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SEPARATION OF PHOSPHORYLATED GLYCOLYTIC INTERMEDIATES, NUCLEOTIDES AND GLYCOGEN IN BLOOD CELLS, USING DIETHYLAMINOETHYL-SEPHADEX IN COLUMNS

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SUMMARY

1. A method is presented for chromatographic separation of glycogen, glucose, phosphorylated glycolytic intermediates and nucleotides, using DEAE-Sephadex columns with linear gradient NaCl as eluent.

2. The technical procedure is described in detail and results of model and recovery experiments are reported.

3. When neutralized trichloroacetic acid extracts from blood cells were chromatographed and the fractions were analyzed by various chemical and enzymic methods, up to eleven different peaks were seen. The main contents of the respective peaks were glycogen, glucose, nucleotide(s), *e.g.* AMP, hexose monophosphates, inorganic phosphate, unknown nucleotide, ADP, hexose diphosphates, 2,3-diphosphoglycerate, unknown nucleotide and ATP.

INTRODUCTION

The anion exchanger DEAE-Sephadex, a diethylaminoethyl ether derivative of cross-linked dextran, is widely used for column chromatography. Separation of basic polypeptides from the pituitary[1], neutral amino acids and oligopeptides[2], RNAase (EC 2.7.7.16) digests of soluble ribonucleic acids[3], 19-S and 7-S serum γ-globulins[4] and protease and esterase activity in serum[5] on DEAE-Sephadex columns has been reported. DEAE-Sephadex has also been used for purification of valylribonucleic acid[6,7], bovine vitreous hyaluronic acid[8] and intrinsic factor[9]. Recently it was shown to be useful for thin-layer chromatography in the separation of lactate dehydrogenase isoenzymes and adenosine phosphates[10]. HOLLEY *et al.*[3] considered that DEAE-Sephadex gave highly reproducible results and recommended it strongly for separation of nucleotides.

Bearing in mind these known properties of DEAE-Sephadex, the usefulness of this ion exchanger was tested in separation of phosphorylated glycolytic intermediates, nucleotides, glycogen and glucose in neutralized trichloroacetic acid extracts of blood cells. Results obtained in healthy persons and in cases of glycogen storage disease (Cori's type I) and their relatives have been published elsewhere[11,12].

MATERIAL

DEAE-Sephadex A-25 fine (200–400 mesh) was purchased from AB Pharmacia, Uppsala. Aldolase (EC 4.1.2.7), 18 Units (Racker)/mg; glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 25 Units (Racker)/mg; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 70 Units (Racker)/mg; glucosephosphate isomerase (EC 5.3.1.9), 390 Units (Racker)/mg; DPN (free acid), TPN (as sodium salt), ADP (as sodium salt), ATP (as sodium salt), Fru-1-*P* (as crystallized dicyclohexylammonium salt), Fru-1,6-P_2 (as sodium salt), Fru-6-*P* (as barium salt), Glc-1-*P* (as crystallized disodium salt), Glc-6-*P* (as sodium salt), 6-*P*-GlcA (as crystallized tricyclohexylammonium salt), Rib-5-*P* (as barium salt) and Tris were purchased from C. F. Boehringer & Sons.

METHODS

Blood extracts

From persons who had fasted overnight, 50–100 ml blood were collected in heparinized tubes. The samples were immediately cooled in an ice-bath. All subsequent procedures were performed at +4° and as rapidly as possible. The blood was centrifuged in an International model PR-2 refrigerated centrifuge at 2300 rev./min for 15 min and the plasma and upper 5–10 % of blood cells were removed. The remaining cells were washed once with two volumes of ice-cold 0.155 M NaCl. The saline and the upper 5–10 % of cells were then removed, after which the remaining cells were mixed gently. The volume, hematocrit and number of red and white cells were determined. Two volumes of ice-cold 10 % trichloroacetic acid were added with thorough mixing and the precipitate was centrifuged at +4°, 2300 rev./min for 10 min. The clear supernatant was decanted and the precipitate was washed with ice-cold 5 % trichloroacetic acid (same volume as earlier 10 % trichloroacetic acid). The two supernatants thus obtained were pooled and then washed three times with two volumes of ether to free the extract from trichloroacetic acid. The water phase was passed through a glass filter, the remaining ether evaporated at low pressure and room temperature, and finally the solution was neutralized with 5 N KOH to pH 7.0–7.5. The extracts were stored at —20° for up to two weeks before column chromatography was performed.

Column chromatography

DEAE-Sephadex A-25 fine (200–400 mesh) was prepared according to the manufacturer's directions (swelling in water and removal of fines by decantation, washing on filter with 0.5 N HCl followed by water, treatment with 0.5 N NaOH and washing with water, neutralization with HCl followed by washing with 0.155 M NaCl until equilibrium). A glass chromatography column of 21 mm inner diameter was packed to a height of 130–160 mm with a slurry of DEAE-Sephadex in saline. The material was slowly introduced from a reservoir above the column and linked to it by a short glass tube 4 mm in diameter. Continuous stirring was arranged in the reservoir. The column was filled with 0.155 M NaCl at the start of the packing. The packing was performed at room temperature, to avoid air bubbles in the column. This method gave uniform packing. Before the blood extract was introduced, a disc of filter-paper was placed on top of the anion exchanger and all material remaining on top of the anion exchanger and all material remaining on the inner wall of the

column above the disc was removed. The column was connected by polyethylene tubing to a Uvicord absorptiometer (LKB-Products, Stockholm (Sweden)), with a recorder for registration of per cent transmittance at 2537 Å, and to an automatic fraction collector (Radi Rac, LKB-Products, Stockholm (Sweden)).

The sample, 40–80 ml, was pipetted on to the column and after it had entered the gel the walls were washed with 80–100 ml of distilled water which was also allowed to pass down the gel. A linear gradient elution system was subsequently started: Two cylindrical vessels of 1000 ml volume and of the same shape were used, one containing 900 ml 0.67 M NaCl (A), the other distilled water (B). The contents of B were continuously mixed by a magnetic stirrer. This system gave a linear gradient of NaCl from 0 to about 0.3 M after 900 ml had been eluted.

5-ml fractions were collected and were analysed for the following compounds: total carbohydrates[13], ketohexoses[14], pentose[15], total and inorganic phosphorus[16], Fru-6-*P*, Glc-6-*P* and Fru-1,6-P_2 (see ref. 17) and glucose[18]. Absorbancy was measured at 2600 and 2800 Å and per cent transmittance at 2537 Å. Standards were always run for each of the measured substances with all the methods used. Since NaCl inhibits the reaction with glucose oxidase (EC 1.1.3.4), glucose standards containing the same concentration of NaCl as that in the glucose peak were always run together with the samples. The linearity of the gradient was easily checked by Cl^--determinations on the fractions.

In model experiments, 2–5 μmoles of some or all of the following substances in sample volumes of 50 ml were used: Glc-6-*P*, Glc-1-*P*, Fru-6-*P*, Fru-1-*P*, Rib-5-*P*, 6-*P*-GlcA, ATP, ADP, AMP, glucose, glycogen and Na_2HPO_4.

Other eluent systems that were tested included hydrochloric acid, linear gradient 0–0.2 M; ammonium formate (pH 7.25), linear gradient 0–0.5 M; formic acid, linear gradient 0–0.3 M; 0.155 M NaCl and 0.2 M ammonium formate (pH 7.25).

A micro-system using a column 13 × 150 mm and 2-ml fractions was tried in some experiments. Detailed multiple-method analysis of the fractions was not feasible in these experiments.

A flow of 10–15 ml/h was used in all experiments. Thus the chromatographic separation took from 24 to 48 h. It was always done at room temperature. The separated fractions were stored at −20° or, in some experiments, at +4° for up to 4 days before chemical and enzymic analyses were performed.

RESULTS

Model experiments

In a preliminary experiment using glycogen, glucose, Glc-6-*P* and Fru-6-*P* as model substances and 0.155 M NaCl als eluent, 3 well separated peaks were obtained, containing glycogen, glucose and Glc-6-*P* + Fru-6-*P* (Fig. 1). In model experiments with a greater number of test substances, the best separation was obtained by linear gradient NaCl. Using all the substances mentioned under METHODS, 7–8 anthrone-reacting, phosphorus-containing or ultraviolet absorbing fractions were separated. Analysis of each of these fractions, numbered in order of appearance, by several or all of the chemical and enzymic methods, gave the results illustrated in Fig. 2. It should be noted that one batch of DEAE-Sephadex was used for the experiments reported in Figs. 1, 3 and 4 and another batch in Figs. 2, 5 and 6. These two batches

probably did not have the same ion exchange capacity, since acid compounds were eluted earlier with the batch used in Figs. 2, 5 and 6.

Peak 1: The first peak contained an anthrone-reacting substance which gave no or practically no reaction with carbazole, orcinol, glucose oxidase or phosphorus reagent and no increased ultraviolet absorbancy. The amount of anthrone-reacting substance corresponded well to the amount of glycogen in the sample. Peak 1 consequently was considered to contain only glycogen. When only the anthrone reaction was used for differentiation, this peak was not always completely separated from the following peak, if a very small amount of glycogen and a considerably larger amount of glucose were used in the same run.

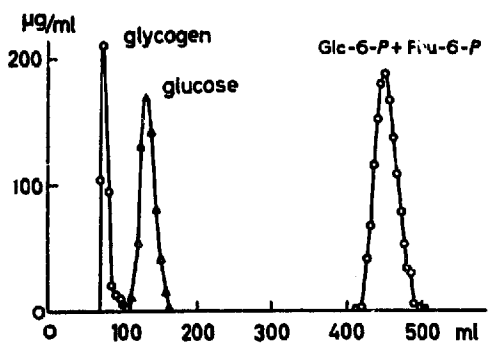

Fig. 1. Model experiment with glycogen, glucose, Glc-6-*P* and Fru-6-*P*. Column packed with DEAE-Sephadex A-25 fine in 0.155 M NaCl. Column size 21 × 160 mm. Eluent: 0.155 M NaCl. O—O, total carbohydrates (anthrone); Δ—Δ, glucose (glucose oxidase).

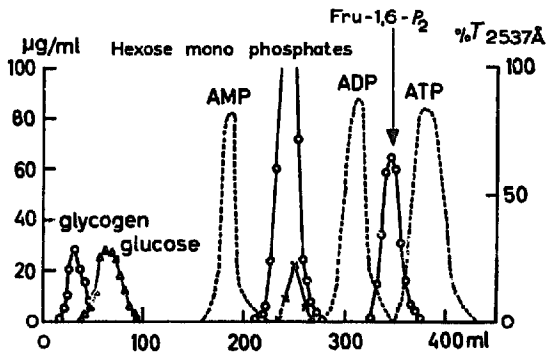

Fig. 2. Model experiment with glycogen, glucose, AMP, Glc-1-*P*, Glc-6-*P*, Fru-1-*P*, Fru-6-*P*, Rib-5-*P*, 6-*P*-GlcA, Na_2HPO_4, ADP, Fru-1,6-P_2, and ATP. Column packed with DEAE-Sephadex A-25 fine in 0.155 M NaCl. Column size 21 × 166 mm. Eluent: 0–0.3 M NaCl in linear gradient. O—O and Δ—Δ as in Fig. 1. -------, per cent transmittance at 2537 Å; ×—×, total phosphorus.

Peak 2: In this fraction there were reactions with both anthrone and glucose oxidase reagents corresponding to the amount of glucose in the sample. No phosphorus reaction or increase in ultraviolet absorbancy was seen, and the carbazole and orcinol reagents were slightly positive. This small degree of reaction was also seen with glucose in pure solution. Peak 2 accordingly was considered to represent only glucose.

Peak 3: This fraction showed an increase in absorbancy at 2600 Å and a reaction with the phosphorus reagent. A strong reaction with orcinol reagent, a very slight reaction with carbazole reagent and none at all with anthrone were observed. From the absorbancy, phosphorus and orcinol reactions and the position in the chromatogram relative to the other ultraviolet-absorbing fractions, it was calculated that this peak contained AMP. Since two other well-defined ultraviolet-absorbing peaks were seen, and since AMP in pure solution gave a slight reaction with the carbazole reagent, it was concluded that no substance other than AMP occurred in Peak 3.

Peak 4: Here the pattern of reactions was more complicated. Very strong reaction was obtained with anthrone and a fairly strong colour was produced by carbazole and orcinol. With enzymic techniques, Glc-6-*P* and Fru-6-*P* could be recovered in expected amounts. No reaction with glucose oxidase occurred. Although direct identification of Glc-1-*P*, Fru-1-*P* or 6-*P*-GlcA was not attempted, these substances were presumed to be present in Peak 4 together with Glc-6-*P*, Fru-6-*P* and Rib-5-*P*.

Peak 5: This was the inorganic phosphate peak. When the column was charged with the above-mentioned large amount of hexose- and pentose-monophosphates, inorganic phosphate was not separated from Peak 4 (Fig. 2). However, when 5 μmoles of Glc-6-*P*, Fru-6-*P* and Na_2HPO_4 were added to a trichloroacetic acid extract of blood cells, part of which had formerly, without any addition, been chromatographed (Fig. 3), some separation of inorganic phosphate from the monophosphate peak was

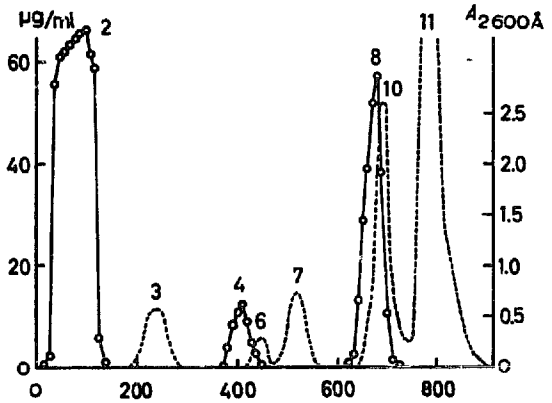

Fig. 3. Trichloroacetic acid extract from 27 ml normal blood cells chromatographed on DEAE-Sephadex A-25 fine, packed in 0.155 M NaCl. Column size 21 × 138 mm. Eluent: 0–0.3 M NaCl in linear gradient. Legend as in Figs. 1 and 2, except ---, absorbancy at 2600 Å. Peak 1 containing glycogen (see *Blood cell extracts*), was not recovered in this experiment, probably because there was too little glycogen in the extract to appear as a distinct peak. Since phosphorus analyses were not made in this experiment, inorganic phosphorus (Peak 5) and diphosphoglycerate (Peak 9) were not measured.

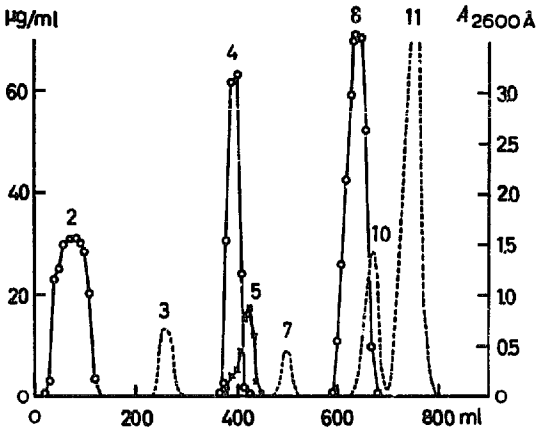

Fig. 4. Trichloroacetic acid extract from 13.5 ml blood cells (same blood sample as in Fig. 3) with addition of Glc-6-*P*, Fru-6-*P*, Fru-1,6-P_2 and ATP chromatographed on DEAE-Sephadex A-25 fine, packed in 0.155 M NaCl. Column size 21 × 152 mm. Eluent: 0–0.3 M NaCl in linear gradient. Legend as in Fig. 3. Peaks 1, 6 and 9 were not recovered. As for 1 and 9 the same reasoning applies as in Fig. 3. Peak 6 was evidently too weak to be seen. (Only half the amount of extract was run here compared with the experiment in Fig. 3.)

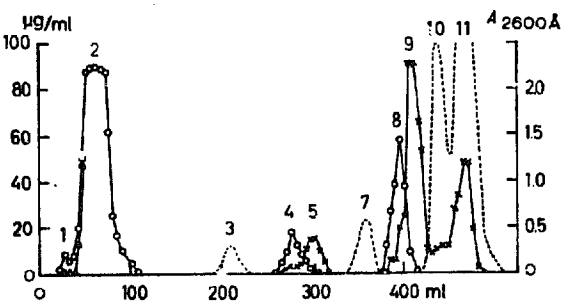

Fig. 5. Trichloroacetic acid extract from 17 ml blood cells from a healthy brother of four siblings with Von Gierke's disease chromatographed on DEAE-Sephadex A-25 fine, packed in 0.155 M NaCl. Column size 21 × 144 mm. Eluent and legend as in Fig. 4. The inconsistent Peak 6 was not seen in this experiment. All other peaks discussed under *Blood cell extracts* were recovered.

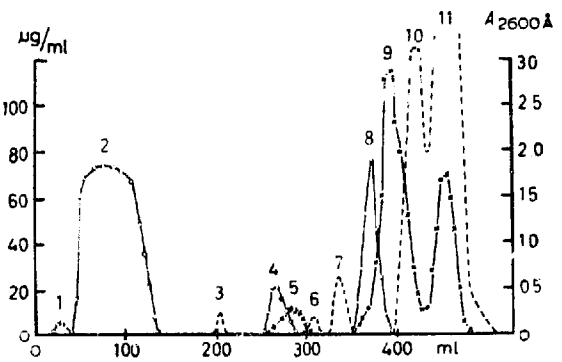

Fig. 6. Trichloroacetic acid extract from 21 ml blood cells from a case of Von Gierke's disease chromatographed exactly as described in Fig. 5. The maximal number of eleven peaks is shown.

seen (Fig. 4). In all experiments with blood cell extracts this separation was also obtained (see Figs. 5 and 6).

Peak 6: In this peak a 2600 Å ultraviolet-absorbing, orcinol- and phosphorus-reacting, non-anthrone-reacting component was observed. A slight reaction was seen with the carbazole reagent. From the phosphate and pentose contents, the absorbancy and the position in the chromatogram relative to the two other ultraviolet-absorbing peaks, it was concluded that this component was ADP. Other substances were presumed to be absent from Peak 6, the reasons being the same as for Peak 3.

Peak 7: This was an organic phosphorus-containing, anthrone- and carbazole-reacting fraction with no increase in ultraviolet absorbancy or reaction for pentoses or glucose. Using enzymic techniques for measurement of Fru-1,6-P_2, it was shown that all Fru-1,6-P_2 added to the original sample was contained in Peak 7. No other component was considered to be included.

Peak 8: An increase in absorbancy at 2600 Å, presence of organic phosphorus and pentose were noted in Peak 8. Although carbazole gave a slight, and anthrone a very slight reaction, it was considered that the only component in this fraction was ATP. The reaction with carbazole and anthrone was of the same intensity as that obtained with a pure solution of ATP.

Recovery of added substrates

The recovery of the different substances measured quantitatively in model experiments is given in Table I. For a discussion of the extraction procedure for blood cells see BARTLETT[14].

Another type of recovery experiment was performed by adding Glc-6-*P* and Fru-1,6-P_2 to part of a mouse-liver homogenate in water. After trichloroacetic acid extraction of this part and another part without added substrates, essentially as

TABLE I

Recovery of glucose, glycogen, Glc-6-*P*, Fru-6-*P* and Fru-1,6-P_2 when 2–5 μmoles of several or all of these substances in a volume of 50 ml water were chromatographed on DEAE-Sephadex using 0–0.3 M linear gradient NaCl as eluent and 5-ml fractions. A, anthrone; GOD, glucose oxidase; C, carbazole.

Substrate	*Column size in cm*	*μg substrate*		*Recovery (%)*	*Method of analysis*
		Added	*Recovered*		
Glucose	21 × 160	1800	1845	102.5	GOD
Glucose	21 × 160	1800	1712	95.1	A
Glucose	21 × 166	720	717	99.6	A + GOD*
Glycogen	21 × 160	1800	1850	102.8	A
Glycogen	21 × 160	1800	1712	95.1	A*
Glc-6-*P* + Fru-6-*P*	21 × 160	2142	2079	97.1	A
Glc-6-*P* + Fru-6-*P*	21 × 152	1800	1694	94.1	A
Fru-6-*P*	21 × 160	2600	2830	108.8	A + C
Fru-6-*P*	21 × 152	650	668	102.8	A + C
Fru-1,6-P_2	21 × 152	3007	3048	101.3	A
Fru-1,6-P_2	21 × 152	2684	2238	83.4	C
Fru-1,6-P_2	21 × 166	1054	1163	110.3	A
Fru-1,6-P_2	21 × 166	1054	1076	104.1	C

* Ammonium formate 0.2 M (pH 7.25) as eluent.

for blood, the part with added Glc-6-*P* and Fru-1,6-P_2 was chromatographed by the standard technique and the original contents of Glc-6-*P* and Fru-1,6-P_2 were determined enzymically on the other extract. Recovery was again calculated, this time by subtracting enzymically determined Glc-6-*P* and Fru-1,6-P_2 in the original liver extract from the amounts chemically obtained (by chromatographic separation) from the extract with added substrates. The results are presented in Table II.

TABLE II

Recovery of Glc-6-*P* and Fru-1,6-P_2 added to a mouse-liver homogenate in water. Trichloroacetic acid extraction as for blood. Chemical measurement after chromatographic separation of the extract. Linear gradient 0–0.3 M NaCl eluent system on DEAE-Sephadex column 21 × 135 mm. Correction made for the original contents of Glc-6-*P* and Fru-1,6-P_2 in the liver (measured enzymically on trichloroacetic acid extract of the liver without additions). A, anthrone; C, carbazole.

Substrate	*μg substrate*		*Recovery (%)*	*Method of analysis*
	Added	*Recovered*		
Glc-6-*P*	383	406	106.0	A
Fru-1,6-P_2	523	486	92.9	C

Other eluent systems

With the hydrochloric acid, formic acid and ammonium formate gradient elution systems, the results were slightly less satisfactory. Confluence of two or more of the peaks obtained with saline gradient was observed in all these systems. For this reason they were not studied in detail.

Nor were the 0.155 M NaCl and 0.2 M ammonium formate systems (pH 7.25) studied in detail. From the experiments that were performed, however, they seemed in no way superior to the other systems studied.

Blood cell extracts

When trichloroacetic acid extracts from red blood cells (containing 10–30 % of the original, normal number of white blood cells) were chromatographed on DEAE-Sephadex using the sodium chloride gradient elution system, up to eleven peaks were seen (Figs. 3, 5 and 6). Some of these were small and did not always appear. The possible contents of the peaks merit discussion.

Peak 1: This was always a small peak when blood extracts were measured. It had the same position as glycogen in the model experiments and gave a reaction with the anthrone reagent but no reaction for glucose, pentose, ketohexoses or phosphorus. Nor did it show any increased absorbancy in ultraviolet light. It was not always well separated from the following peak and at times it could not be seen at all (Figs. 3 and 4). In a few experiments in which liver extracts were used, however, Peak 1 was markedly bigger and always well separated from Peak 2 (glucose). It is probable that most of Peak 1 could be accounted for by glycogen, although the possibility that other anthrone-reacting polysaccharides might contribute to the reaction was not excluded.

Peak 2: Invariably this was a rather large fraction, corresponding to glucose in the model experiments. Measurements with anthrone and glucose oxidase reagents showed that most if not all of Peak 2 was glucose. However, a very slight reaction with carbazole and orcinol was always seen, possibly as a non-specific reaction with

glucose. If any free non-glucose-hexoses or pentoses were contained in this fraction, their amount presumably was insignificant or very small. Consequently, no further attempt was made to estimate non-glucose sugars in Peak 2, and it was accepted as being chiefly glucose. No phosphorus reaction was seen here, but there was always a small increase in absorbancy at both 2600 and 2800 Å. This implies that a small amount of neutral, relatively low-molecular ultraviolet-absorbing substances was present. No studies were made to determine whether these were amino acids, and/or free purine and pyrimidine bases or other substances.

Peak 3: Here a small but fairly constant increase in absorbancy at 2600 Å was observed. When this increase was not too small, a pentose and a total phosphorus reaction were also obtained. Once or twice a very faint anthrone reaction was noted. Peak 3 had the same position in relation to the glucose and hexose monophosphate peaks as AMP had in the model experiments and may contain some slightly acid nucleotide or nucleotides, possibly consisting wholly or partly of AMP. Other components could not be excluded, however.

Peak 4: This was always a rather big peak, with positive reactions for total carbohydrates, total phosphorus and ketohexoses. Some orcinol-reacting component was also noticed. By enzymic methods, Glc-6-*P* and Fru-6-*P* could be estimated quantitatively and were shown to be the main components of this fraction. Figs. 3 and 4 give further proof that Glc-6-*P* and Fru-6-*P* were contained in Peak 4. The orcinol reaction could be accounted for, at least partly, by the presence of Rib-5-*P*. Other monophosphates known to occur in blood cells presumably also were present in this fraction, but their concentration must have been comparatively low.

Peak 5: Inorganic phosphorus was found here. It was usually, but not invariably separated from the monophosphate fraction.

Peak 6: This was a small and inconsistent ultraviolet-absorbing fraction (chiefly at 2600 Å), probably containing some organic phosphorus. A reaction with the orcinol reagent was also noticed. It did not contain measurable amounts of anthrone-reacting carbohydrates. Its significance was not further studied.

Peak 7: Here a clear increase in absorbancy at 2600 Å was noted together with a reaction for phosphorus and pentoses. The relative position was the same as that for ADP in model experiments. The relationship between absorbancy, phosphorus content and orcinol reaction was close to that found for ADP. Accordingly, it was considered probable that ADP accounted for most of the observed reactions. No anthrone reaction was noted.

Peak 8: Tests using the enzymic technique of NEWSHOLME *et al.*[17] showed this fraction to contain Fru-1,6-P_2 and a rather large amount of anthrone-reacting substance(s) that could not have been Fru-1,6-P_2. Figs. 3 and 4 also provide evidence that Fru-1,6-P_2 was contained in Peak 8. Anthrone, carbazole and total phosphorus always gave positive reactions, but no pentose (orcinol) or ultraviolet-absorbing substance was found. According to BARTLETT[14], human erythrocytes contain more Glc-1,6-P_2 than Fru-1,6-P_2. It is therefore probable that some or most of the measured non-Fru-1,6-P_2, anthrone-reacting substance was Glc-1,6-P_2. Other known diphosphates in blood cells may also have formed part of the peak.

Peak 9: By total phosphorus analysis this was the largest peak of all. No reaction was seen with anthrone or orcinol and no increase in ultraviolet absorbancy was noted. The only substance in blood cells that could account for this pattern of reactions

is 2,3-diphosphoglycerate. The expected position of that compound in the chromatogram is in good correspondence with Peak 9.

Peak 10: This was a rather large ultraviolet-absorbing, orcinol- and phosphorus-reacting peak immediately preceding ATP (Peak 11). No colour was obtained with anthrone or carbazole. The contents of Peak 10 have not yet been identified.

Peak 11: Always a very big fraction, Peak 11 had the same relative position as ATP in the model experiments. Its strong orcinol and total phosphorus reactions, very high absorbancy at 2600 Å and very weak reaction with the anthrone reagent all indicate that it contained ATP.

Blood cell extracts with added substrates

One of the trichloroacetic acid extracts from blood cells was divided into two parts. Two-thirds of the volume were chromatographed directly, the other third after addition of Glc-6-*P*, Fru-6-*P*, Fru-1,6-P_2, and ATP in volumes which made the sample volume equal in both cases. The results are shown in Figs. 3 and 4. No unexpected distortion or increase in the number of peaks resulted. A clear increase in the height of the hexose mono- and diphosphate peaks, and ATP peaks was demonstrated. A very slight increase in Peak 3 could be explained by contamination of the ATP preparation by a small quantity of AMP.

Small-column system

The small-column system, mentioned previously, was used for model experiments and for blood cell and mouse-liver extracts with and without addition of phosphates. The fractions collected (2 ml) were too small to allow complete analysis by all the methods used, but in the model experiments the expected separation was noted. This was slightly less complete than with the larger column, probably mainly because 2-ml fractions in the small-column system correspond to about 10-ml fractions in the ordinary system. With the liver extracts a very clear separation of glycogen from glucose was always obtained and the added substrates (hexose mono- and diphosphates) appeared at the expected tube numbers. No attempt was made to identify observed peaks in the chromatogram from liver extracts. The results were considered to indicate that in most situations the larger column is preferable, and the "micro-system" consequently was not used for further studies.

DISCUSSION

Chromatographic separation of nucleotides and phosphorylated glycolytic intermediates in blood cells has been described by several writers who used Dowex anion exchangers. Good separation of several nucleotides was often achieved[19-21] with this method, but satisfactory separation of phosphorylated glycolytic intermediates has previously been obtained only with rather complicated and time-consuming procedures[22-25].

The available literature hitherto contains no report on the use of DEAE-Sephadex for separation of glycolytic intermediates.

From the results of the experiments reported here, it is clear that no simple gradient system alone can give the very high degree of separation described by BARTLETT[22] and SCHMITZ *et al.*[23].

Possibilities of obtaining further information using the described system probably exist. All the observed peaks have not yet been identified and a detailed study may be valuable. In addition, more complicated elution systems might help to give a better separation, *e.g.* within the hexose monophosphate peak. The system here presented is, however, considerably simpler than those used by BARTLETT[22] and SCHMITZ *et al.*[23] for their best separations. The results of the quantitative analyses on blood cells, for which this system has been used[11,12], agree very well with the values given by BARTLETT[22] after extensive experiments. All substances measured quantitatively after chromatography on DEAE-Sephadex columns have been identified in the described system. As an alternative to other ion-exchange chromatographic methods for the measurement of phosphorylated glycolytic intermediates and nucleotides the NaCl gradient elution on DEAE-Sephadex columns represents a technically simple, yet very reproducible method, giving excellent recovery. If it is desired also to measure polysaccharides (glycogen) in the same procedure the DEAE-Sephadex method has the advantage over earlier methods that it permits separation of high-molecular neutral polysaccharides from glucose. The probable reason for this separation is that DEAE-Sephadex retains some of the molecular sieving effect of the unsubstituted Sephadex.

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