ACTA CHEMICA SCANDINAVICA 16 (1962) 1770–1778

The Incorporation of Phosphorus into Human Erythrocyte Ghosts

GUNNAR ÅGREN, BIRGITTA HALLBERG and GUNNAR RONQUIST

Institute of Medical Chemistry, University of Uppsala, Sweden

About 30 labelled phosphopeptides and phosphorylserine have been isolated from red-cells ghosts incubated with radioactive inorganic phosphate. The rate of incorporation and the pH-dependence of the labelling have been determined.

It was demonstrated in a previous paper that the main part of the phosphoproteins in *Escherichia coli* are located in the cell-wall preparations [1]. It was therefore of interest to carry out a similar investigation with some animal cell-wall material. The erythrocyte ghosts seemed to be appropriate for this purpose. It was also shown [2] that labelled phosphorylserine could be isolated from erythrocyte ghosts which had been suspended in 0.1 M tris * at pH 7.4 in the presence of ^{32}P. These experiments indicated that the ghosts contain phosphoproteins which may be active in the phosphorus metabolism of the erythrocytes.

It has now been possible to isolate several phosphopeptides from the acid-hydrolyzed protein residue of the ghosts, and the qualitative amino acid composition of the phosphopeptides has been studied in greater detail. A study has also been made of the initial rate of ^{32}P-labelling of the phosphopeptides, as well as of the pH-dependence of the labelling.

METHODS

Incubation of red cell ghosts with ^{32}P. After some preliminary work with earlier methods, the ghosts were finally prepared in the following way.

2000–3000 ml samples of human blood obtained from the University Hospital, Uppsala, were centrifuged and washed at least twice with cold physiologic sodium chloride solution, in a refrigerated Stock centrifuge taking 7.5 l. All further procedures were carried out at 2°C. After each centrifugation, the leucocytes were removed by skimming and suction. Nine volumes of cold distilled water were added, and the pH adjusted to 6.0 by means of CO_2. The ghosts settled to the bottom during storage overnight in the cold room. The clear supernatant was removed by decantation and suction.

* Tris = tris(hydroxymethyl)aminomethane; ^{32}P = radioactive inorganic phosphate; TCA = trichloroacetic acid; SerP = phosphorylserine; cpm. = counts per minute; UV = ultraviolet.

The ghosts from 1000–1500 ml samples of red cells were washed mainly according to Post *et al.* [3] They were first suspended in 7.5 l of 10^{-3}M tris-acetate buffer, adjusted to pH 6.5 by CO_2. Centrifugation at 3000 r.p.m. for 20 min in the Stock centrifuge, and resuspension in the dilute buffer, were repeated 6 or more times until the supernatant was colourless. The volume of the faintly pink ghosts usually diminished to 50–80 % of the corresponding red cells. The average dry weight of ghosts from 1000 ml of red cells was 6 g.

To the ghosts (about 1000 ml) were added 2 volumes of 2×10^{-2} M tris-glycylglycine buffer, pH 8.2. After mixing, the suspension was centrifuged at 20 000 r.p.m. for 60 min in the Spinco ultracentrifuge (Model L, rotor No. 21). The precitate was washed about 6 times by resuspension and centrifugation. Tris-glycylglycine, 10^{-2} M, pH 8,2 was used twice, and 2×10^{-4} M buffer was used in the final washings (4 times) until the supernatant was colourless. The volume of the practically colourless ghosts, when prepared from about 1000 ml of red cells, usually amounted to around 300 ml, with a decrease in the dry weight from about 6 to 2 g. Ghosts from totally 25 l of human red cells were prepared in this way.

For labelling with ^{32}P, the ghosts (300 ml) were diluted to about 2 l with 2×10^{-4}M tris-glycylglycine buffer at pH 7.4, and incubated with approximately 10 mC of ^{32}P for 30 min at 20°C. The ghosts were then precipitated by addition of cold TCA to a final concentration of 10 % (w/v). The precipitate was centrifuged, washed with 10 % TCA and dried with ethanol. The lipids were removed by extraction twice with ethanol/ether at 45°C, followed by ether. The residue contained the Schneider protein fraction and nucleic acid.

Isolation of SerP and phosphopeptides. To obtain these substances, the protein fraction was partially hydrolyzed according to Lipmann [4], with 10 times the protein weight of 2 N HCl under reflux on a water bath for 20 h. After most of the HCl had been removed by repeated evaporation *in vacuo*, the hydrolysate of ghosts from a 1000 ml sample of red cells was applied to a Dowex 50 column (40 × 2 cm), and eluted with 6 column volumes of 0.01 N HCl. The four ^{32}P-labelled fractions following inorganic phosphate were further separated into several fractions on 25 × 0.9 cm columns of Dowex (2 % DVB) formate with gradient elution 0 → 1 M formic acid, using the procedure of Busch *et al.* [5] The volume in the mixer was 150 ml, and the eluate was collected in fractions of 5 ml every 20 min.

Analytical methods. The purity of the labelled subfractions from the Dowex 1 formate columns was analyzed by high-voltage electrophoresis, using Whatman No. 3 paper. The electropherograms were run in the Wieland-Pfleiderer apparatus at 2650 V and 35 mA for 60 min at 4–6°C, using 1 N acetic acid as solvent. Several of the subfractions were found to contain up to five non-labelled ninhydrin-positive fractions, and one or several labelled fractions. All fractions from the Dowex 1 formate columns were therefore further purified by preparative paper electrophoresis. Scanning of the electropherograms was performed according to Ågren *et al.* [6] The labelled spots were eluted with water, and the solutions concentrated to dryness *in vacuo*.

The radioactivity of the different fractions from column chromatography of the hydrolyzed protein residues was measured in glass cups, using an L. K. B. Robot Scaler. Determinations of the specific activity were made on stainless steel planchettes with the Tracerlab Superscaler. At least 1000 counts were measured. The specific activity of the different phosphorylated compounds was calculated as cpm./μg phosphorus. All activity values were recalculated to the time of incubation.

The qualitative amino acid composition of the purified phosphopeptides was determined by one-dimensional high-voltage electrophoresis at 2600 V and 40 mA for 60 min using 1 N acetic acid as solvent, and Whatman No. 1 paper. The phosphopeptides were hydrolyzed with 2 N HCl in sealed tubes for 20 h at 120°C.

RESULTS

Isolation of phosphopeptides. Fig. 1 illustrates a typical elution diagram of the hydrolyzed protein residue of ghosts from 1150 ml of red cells passing through a Dowex 50 column with 0.01 N HCl. A large peak containing nin-

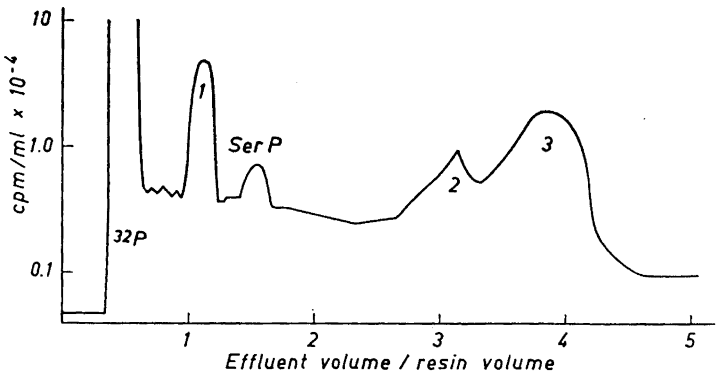

Fig. 1. Radioactivity curve from a hydrolysate of red cell ghosts separated on a Dowex 50 (8 % DVB) column. The curve was obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The ghosts had been incubated for 30 min with ^{32}P.

hydrin-positive material was eluted in a position between ^{32}P- and SerP. Two additional large peaks containing ninhydrinpositive material appeared after SerP, the first after 3.0 column volumes and the second after 4.0 column volumes of eluate (effluent volume/resin volume). The following total amounts of material were obtained from 21 similar experiments: No. 1 fraction after ^{32}P = 98 mg; SerP = 43 mg; fraction No. 1 after SerP = 38 mg; fraction No.2 after SerP = 27 mg.

The material from each of the four peaks after ^{32}P was collected, and run through small Dowex 1 colums with gradient elution 0 → 1 M formic acid. The results were as follows.

Fig. 2 shows the elution chromatogram of 34 mg of radioactive material from peak No. 1 in Fig. 1, after running through the Dowex 1 column. Three

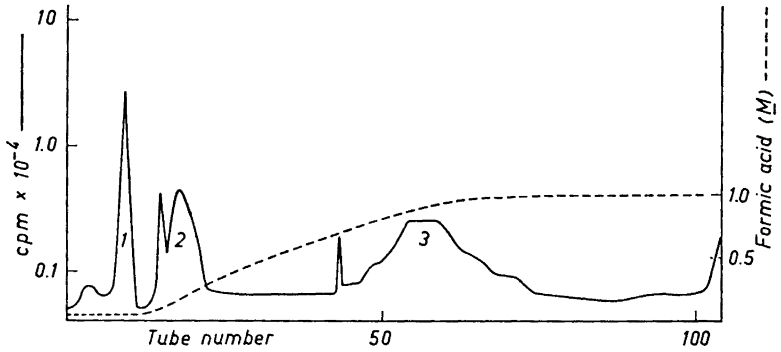

Fig. 2. Radioactivity curve of the material from peak No. 1, Fig. 1, separated on a Dowex 1 (2 % DVB) column. The radioactivity curve (the continuous line) was obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The broken line represents formic acid concentration.

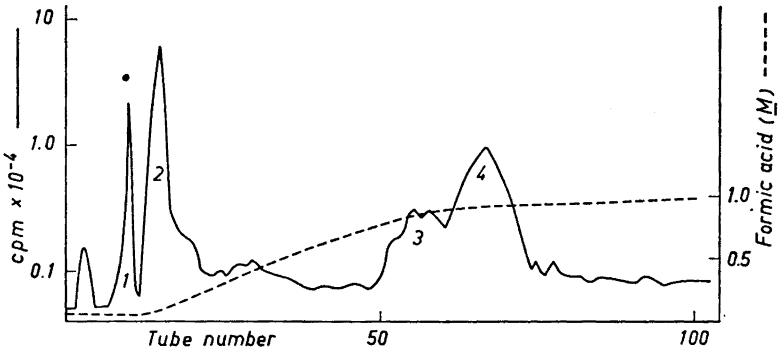

Fig. 3. Radioactivity curve of the material from the SerP-peak, Fig. 1. See legend for Fig. 2.

large labelled peaks were obtained. Their weight was 3.0, 3.9 and 0.8 mg, respectively. The material from each fraction was further purified by preparative paper ionophoresis. In many cases, nonlabelled ninhydrinpositive material could be separated from a labelled compound. This was eluted from the electropherogram, hydrolyzed and analyzed by paper electrophoresis. The amino acid composition of the phosphopeptides is given in Table 1. The low recovery of labelled material must imply that a considerable proportion of it was not eluted with the gradient system used. By succeeding gradient elution 1 → 4 M formic acid 2 large labelled peaks were obtained, which contained phosphopeptides.

The elution chromatogram of 14 mg of radioactive material from the SerP peak in Fig. 1, when run through the Dowex 1 column with gradient 0 → 1 M formic acid, is presented in Fig. 3. This material, as well as the other amounts used in the experiments illustrated in Figs. 2—5, was obtained from 6 samples of red cells, representing approximately 8 l of cells. Four large labelled peaks were obtained from the SerP fraction. They weighed 3.0, 0.5, 1.0 and 2.5 mg, re-

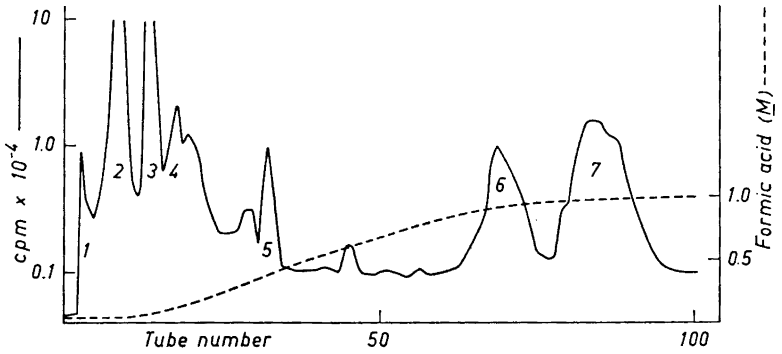

Fig. 4. Radioactivity curve of the material from peak No. 2, Fig. 1. See legend for Fig. 2.

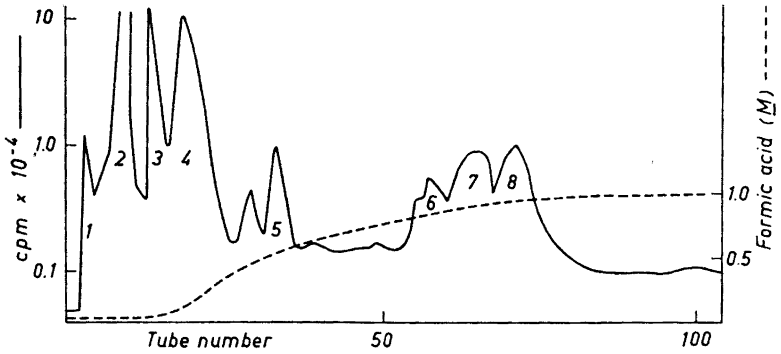

Fig. 5. Radioactivity curve of the material from peak No. 3, Fig. 1. See legend for Fig. 2.

spectively. The last fraction contained mainly SerP. The amino acid composition of the other three phosphopeptides, which had been further purified by paper electrophoresis, is given in Table 1. A purer SerP sample was obtained when the SerP fraction from the Dowex 50 fraction was instead run through a Dowex 1 (10 % DVB) chloride column with 0.01 N HCl.

Fig. 4 shows the elution chromatogram of 21 mg of radioactive material from peak 2 in Fig. 1, when run through a Dowex 1 formate column. Seven large labelled peaks were obtained. Their weight was 3.2, 3.2, 2.0, 3.0, 2.2, 1.5 and 1.4 mg, respectively. The recovery was satisfactory. The material from the 7 fractions was further purified by preparative paper electrophoresis, hydrolyzed and analyzed for amino acids. The results are given in Table 1.

In a similar way, Fig. 5 shows the elution chromatogram of 12 mg of radioactive material from peak 3 in Fig. 1, after running through the Dowex 1 formate column. Eight large labelled peaks were obtained, and their pattern seemed to be about the same as in Fig. 4. The weight of the different fractions was 3.4, 4.8, 2.2, 1.5, 1.0, 1.2, 0.8 and 1.4 mg, respectively. As before all fractions were further purified by preparative paper ionophoresis, and analyzed for amino acids. The results are given in Table 1.

It is evident from the table that more than 30 phosphopeptides could be prepared in this way. The simplest phosphopeptide consisted of serine and glutamic acid. Three different phosphopeptides containing glutamic acid and serine, and either glycine, aspartic acid or alanine were also identified. In addition, several tetra- and pentaphosphopeptides were obtained which had the same amino acid pattern, but which differed with respect to their content of the individual amino acids. In the pentapeptides, the most usual combination was aspartic acid, glutamic acid, serine, glycine and alanine. Proline was present in one hexapeptide, and threonine in another. The two phosphopeptides containing 8 amino acids and hexosamine had the same amino acid pattern. In some fractions, the amount of material was insufficient for analysis.

In some experiments, the two first fractions from the Dowex 50 columns were run through a Dowex 1 (10 % DVB) chloride column. A hexapeptide containing glutamic acid, alanine, proline, β-alanine, threonine and glycine

Table 1. Amino acid pattern of phosphopeptides separated on Dowex 50 columns with 0.01 N HCl in four fractions (Fig. 1). Each fraction subsequently separated into several fractions first on Dowex 1 formate columns (Figs. 2—5) and in a few cases by the succeeding paper electrophoresis (a, b, c).

Amino acid	Fraction No. 1 (Fig. 1) divided into fractions				SerP fraction divided into fractions			Fraction No. 2 (Fig. 1) divided into fractions on Dowex 1 columns												Fraction No. 3 (Fig. 1) divided into fractions on Dowex 1 columns																	
	1	2	4	5	1	2	4	1	2	3	4	5			6			7				1	2	3	4	5			6			7			8		
												a	b	a	b	c										a	b	a	b	c	a	b	a	b	c		
Asp	+	+	+	+	+			+	+	+	+	+	+			+	+					+	+	+	+		+	+		+	+	+		+	+	
Glu	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					+	+	+		+	+	+	+	+	+	+	+	+	+	
Ser	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Ala	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					+	+		+	+	+	+	+		+	+	+		+	
Gly	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+					+	+		+	+	+	+	+		+	+	+	+	+	
Pro	+	+							+	+													+		+											
Lys									+														+													
Thr	+																																		+	+	
Hexos-amine	+				+	+		+	+														+													
β-Ala									+														+													

Table 2. Specific activities of red cell ghosts incubated for different times with ^{32}P. Phosphopeptides and SerP separated first on Dowex 50 and subsequently on Dowex 1 (10 % DVB) 10 columns.

Time of incubation	Fraction No. 4 Fig. 2	Fractions No. 1 & 2 Fig. 3	Fraction No. 4 Fig. 3 (SerP)	Fraction No. 2 Fig. 4	Fraction No. 2 Fig. 5
10 sec	496*	—	34	—	203
5 min	1 860	1 990	930	9 940	14 300
30 min	19 700	6 800	3 410	67 100	93 100

* Counts per min and μg of phosphorus.

was isolated from fraction No. 1 after ^{32}P. Since serine was present in all the other phosphopeptides isolated, the acid-stable phosphorus must have been linked to this amino acid.

Rate of incorporation of ^{32}P. Washed red-cell ghosts from 1500 ml of cells were suspended in 1000 ml of this-glycylglycine buffer at pH 7.4, and 3 equal parts of the suspension were incubated with 10 mC of ^{32}P for 10 sec, 5 min, and 10 min, respectively. The ghost suspensions were precipitated with TCA, and prepared and treated as previously described. The fractions from the Dowex 50 columns were further separated, using Dowex 1 (10 % DVB) chloride columns and 0.01 N HCl, without a gradient system. The values for the specific activity are given in Table 2.

Only the first part of fraction No. 4 (from fraction No. 1, Fig. 1) is visible at the end of the elution curve in Fig. 2. It was eluted with gradient 1 → 4 N formic acid on a Dowex 1 (2 % DVB) formate column. When the original fraction (No. 1, Fig. 1) was purified on a Dowex 1 (10 % DVB) chloride column, it appeared comparatively rapidly in the eluate. When further purified by paper electrophoresis, it could be separated into three fractions, *i.e.*, one unlabelled UV-absorbing fraction, one labelled UV-quenching fraction, and one labelled fraction without UV absorption. In view of the small amount of material in the latter two fractions, no detailed analysis could be made of the rate of ^{32}P incorporation.

It is evident from the figures in Table 2 that the maximal incorporation of activity may not be attained even after 30 min incubation. Another interesting feature is the low values for the specific activity of the SerP isolated (fraction No. 4, Fig. 3).

Table 3. Total radioactivity of phosphopeptides and SerP calculated as cpm per gm dry weight of ghosts incubated with ^{32}P at three different pH for 30 min.

pH	Fraction No. 1 Fig. 1	SerP Fig. 1	Fraction No. 2 Fig. 1	Fraction No. 3 Fig. 1
7.2	76.6×10^5	14.5×10^5	24.0×10^5	14.2×10^5
6.2	22.9×10^5	19.5×10^5	23.0×10^5	44.7×10^5
5.5	2.15×10^5	3.35×10^5	4.1×10^5	7.3×10^5

Table 4. Specific activity of phosphopeptides and SerP isolated by Dowex 50 chromatography (Fig. 1). The material from each peak was resolved into several fractions by Dowex 1 (2% DVB) chromatography (Figs. 2–5). The ghosts had been incubated with ^{32}P at three different pH.

Fraction No. (Figs. 2–5)	Peak No. 1 (Fig. 1)			SerP–Peak (Fig. 1)			Peak No. 3 (Fig. 1)		
	pH 7.2	pH 6.2	pH 5.5	pH 7.2	pH 6.2	pH 5.5	pH 7.2	pH 6.2	pH 5.5
1	223 000*	148 000	9 100	282 000	—	29 500	792 000	18 500	7 770
2	51 000	18 000	7 800	34 700	—	4 100	16 600	435 000	210 900
3	24 500	11 800	9 030	94 500	—	1 600	16 700	—	2 700
4	101 000	—	4 800	413 000	10 700	1 140	16 900	—	2 600
5	631 000	10 700	24 200				3 500	—	4 800
6							4 870	11 100	26 600
7							60 000	20 700	22 200
8							520	10 100	2 900

* Counts per min and μg of phosphorus.

pH dependance of ^{32}P incorporation. Washed red-cell ghosts from 1200 ml of cells were suspended in 1000 ml 2×10^{-2} M tris-glycylglycine buffer at pH 7.2, and divided into three equal parts. The pH of two of the samples was adjusted to 6.2 and 5.5, respectively. Each sample was incubated with 8 mC of ^{32}P for 30 min. The suspensions were precipitated with TCA. Phosphopeptides and SerP were isolated by Dowex 50 chromatography. The total activity of the 4 fractions isolated in these three experiments is given in Table 3 (*cf.* Fig. 1). These values indicate that the total activity was maximal at pH 7.2 and minimal at pH 5.5. The ghosts had, however, a tendency to precipitate rapidly from the suspension when the pH fell from 6.2 to 5.0. The four fractions were resolved into several subfractions by Dowex 1 chromatography. Their specific activity is shown in Table 4. It is evident from this table that the highest values for specific activity were recorded chiefly in the samples isolated from the fraction incubated with ^{32}P at pH 7.2, with intermediate values in incubation at pH 6.2, and minimum values at pH 5.5.

DISCUSSION

Several experiments were carried out to compare the labelling of ghosts which had been washed 6 or 12 times with the two types of buffer. The more thoroughly washed ghosts showed greater incorporation of ^{32}P in the different peaks. Similar determinations were made when part of the fresh blood had been stored for 3 weeks at +2°C. These experiments also showed more marked labelling of the ghosts washed 12 times. A comparison indicated that the degree of labelling of the ghosts was about the same when they were prepared from fresh or from stored blood.

The phosphorus content of the protein residues from the ghosts was low, *i.e.* 0.06 %. The dry weight of ghosts decreased considerably when they were washed with the more alkaline buffer. Several experiments showed that the clear centrifugates from the washings contained material which incorporated

^{32}P in about the same way as the ghosts. An account of these results will be given in a later paper.

Although the amino acid sequence in the isolated phosphopeptides was not determined, it can be pointed out that the composition of each peptide in Table 1, as well as the comparative order of lower and higher peptides (Asp Ser Glu Ala Gly Pro), is almost the same as the sequence for chymotrypsin [7] or phosphoglucomutase [8]. The probability that so many phosphopeptides isolated from the ghosts would nearly agree with this sequence by chance is fairly small. Consequently, the data indicate that the amino acid sequence of the active site is almost the same for at least 5 amino acids as for some enzymes which specifically hydrolyze proteins, or transfer a phosphate group between glucose donator and acceptor molecules.

The rate of ^{32}P-labelling is a comparatively slow process, and of the same order as that previously found for several rat organs [10]. It is conceivable that enzymes, as phosphatases, are engaged in the labelling of the phosphoproteins of the red-cell wall. It has previously been shown that dephosphorylated phosphopeptone can, in the presence of alkaline phosphatase and ^{32}P-labelled glycerophosphate, act as a phosphate acceptor [10]. Some preliminary results obtained in this work seem, however, to indicate that a labelled nucleotide can also be isolated from the hydrolyzed cell ghosts. Schauer and Hillman [11] recently obtained some evidence of the presence in the red-cell ghosts of an acid-labile, UV-absorbing substance which may play a role in the labelling of the membrane.

The function of cellular phosphoproteins of a non-enzymic type is not known, although it has been suggested that they may be involved in ion transport [12]. The localization of the labelled phosphoprotein to the red-cell membrane, as well as the possibility of extracting it from this site, suggest that it may operate in the cell as a metabolically activated natural ion-exchange material, the results of this activation being the transport of phosphorus across the cell membrane.

Acknowledgement. The authors are indebted to Mr. Roman Bijan and Mr. Tore Persson for skilful assistance. This investigation forms a part of a programme supported by grants from the *Swedish Medical Research Council.*

REFERENCES

1. Ågren, G. *Acta Chem. Scand.* **10** (1956) 152.
2. Ågren, G. and Engström, L. *Acta Chem. Scand.* **10** (1956) 876.
3. Post, R. L., Merrit, C. R., Kinsolving, C. R. and Albright, C. D. *J. Biol. Chem.* **235** (1960) 1796.
4. Lipmann, F. *Biochem. Z.* **262** (1933) 3.
5. Busch, H., Hurlbert, R. B. and Potter, V. R. *J. Biol. Chem.* **196** (1952) 717.
6. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 1570.
7. Turba, F. and Grundlach G. *Biochem. Z.* **327** (1955) 186.
8. Koshland, D. E. and Erwin, M. Y. *J. Am. Chem. Soc.* **79** (1957) 2657.
9. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 503.
10. Ågren, G. *Acta Chem. Scand.* **13** (1959) 1048.
11. Schauer, R. and Hillman, G. *Z. physiol. Chem. Hoppe-Seyler's* **325** (1961) 9.
12. Heald, P. J. *Biochem. J.* **80** (1961) 510.

Received March 2, 1962.