatographic constants of amino acids and peptides in butanol–acetic acid–water–pyridine*

R_F values were obtained after development for 48 hr. Values of $A+B$ and of $2A+B$ were calculated from equation (1).

Sample	R_F	$A+B$
Arginine	0·13	.
Histidine	0·13	.
Glutamic acid	0·14	.
Aspartic acid	0·09	.
Proline	0·25	.
Glycine	0·12	.
Alanine	0·19	.
Valine	0·36	.
Leucine	0·55	.
Threonine	0·23	.
Serine	0·12	.
Serine phosphate	0·03	.
Leu.SerP	0·18	−1010
Gly.SerP	0·03	−1140
SerP.Gly	0·04	−1290
SerP.Ala	0·07	−1360
Ala.SerP	0·06	−1250
Glu.SerP	0·05	−1360
Thr.SerP	0·05	−1325
Mean value ($A+B$)		−1240±125
Peptide 7A (SerP.His)	0·04	−1270
		$2A+B$
Leu.Gly.Gly	0·40	−1940
Gly.Gly.Gly	0·09	−2050
Mean value ($2A+B$)		−1990
Peptide 4	0·06	.
Peptide 5	0·08	.
Peptide 6A	0·03	.

treated with periodate (Sanger & Shaw, 1960). The result is shown in Fig. 11. Peptide 5 has completely disappeared and has been replaced by a main spot moving faster than peptide 4 and two minor spots. Peptide 4, on the other hand, has remained largely unchanged. This indicates that peptide 5 has a *N*-terminal serine or threonine residue, whereas peptide 4 has not. The sequence around the serine phosphate residue may thus now be written:

$$\begin{pmatrix}\text{Thr}\\ \text{Ser}\end{pmatrix}.\text{B.SerP.His.}\begin{pmatrix}\text{Asp}\\ \text{Glu}\end{pmatrix}$$

where B is some neutral amino acid other than threonine or serine.

Table 5 shows the R_F values in one solvent system for a number of peptides of known constitution and for the main radioactive bands. The values of the constant $(n-1)A+B$ [equation (1)] for the known peptides have also been calculated. For the butanol–acetic acid–water–pyridine system the values for the constant are within the expected limit of errors, whereas for the phenol–water system there was much more spreading of the values. Much more reliance therefore can be placed on the values obtained in the former system.

The R_F of peptide 4 in the butanol–acetic acid–water–pyridine system was 0·06. Assuming a value of -1240 for the constant $(n-1)A+B$ it can be calculated from equation (1) that the R_F of the unknown amino acid B is 0·20. If we assume that the error in R_F is not more than 0·05 (Moore & Baker, 1958), the R_F of B must lie between 0·15 and 0·25. The only neutral amino acids to be considered are alanine, proline and possibly threonine (R_F 0·23). The results with the periodate treatment indicate that it is not threonine. Alanine and proline have very different R_F values on the phenol–water system (0·49 and 0·81 respectively), although there was considerable variation in the values of $(n-1)A+B(-300\pm260)$ in this system it seems reasonably certain that the unknown amino acid is not proline. Proline has an R_F slightly higher than leucine, whereas peptide 4 (R_F 0·06) moves much more slowly than Leu.SerP (R_F 0·37). Further evidence that peptide 4 was Ala.SerP was obtained by comparison with a sample of radioactive Ala.SerP prepared by another method from ovalbumin (D. C. Shaw, personal communication). SerP.Ala was obtained from a partial acid hydrolysate of ovalbumin (Flavin, 1954) and partly converted into Ala.SerP by inversion of the dipeptide sequence in hot dilute acid. This material ran at the same rate as peptide 4 on ionophoresis at pH 6·5 for 1 hr. 45 min. at 40 v/cm.

Assuming that peptide 4 was Ala.SerP, it was calculated that the R_F of residue A (Fig. 8) was 0·27. The periodate reaction indicated that it is either serine or threonine: serine has R_F 0·12 and threonine 0·23, suggesting that the residue is most

Table 6. *Amino acid composition of purified peptides*

Figures in parentheses represent the approximate amount present as estimated visually from the strength of the ninhydrin colour; tr., trace.

Peptide	Purification procedure: Chromatography on Dowex 50	Ionophoresis pH 2·1	Ionophoresis pH 3·5	Ionophoresis pH 6·5	Chromatography in butanol–acetic acid–water (3:1:1, by vol.)	Amino acid composition
5	+	.	+	.	.	Glu (3), Ser (2), Ala (2), Thr (1), Val (1), Ileu (1), Leu (1)
5	+	+	+	.	+	Ala (1), Ser (1), Thr (1)
7A	+	+	+	+	.	His (2), Ser (2), Glu (1), Asp (1), Thr (tr.), Ala (tr.)
7B	+	+	+	+	.	His (1), Ala (1), Ser (1), Thr (tr.), Glu (tr.), Asp (tr.)
6A	.	+	+	+	+	His (1), Ser (1), Asp (1), Glu (tr.), Ala (tr.), Gly (tr.)
6A	+	+	+	+	.	His (1), Ser (1), Asp (1), Gly (1), Glu (0·5)

probably threonine. From the R_F of peptide 6A in butanol–acetic acid–water–pyridine a value of 0·18 for the acidic residue (Y in Fig. 8) was calculated. Glutamic acid has R_F 0·14 and aspartic acid 0·09. This result would suggest that glutamic acid is the most probable. However, further work (see below) showed that residue Y was in fact aspartic acid. It seems probable that equation (1) will be less reliable where amino acids with charged side chains are concerned, since variations in pK values between amino acids and peptides would be expected to affect the partition coefficients, whereas this is not taken account of in the equation.

It may be concluded from the above results that the sequence around the serine phosphate residue is most probably:

$$\text{Thr.Ala.SerP.His.}\begin{pmatrix}\text{Asp}\\ \text{Glu}\end{pmatrix}.$$

Table 6 shows the results of the large-scale experiments in which the amino acids present in the peptides were identified by the standard ninhydrin technique. Several preliminary attempts failed to give pure peptides and extensive purification was necessary. Glutamic acid, aspartic acid and glycine were often present as impurities. The spot identified as serine phosphate (band 2) contained more glutamic acid than serine after purification by ionophoresis at pH 3·5 and 6·5.

Since the pentapeptide sequence could be deduced from the composition of the two tripeptides 5 and 6A, special care was taken to obtain these in a pure form, and the results with these two (Table 6) were clear and demonstrate that the pentapeptide sequence is Thr.Ala.SerP.His.Asp. The results with the other peptides are consistent with this sequence, although in most cases they are clearly not completely pure. Thus peptide 7A contained glutamic and aspartic acids in about half the amount of the true components, histidine and serine.

DISCUSSION

In this work we have attempted as far as possible to use methods that depend only on radioactive techniques for the identification of peptides and their degradation products rather than those that depend on the more conventional ninhydrin reaction. Such methods which can be employed on a much smaller scale have the advantage that they can be applied to isotopic peptides that are contaminated with other non-isotopic substances provided they are free from other isotopic substances. The extreme difficulty of purifying peptides from an acid hydrolysate of a large protein is evident from this work, and, if the ninhydrin method had been used by itself, it would have been very difficult to derive the correct sequence. On the other hand, it is relatively easy to obtain isotopic peptides free from one another, and it is hoped that the techniques described here may prove of value in other cases where it is desired to know the sequence around a given labelled amino acid residue.

The method of deduction of the interrelationships of the various peptides from the partial acid hydrolysate (Table 5) depends on the assumption that all the peptide bonds are to some extent hydrolysed by acid. Thus, for instance, peptide 7A was said to be a dipeptide since it gave only serine phosphate on partial hydrolysis. It could possibly have been a tripeptide SerP.X.Y where the bond X–Y was extremely stable so that SerP.X was never produced in appreciable amounts. In fact

Table 7. *Amino acid sequences around reactive serine in enzymes*

Enzyme	Sequence	Reference
Trypsin, Chymotrypsin, Elastase, Thrombin	Gly.Asp.Ser.Gly	Dixon, Kauffman & Neurath (1958); Turba & Gundlach (1955); Schaffer *et al.* (1957); Hartley, Naughton & Sanger (1959); Gladner & Laki (1958)
Liver ali-esterase, Pseudocholinesterase	Gly.Glu.Ser	Jansz, Posthumus & Cohen (1959); Jansz, Brons & Warringa (1959)
Subtilisin	Thr.Ser.Met.Ala	Sanger & Shaw (1960)
Phosphorylase A	Lys.Glu-NH_2.Ileu.SerP.Val.Arg	Fisher, Graves, Grittenden & Krebs (1959)
Phosphoglucomutase	Thr.Ala.SerP.His.Asp	This paper

the further confirmatory experiments did show that all the deductions made from the partial hydrolysis experiments were correct, and whether or not such methods will be entirely reliable can only be decided by further experience. From the partial acid hydrolysis the relationships of the peptides could have been as shown or in the reverse order. The Edman degradation studies showed which was the correct order and confirmed some of the relationships.

The pH–mobility curves have proved useful for identifying charged residues in peptides. It was hoped that they could be used further, for instance to obtain information about the exact pK values of amino and carboxyl groups and about the size of residues involved in peptides. However, preliminary experiments with known serine phosphate peptides and other neutral peptides suggested that accurate results could not easily be obtained. It appeared that other factors, such as adsorption on the paper and hydration, may determine the rate of migration at different pH values, since many of the pH–mobility curves obtained did not agree with any predicted formula (e.g. Alberty, 1953). Thus for instance at all pH values the difference in mobility between SerP.Gly and SerP.Ala was considerably greater than would be expected. At high pH values the migration of serine phosphate peptides was frequently slower than expected, suggesting increased hydration with the increased charge. Perhaps by a more intensive study of the relationships of the structures of peptides to their ionophoretic mobilities, it may be possible to devise methods for identifying residues in peptides in this way. However, at present it seems that more reliable information can be obtained by the use of specific reactions, such as the periodate and the photo-oxidation reaction, and by studying the R_F values of the peptides as described above. With the two chromatographic systems we have used, it was not possible to identify with certainty all the amino acids present in the peptides, though a large number of possibilities could be eliminated. In order to make such a method completely reliable, it would be necessary to use more chromatographic systems and to have more known peptides to use as markers. The formula of Pardee (1951) is not completely accurate and certain peptides may be exceptional (Moore & Baker, 1958). It is to be hoped that with further experience it may be possible to improve the reliability and to develop it as a general method of identifying the amino acid sequence around a given labelled amino acid residue.

The results reported here demonstrate that the structure around the serine phosphate residue is Thr.Ala.SerP.His.Asp (or Asp-NH_2). There seems little doubt that the serine phosphate is involved in the 'active centre' of the enzyme since the phosphate can be exchanged with phosphate in the substrates and, also, since the inactive enzyme does not become labelled. Whether or not the other residues in this sequence are concerned in the enzymic activity cannot be said; but the presence of a histidine residue next to the serine phosphate is of particular interest, since there is some evidence that such a residue is involved in the activity (Koshland *et al.* 1958). There are only three histidine residues in PGM, but whether in fact this particular one is involved in the activity cannot be said with certainty at present.

It is clear that the amino acid sequence around the serine phosphate residue of PGM is in no way similar to that found around the reactive serine residue in other enzymes which react with di*iso*propyl phosphorofluoridate. Some sequences in the immediate vicinity of reactive serine or serine phosphate residues in various enzymes are listed in Table 7. Although there are similarities between different enzymes as between trypsin and chymotrypsin or between liver ali-esterase and pseudocholinesterase, these similarities are not as universal as has sometimes been suggested and cannot at present be made the basis of any general hypothesis.

SUMMARY

1. Phosphoglucomutase labelled with ^{32}P was prepared by two methods involving reaction of the enzyme with labelled substrates.

2. The [^{32}P]phosphoglucomutase was subjected to partial hydrolysis with trypsin and with acid and the [^{32}P]peptides were purified by ionophoresis.

3. The interrelationships of the peptides in the acid hydrolysate were determined by subjecting them to further partial acid hydrolysis and to the Edman degradation procedure.

4. The individual residues in these peptides were identified from the ionophoretic and chromatographic mobilities of the peptides, by specific reactions and from the amino acid composition of highly purified peptides.

5. It was concluded that the amino acid sequence around the serine phosphate (SerP) residue was Thr.Ala.SerP.His.Asp (or Asp-NH_2).

We are grateful to Dr G. Fölsch for a gift of Leu.SerP and Gly.SerP and to Miss R. M. Carpenter for the mitochondria and hexokinase. One of us (C.M.) wishes to acknowledge a scholarship from the British Council.

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A Native Cobalamin–Polypeptide Complex from Liver: Amino Acid Composition and Terminal Amino Acid Analyses of the Peptide Part

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The isolation of a cobalamin–polypeptide from bovine liver was described by Hedbom (1955, 1960). In view of the current interest in protein or peptide conjugates of corrinoids for basic metabolic studies (Wagle, Mehta & Johnson, 1958*a*, *b*; Helleiner & Woods, 1956; Barker, Weissbach & Smyth, 1958) and for the oral treatment of pernicious anaemia (cf. Heathcote & Mooney, 1958) it has seemed desirable to investigate this complex in more detail. Knowledge of its composition and structure might form a basis for general conclusions regarding relationships between structure and biological activity of the corrinoids. The present paper deals with the quantitative amino acid analysis and the determination of the terminal amino acids of the peptide part of the complex.