differing strength as potassium hydroxide and quinoline give about the same reaction rates with cymene and sulfur. The nature of the intermediates has not been determined. Levi[5] has found that amines form definite compounds with sulfur. These compounds are too unstable to exist at the temperatures used in this study. The reaction of α-methylstyrene with sulfur to give the dithiolethione may be by a free radical mechanism.[6]

The relative reaction rates in Table IV, relative to cumene (taken as 1.00), show the order: methyl 4.8, ethyl 1.8 and *t*-butyl 0.5. This sequence lines up with that obtained by Baker and Nathan[7] in the hydrolysis of *p*-substituted benzyl halides, and by Hughes, Ingold and Taher[8] in the hydrolysis of *p*-substituted benzhydryl halides and ascribed by them to hyperconjugation. Good agreement in the rates obtained at constant time and constant percentage conversion indicates the same mechanism is operating in all cases.

TABLE IV

RELATIVE RATES OF REACTION OF CUMENES WITH SULFUR

Catalyst: 0.00165 mole of di-*o*-tolylguanidine

	Cumene	Cymene	*p*-Ethyl-cumene	*p*-*t*-Butyl-cumene
Temperature 151°				
Reacn. in 24 hr., %	5.7	28	10	...
Rel. reacn. rate	1	4.9	1.8	...
Time for 40% reacn., hr.	144	30	82	...
Rel. reacn. rate	1	4.8	1.8	...
Temperature 178°				
Reacn. in 8 hr., %	...	82.3	29.2	8.56
Rel. reacn. rate	...	9.6	3.4	1
Time for 80% reacn., hr.	...	7.5	20.25	71
Rel. reacn. rate	...	9.5	3.5	1

Aryldithiolethiones can now be made easily from readily available saturated hydrocarbons.

WHITING, INDIANA

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[CONTRIBUTION FROM THE RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Fractionation of Phosphates from Scenedesmus by Anion Exchange[1]

BY M. GOODMAN,[2] A. A. BENSON AND M. CALVIN

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The phosphorylated compounds involved in algal metabolism have been extracted and fractionated on Dowex 1 and 2 columns. The separation of hexose monophosphates, phosphoglycerate, hexose diphosphate and ribulose diphosphate is described. The elution characteristics of trace amounts of radioactive phosphate esters were found identical to those of macro amounts. Using P^{32}-labeled algal extracts, relatively large amounts of material have been fractionated. The ion exchange elution data of ribulose diphosphate are described.

Introduction

The fractionations of phosphorylated compounds from algae have been reported by Umbreit, *et al.*,[3] Kamen and Spiegelman,[4] Emerson, *et al.*,[5] Gest and Kamen[6] and Simonis and Grube.[7] The techniques described involved gross fractionation methods. The object of this investigation is to present a preliminary scheme for extending the gross fractionations to permit chemical characterization of compounds in the alcohol–water extracts of algae.

Paper chromatography[8-12] has been used extensively to characterize compounds occurring in algae. Ion exchange chromatography, however, offers the possibility of larger scale isolation for chemical analysis. The purpose of this work is to develop separations of photosynthesis intermediates, particularly ribulose diphosphate.

The separation of phosphorylated compounds by anion exchange has been demonstrated by Khym and Cohn,[13] who used borate complexing to achieve separation. Benson, *et al.*,[8] separated fructose-6-phosphate from fructose-1,6-diphosphate. Horecker and Smyrniotis[14] separated ribose-5-phosphate from ribulose-5-phosphate. The methods developed using pure compounds have been applied to the rather complex mixture in plant extracts.

Experimental

Preparation of Algal Extracts.—Two methods were used to obtain the algae: 1. *Scenedesmus* was grown as a continuous sterile culture under controlled conditions[15] with the addition of ferric Versenate.[16] The algae were harvested, centrifuged from the growth medium, resuspended in water and killed in 80% boiling ethanol while photosynthesizing with 4% carbon dioxide bubbling through the suspension. After an accumulated total of 75 ml. (packed volume) of

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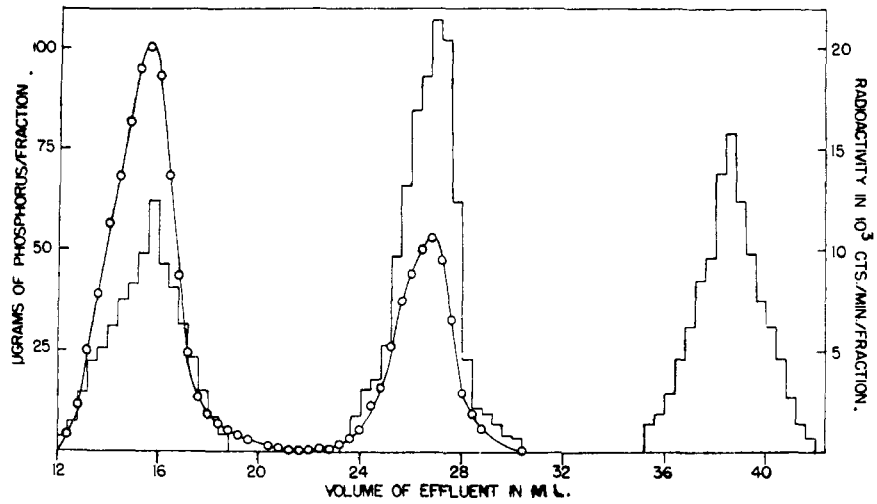

Fig. 1.—Glucose-6-phosphate (first peak), phosphoglyceric acid (second peak) and fructose diphosphate (third peak) + carbon labeled phosphoglycerate and monophosphate areas eluted from 40-second barley leaf chromatograms, eluted by acid from a Dowex 1 (200–400 mesh)–chloride column (29 × 0.6 cm.). Smooth curve denotes radioactivity. Block curve is obtained from phosphorus analysis. Recovery was quantitative. Eluting agent: 0.15 *N* NaCl + 0.05 *N* HCl. Elution rate: 0.1 ml./min. Fraction volume: 0.4 ml.

algae had been killed thus, the cell residues were filtered off and extracted with 20% boiling ethanol. The 80 and 20% extracts were combined and concentrated to 100 ml. under vacuum at 25°. The solution then was extracted with ethanol–petroleum ether (3:1 by volume), to remove the phospholipids and chlorophyll.

2. Larger quantities of *Scenedesmus* were obtained from 40-liter cultures grown rapidly but under non-sterile conditions. Starting with an inoculum of 1 g., in one week 80 ml. (packed volume) of algae were harvested. The extract prepared as before was mixed with small amounts of labeled extracts of 10- and 25-minute photosynthetic experiments with radiophosphate. The purified alcohol–water extracts were used for further fractionation.

Preparation of Materials.—Two methods were used to obtain pure 3-phosphoglyceric acid. Commercial 3-phosphoglyceric acid was recrystallized by the Neuberg procedure[17] and Neuberg's[18] yeast fermentation method was employed. Commercial fructose-1,6-diphosphate was contaminated with fructose-6-phosphate and ortho-phosphate. Ion exchange treatment of the crude material[3] gave pure fructose-1,6-diphosphate. The hexose monophosphates were recrystallized several times as the barium salts from water and ethyl alcohol. The glucose-6-phosphate was contaminated with 10% fructose-6-phosphate while the fructose-6-phosphate contained 5% glucose-6-phosphate. Glucose-1-phosphate was free of any other phosphates.

The pure radioactive materials required were eluted from paper chromatograms[6,19] of photosynthetic experiments which were carried out according to the usual methods.[5,10,15,20]

Detection and Identification.—Total phosphorus was determined colorimetrically by the technique of Allen.[21] Aliquots were taken containing between 5 and 40 micrograms of phosphorus, the range of this method. The color intensity was read in a Klett–Summersen colorimeter with a 660 mμ filter. The actual amount of phosphorus was obtained from a calibration curve.

Carbon- or phosphorus-labeled compounds were used in these experiments. Aliquots of radiocarbon-labeled phosphates were plated on aluminum discs and counted with a thin mica-windowed G–M tube. Radiophosphorus also was counted in this way. In addition, since the β-particle from P^{32} (1.7 Mev.), is stronger than that from C^{14} (0.126 Mev.), the P^{32} could be counted in polyethylene cups.

Paper chromatography was used in the identification of the radioactive compounds.[5,9,19,15] The compounds were desalted with Dowex 50 and lyophilized. In some cases, acid phosphatase was added to the desalted phosphates. The resulting dephosphorylated compounds were identified on paper chromatograms.

Preparations of Resins and Columns.—The separations were carried out by using Dowex 1 and Dowex 2 resins (200–400 mesh). Fine particles were removed by back washing or repeated decantation.[22] The columns were prepared by the gravity method, *i.e.*, the resin was slurried into the column, the size of the column depending on the amount of material and the number of components in the mixtures. After the ion exchanger was thus constructed, it was washed with at least 20 column volumes of 1 *N* HCl. This was followed by an equal volume of distilled water. If the resin was used in the chloride form it was ready for use after water washing. To convert the resin to the borate form, 0.2 *M* $Na_2B_4O_7$ was run through the column, followed by distilled water.

Column Chromatography of Authentic Phosphorylated Compounds.—The two eluting conditions which were employed achieved the desired separations. Figure 1 shows the separation of glucose-6-phosphate, 3-phosphoglyceric acid and fructose-1,6-diphosphate and the partition of the radioactivity among the peaks. The graph shows that the radioactivity eluted with the monophosphates and 3-phosphoglyceric acid and that no radioactivity appeared in the diphosphate area. It remained then to analyze the radioactive constituents of the first two peaks in order to identify the compounds.

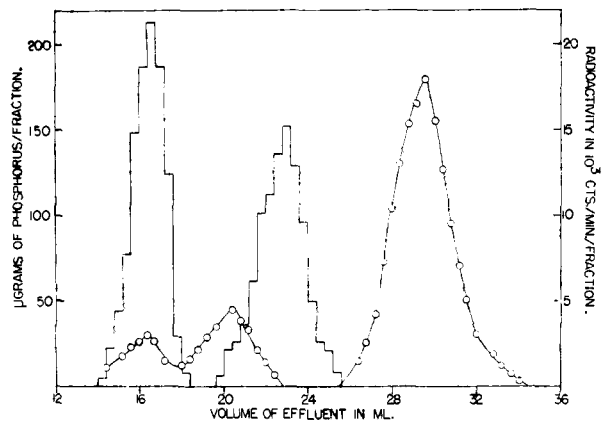

Fig. 2.—Acid elution of phosphoglyceric acid, fructose diphosphate and radioactive ribulose diphosphate eluted from a unidimensional paper chromatogram of a 5-minute photosynthetic experiment on *Scenedesmus* with $C^{14}O_2$, from a Dowex 2 (200–400 mesh)–chloride column (28 × 0.6 cm.). Recovery was quantitative.

Eluting agent:	0.15 *N* NaCl + 0.05 *N* HCl
Elution rate:	0.11 ml./min.
Fraction volume:	0.4 ml.

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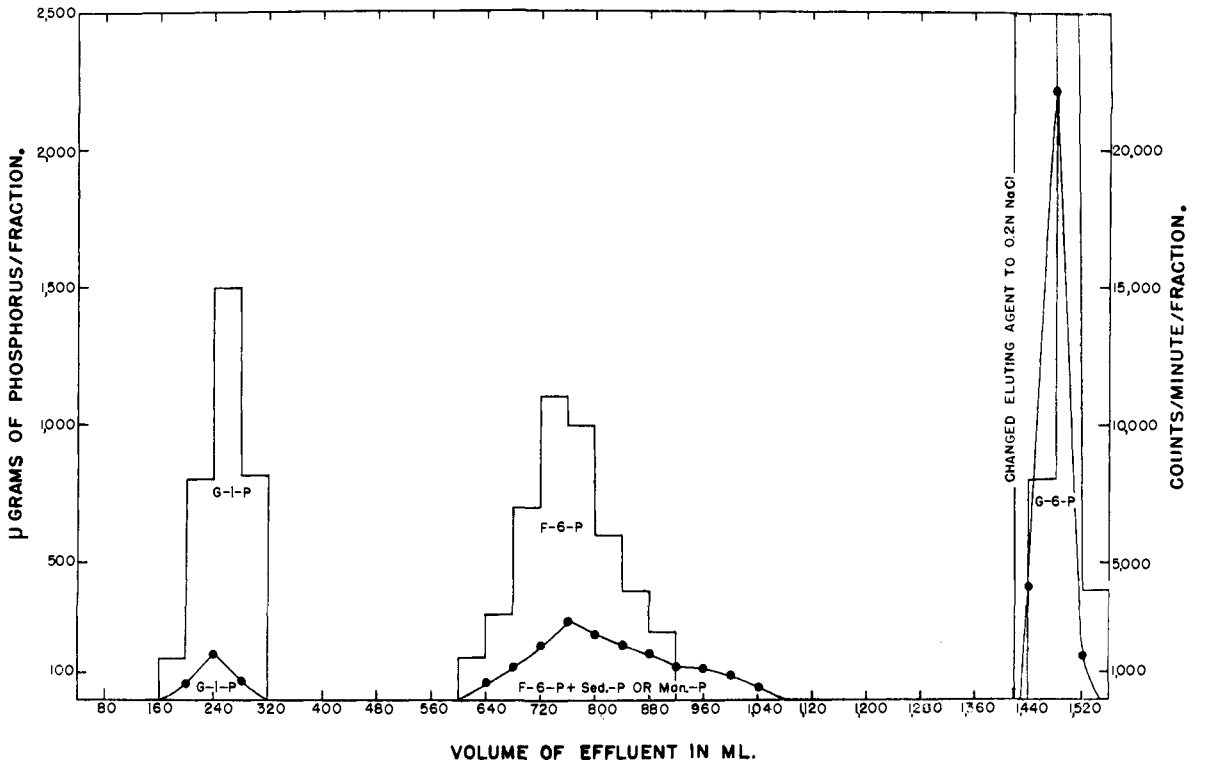
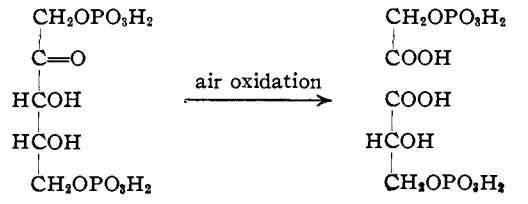


Fig. 3.—Borate elution of glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate plus phosphorus labeled hexose monophosphate area from *Scenedesmus* chromatograms (10-minute photosynthetic experiment), from a Dowex 1 (200–400 mesh)–borate column (28 × 0.8 cm.). Recovery was quantitative. Smooth curve denotes radioactivity while the block curve is obtained from phosphorus analysis. Eluting agent: 0.1 *M* $Na_2B_4O_7$. Elution rate: 1.3 ml./min. Fraction volume: 40 ml.

In a given peak, if the radioactivity and carrier phosphate were identical, the ratio of counts/minute/fraction *versus* the micrograms of phosphorus/fraction would be constant over the entire peak. The phosphoglyceric acid peak gave a constant ratio while the hexose monophosphate peak did not. The conclusion is that radioactivity in the monophosphate peak is made up of many compounds while the radioactivity in the second peak is essentially all phosphoglyceric acid. Aliquots of both peaks were treated with phosphatase and chromatographed on Whatman No. 1 paper two dimensionally in phenol saturated with water and *n*-butyl alcohol, propionic acid, water.[8] Peak 2 gave only radioactive glyceric acid while peak 1 gave many compounds, among them glucose, fructose, sedoheptulose and mannose.

Figure 2 shows an elution of phosphoglyceric acid, fructose-1,6-diphosphate and radioactive ribulose diphosphate. The ribulose diphosphate separated from the fructose diphosphate indicating that this might offer a method of obtaining ribulose diphosphate in pure form for chemical characterization. The activity in the phosphoglyceric acid peak was identified as phosphoglyceric acid by paper chromatography. The second radioactive peak contained phosphoglycolic acid. This was determined by co-chromatography (after the phosphatase hydrolysis) with authentic glycolic acid. The origin of these compounds can be explained by assuming the air oxidation of ribulose-1,5-diphosphate. If the compound split between carbons 2 and 3, phosphoglyceric acid and phosphoglycolic acid would arise.



The use of borate for the separation of sugar phosphates has been demonstrated amply in the paper by Khym and Cohn.[13] We employed quite different eluting conditions as far as concentration of borate. Figure 3 shows the resulting separation. Peak 2 gave a positive ketose test with Roe reagent[23] while peaks 1 and 3 gave negative tests. It is interesting that radioactivity appeared in the glucose-1-phosphate peak in addition to the fructose-6-phosphate and glucose-6-phosphate peaks. From other experiments,[24] the radioactive constituent of the fructose-6-phosphate peak has been shown to be mostly mannose-6-phosphate.

Identification of Metaphosphates.—The last phosphate peaks obtained from acid elution of algae extracts were taken to dryness in vacuum and found to contain no carbon. These compounds hydrolyzed completely in seven minutes at 100° in 1 *N* HCl and gave a purple color with toluidine blue[25] in acid solution. In this manner their identity as metaphosphates or polyphosphates was established.

Column Chromatography of Phosphorylated Compounds from *Scenedesmus*.—The 80 and 20% ethanol extracts of *Scenedesmus* were combined and clarified by high-speed centrifugation, and to this was added an extract from a smaller volume of radiophosphate-labeled (25 minutes in radiophosphate) extract.[10]

TABLE I

PHOSPHORUS CONTENT OF VARIOUS EXTRACTS OF *Scenedesmus*

		P, mg.
1	Total (75 cc. of algae)	325
2	In 80 and 20% EtOH extracts before petr. eth. extr.	160
3	In 80 and 20% EtOH extracts after petr. eth. extr.	90

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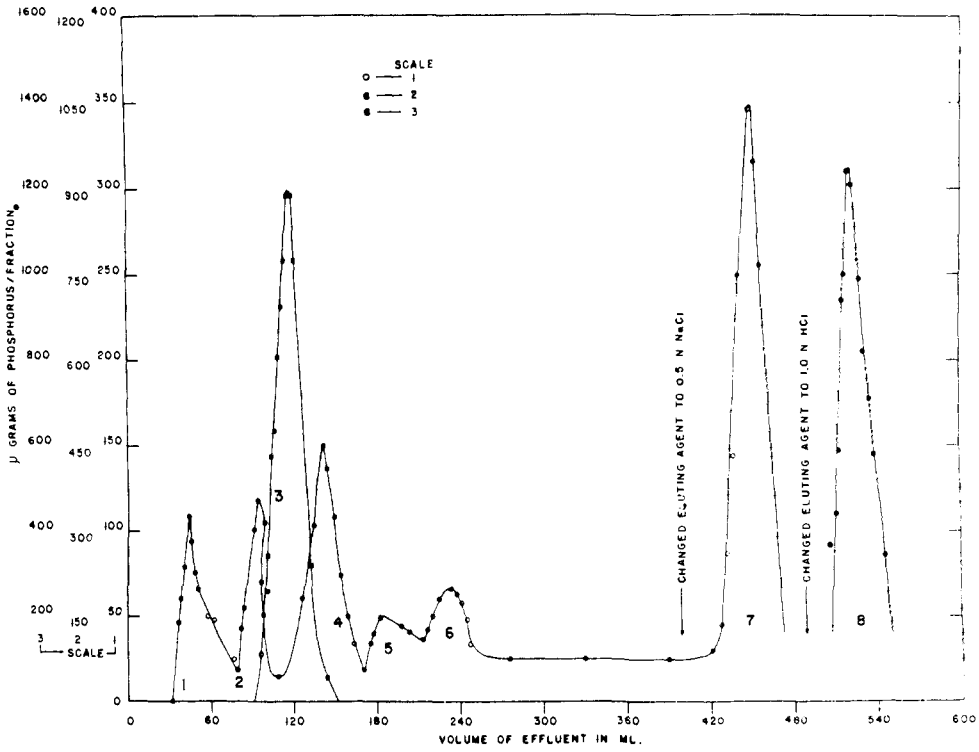

Fig. 4.—Acid elution of algal extracts. I. From Dowex 1-chloride column (3.5 × 1.6 cm.) (200–400 mesh). In order of elution: 1 and 2, monophosphates; 3, orthophosphate; 4, phosphoglyceric acid; 5 and 6, diphosphates; 7 and 8, metaphosphates. Eluting agent: 0.15 *N* NaCl + 0.05 *N* HCl. Elution rate: 0.22 ml. /min. Fraction volume: 1.2 ml.

Solubility Fractionation of Algal Phosphates.—The algae which were grown and harvested under the first conditions described above exhibited the total phosphorus analysis shown in Table I. The third fraction (*i.e.*, after petroleum ether extraction) was used in the ion exchange separations, whereupon the resin elution curve, Fig. 4, was obtained.

Table II shows the compounds or groups of compounds identified and their extractable phosphorus content. The very large metaphosphate fraction consists of polymerized phosphate residues. These have been isolated from many organisms.[26–29] Figure 5 describes ion exchange separation for algae grown under mass culture conditions.

The radioactivity followed the phosphorus analyses closely. Table III presents the over-all fractionation of the phosphates in the algae.

TABLE II

FRACTIONATION OF CERTAIN PHOSPHATES IN *Scenedesmus*

		% of total phosphorus placed on col.	In algae
Peak 1 + 2	Monophosphates	3.5	1.0
Peak 3	Ortho-phosphates	28.8	9.8
Peak 4	Phosphoglyceric acid	3.5	1.0
Peak 5 + 6	Diphosphates	1.2	0.3
Peak 7 + 8	Metaphosphates	63.0	21.0

Discussion

The use of acid in the chloride eluting agent maintained the carboxyl group of phosphoglyceric acid in the un-ionized form. Thus, it was held on the column by its phosphate group only. Under these circumstances, it was separated easily from the diphosphates and hexose monophosphates. Khym and Cohn[13] also have achieved this separation in their borate complexing scheme.

TABLE III

FRACTIONATION OF PHOSPHORUS IN *Scenedesmus* GROWN BATCHWISE (80 CC. OF CELLS)

	Mg. of phosphorus extracted Total	Ortho-
80% EtOH extr.	5	..
20% EtOH extr.		
a	28	20
b	24	19
c	16	11
d	10	8
e[a]	8	8
Ether–ethanol (1:3)	16	..
Trichloroacetic acid	88	68
Residual	200	..

[a] Since "e" contained only ortho-phosphate, it was discarded.

It has been demonstrated that *cis*-hydroxyls are involved in the structure of the borate complexes of sugars.[30–33] Under our conditions of concentrated borate (0.1 *M*) and Dowex 1 borate form, the glucose-1-phosphate came off the column rapidly, indicating it did not form a borate complex. However, the order of elution of fructose-6-phosphate and glucose-6-phosphate was inverted from the order obtained by Khym and Cohn.[13] In fact, the glucose-6-phosphate was held so tightly by the

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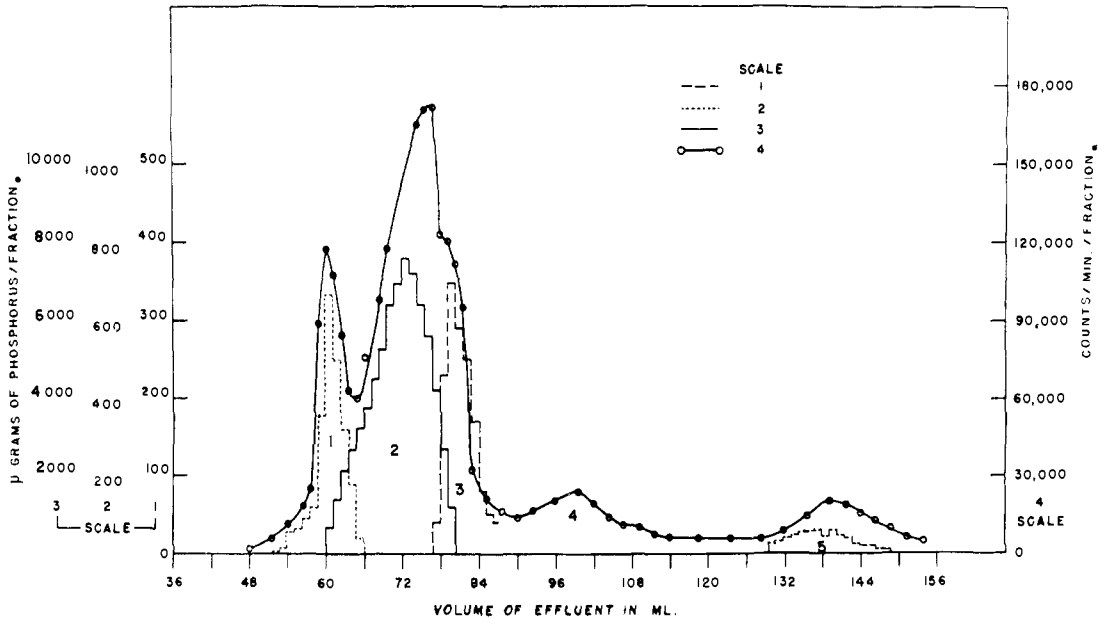

Fig. 5.—Acid elution of algal extracts from a Dowex 1-chloride column (200–400 mesh). In order of elution: 1, monophosphates and nucleotides; 2, ortho-phosphate; 3, phosphoglyceric acid; 4 and 5, diphosphates. Eluting agent: 0.15 *N* NaCl + 0.05 *N* HCl. Elution rate: 0.22 ml./min. Fraction volume: 1.2 ml.

resin that 0.2 *N* NaCl was used as an eluting agent to bring it off the column.

A possible explanation for the inversion was presented by Khym and Cohn.[13] Ribose-5-phosphate and fructose-6-phosphate can only form furanose structures while glucose-6-phosphate can form both furanose and pyranose rings. At low levels of borate ion concentrations (10^{-2}–10^{-5} *M*), the pyranose form of glucose-6-phosphate predominates. Since the ribose-5-phosphate and the fructose-6-phosphate elute from the resin much slower than the glucose-6-phosphate, the furanose forms are assumed to give a borate complex which is bound more firmly to the resin. At high borate ion concentrations, the glucose-6-phosphate assumes the furanose structure and is thus held more strongly by the resin.

The elution position of ribulose diphosphate suggests the feasibility of using the chloride elution conditions which have been described in this paper on extracts of photosynthetic algae to obtain enough ribulose diphosphate for chemical and physical characterization.

It is very difficult to explain the large concentration of ortho-phosphate in both the 20% alcohol extracts and in trichloroacetic acid extracts from the algae grown under batchwise conditions without assuming that the metaphosphates have been hydrolyzed. Since these algae were grown and harvested under different conditions from those used in the first algae experiment described above, no direct correlation is possible. At present, no experimental explanation can be offered to explain why the metaphosphates should hydrolyze so completely in the batchwise experiment. It can be suggested that in the batchwise technique the algae are relatively phosphate starved. As a result, they may have utilized the metaphosphate as a source of energy.

The conditions for the accumulation of metaphosphates are being investigated, for this will certainly give clues as to their function in the cell. It has been suggested[29] that these polymerized compounds are storage reservoirs for phosphate. However, there remains the possibility that they are actually energy storage points from which the cell can draw energy when needed. Experiments to determine molecular weights and titration curves are also being undertaken.

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