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Berkeley, California

SUGAR PHOSPHATES, PAPER AND COLUMN CHROMATOGRAPHY[1]

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The impetus of application of paper and ion exchange chromatography upon the identification and purification of phosphorylated metabolic intermediates can scarcely be underestimated. Standards of purity have been re-evaluated and the identification of unanticipated sugar phosphates has exposed new pathways for synthesis and degradation.

These two complementary techniques allow rapid separation of a great variety of phosphate esters on practically any scale. Trace amounts of high specific radioactivity compounds may be separated as well as multigram amounts of nucleic acid degradation products. This new range of sensitivity demands the establishment of new criteria of purity, a factor which has often received too little attention in commercially available products. We may expect that sugar phosphates of reliable purity will become increasingly available as commerical products.

Both paper and column chromatography of sugar phosphates are natural extensions of the original applications of these techniques. In each case the initial difficulties have been overcome and their use is expanding as rapidly as their value is comprehended.

FILTER PAPER CHROMATOGRAPHY

Chromatography on pure paper may be considered an establishment of a dynamic equilibrium or partition between solution (adsorption) in a fixed semi-aqueous layer upon the cellulose fiber and solution in the moving

organic phase. Although the role of adsorption is certainly greater for sugar

phosphates than for the free sugars the general principles of relative solubili-

ties may be applied in predicting their movement in a particular solvent system.

The phosphate group determines much of the solubility properties of the compound.

This requires that the chromatographic method be particularly effective in order

to separate compounds by virtue of the less polar carbohydrate moiety.

The hydrophilic nature of the phosphate esters results in relatively low

R_f values with solvents normally used for amino acids and sugars. A number of

stronger solvents have been developed, either by increasing water content, or

by increasing acidity. The picric acid (pK = 0.38) solvent developed by Hanes

and Isherwood[2] successfully represses the dissociation of the phosphate group

(pK = 2.12) to such an extent that it offers almost no retarding influence

upon the movement of the sugar.

Limitations of the Method

Salt toleration is a serious handicap in application of the method to bio-

chemical preparations which are rarely salt-free. The paper may not be over-

loaded with any solute, salts, fats or phosphates without serious distortion.

Salts may be avoided in preparation of the sample or preliminary barium preci-

pitation or anion resin adsorption may be used to effect suitable purification.

Barium salts are readily decationized with a cation exchange resin such as 400

mesh Dowex 50. Some degree of salt toleration has been obtained by using sol-

vents saturated with the salt.

Readily hydrolyzable esters are labile in many of the acidic or basic sol-

vents commonly used. Reduced temperature or faster development with a less

acidic solvent should be considered.

Paper Purification

Calcium and magnesium salts in paper cause irreversible adsorption of phos-

phates which cannot be tolerated in their chromatography. Whatman Nos. 1, 4 and

52 paper and Schleicher and Schuell No. 589 have been used with suitable pre-washing.

Cations have been removed by washing with $\underline{N}$ hydrochloric acid and 0.02% aqueous versene (Versenes, Inc., formerly Bersworth Chemical Co., Framingham, Mass.).[3] Oxalic acid solutions (1%) have been successful in reducing phosphate adsorption and yield an "acidic" paper upon which carboxylic acids and phosphate esters move relatively faster than the neutral compounds.

Method: Large quantities of paper (100-200 sheets) are washed in a rectangular lucite filter with a perforated bottom for applying suction.[2] The paper is saturated with oxalic acid solution and washed thoroughly with distilled water. The pile may be dried on a screen in a warm-air oven and the outer sheets discarded. Single sheets may be effectively prewashed by hanging them from a trough of water by which the paper is washed chromatographically.

Application of Solutions to Origin

Phosphate esters are strongly adsorbed by polysaccharide material present in many extracts and cannot be moved by the solvent. Such adsorption is serious in handling tracer quantities but can be prevented in many cases by observing certain precautions in applying the solution to the origin.

1. The paper should be moist or at least hydrated before the sample is applied.
2. The origin should never be overheated enough to cause adsorption or chemical transformation of the compounds involved. When the air velocity is high enough to maintain rapid surface evaporation it is possible to use quite hot (100° C.) air without deleterious effects. Such heating should cease when the origin approaches dryness. With adequate ventilation an aqueous solution may be dried at the rate of 0.25 ml./min./cm.[2] A convenient technique uses compressed air heated by passing in a metal tube through a steam bath and emerging from an 8 mm. diameter tube placed directly beneath the

origin of the paper held by two glass plates with circular holes for exposing the origin. The sample is applied from above as it dries.

3. <u>Overloading</u> occurs at points where the amount of material exceeds the capacity of the paper and solvent for satisfactory separation. An elongated origin will give "round" spots under conditions in which a round origin gives an oval spot or a streak due to overloading.

QUANTITIES DETECTABLE AND SEPARABLE

A minimum of 0.1 µg. P is generally needed for phosphate detection on paper. Amounts of ester containing 3 to 10 µg. P (0.2-0.6 micromoles) are suitable for separation and detection from an origin of 5 mm. diameter. Larger amounts may be separated by chromatography as a 55 cm. x 0.5 cm. stripe. Proper loading may be calculated from the area of the stripe and that of a satisfactory unidimensional origin loading. The use of thick papers such as Whatman 3 MM for this purpose has not been reported.

The quantities of material may be very much smaller when the compounds are detected by virtue of C^{14} or P^{32} radioactivity. With suitable specific radioactivity 10^{-9} to 10^{-10} mole quantities may be separated, detected and accurately measured. The limiting factor in chromatographing such small quantities is irreversible adsorption caused by impurities in the paper. It may be possible to prevent such losses by previously washing the paper with a solution of the unlabeled compound.

DETECTION OF SUGAR PHOSPHATES ON PAPER

Acidity and sugar color reactions as well as phosphate content may be used to detect these compounds on paper. Neutron activation is not yet well developed but may attain an importance comparable to chemical identification methods.

Phosphate Spray Test

Hanes and Isherwood[2] developed a suitable spray reagent for difficultly hydrolyzable phosphate esters. Bandurski and Axelrod[4] simplified the reduction step by using UV-stimulated reduction of the ammonium molybdate to molybdenum blue.

Principle: Perchloric acid is used as an extremely strong acid for rapid hydrolysis of difficultly split esters like PGA. The freed orthophosphate forms ammonium phosphomolybdate which is reduced by saturation with H_2S or by organic reducing agents formed upon UV-irradiation.

Method:[4] The paper is dried in warm air and evenly sprayed with the following solution:

$$25 \text{ ml. } 4\% \text{ (W/V)}(NH_4)_6Mo_7O_{24} \cdot 4H_2O$$

$$10 \text{ ml. N/1 HCl}$$

$$5 \text{ ml. } 70\% \text{ HClO}_4$$

$$60 \text{ ml. } H_2O$$

The paper is then heated in an oven at 100° for 5 minutes. It should not begin to char or darken significantly. The paper becomes rather fragile and must be handled carefully. After briefly humidifying with steam to restore flexibility the paper is uniformly irradiated with a 15 watt germicidal ultraviolet (2537 Å) lamp for 1 to 10 minutes at 10 cm. All sugar phosphates give blue spots.

Sensitivity. - The intensity of the blue spot is dependent upon the paper, size of origin, distance traveled and initial phosphorus content. Spots of 0.1 to 1.0 μmole (25 γ Na_3PGA) are satisfactory.

It must be borne in mind that the sensitivity is a function of the background color and the concentration of phosphorus (P/cm.^{2}) in the final spot.

Blue background due to insufficient heating or to excess organic solvents may be destroyed by ammonia vapors. Organic impurities in the original material often reduce sensitivity and necessitate the use of the larger amounts of compound for satisfactory detection.
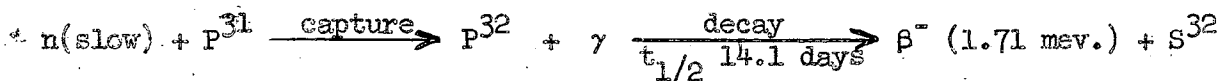
Acid Spray

Bromcresol green (0.05% in 90% ethanol) adjusted to a blue tint (ca. pH 5.5) gives yellow spots for PGA and other acids after development in an acid solvent. It may be necessary to autoclave the paper briefly in order to remove acidic solvents effectively.

Sugar Reagents

Aniline-TCA or phthalic acid[3]-acetic acid (glucose-1-P), resorcinol-HCl-ethanol (fructose, sucrose phosphates), orcinol-TCA acetic acid (heptulose phosphates) or Tollens' reagent may be used to detect the sugar moiety in these compounds. Their sensitivity is generally not as great as that of the molybdenum blue reaction and their use may be limited to cases where their specificity is of importance.

Detection of Chromatographed Phosphate Esters by Neutron Activation

Radiophosphorus is obtained by absorption of slow neutrons by ordinary phosphorus.

$$n(\text{slow}) + P^{31} \xrightarrow{\text{capture}} P^{32} + \gamma \xrightarrow[t_{1/2} \ 14.1 \ \text{days}]{\text{decay}} \beta^- (1.71 \ \text{mev.}) + S^{32}$$

The resulting P^{32} activity depends upon time and flux in which the paper is exposed. It can be seen that it will be possible to convert a small fraction of the P of the sugar phosphate to P^{32} for location of the spots and use the rest for further work. Assay may be done by comparison to the P^{32} activity induced in a known amount of phosphate compound on the same paper.

Certain difficulties are inherent in this technique. High gamma flux of nuclear reactors is damaging to paper. Traces of impurities (Na, K) produce

other activities in which P^{32} is not easy to measure. The presence of elements

with large neutron-capture cross sections, even in trace quantities, results

in a variety of radioactive species. Absorption of a neutron results in des-

truction or at least cleavage of the phosphate group from the sugar. In this

respect, however, the detection method is as disastrous for the molecule as

chemical tests.

Schmeiser and Jerchel[5] have developed this technique for fast neutron

bombardment obtainable with the cyclotron (D^+ bombardment of Be). They obtained

good yields of Si^{31} (2.8 hr.) and good sensitivity. They were able to detect

1 µg. P/cm.^{2} on paper. This activation is much faster than slow neutron acti-

vations but also suffers from the short half life of the radiosilicon formed.

Radioautographs can be made when the activities are high enough.

$$n(\text{fast}) + P^{31} \xrightarrow{\text{capture}} Si^{31} + P \xrightarrow{t_{1/2} \ 2.8 \ hr.} \beta^- \ (1.8 \ \text{mev.}) + P^{31}$$
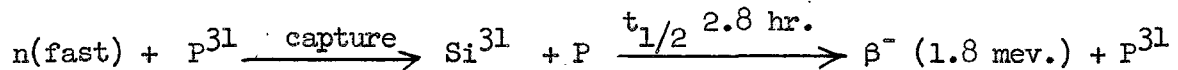
Inorganic substances in the original mixture are generally separated by paper

chromatography. Hence their induced radioactivity can be cut out to facilitate

counting of the desired P^{32} or S^{31} activity.

PROCEDURE FOR HYDROLYSIS OF PHOSPHATE ESTERS

Method for Elution from Paper Chromatograms

The areas of the paper chromatogram defined by the radiogram if radio-

active or by other means if not, can be eluted with water and used directly for

identification or chemical degradation. These are cut out as wedge-shaped

pieces with a sharp point for collecting the effluate and attached by the

straight end by capillarity to a wet filter-paper wick hanging over the edge

of a small water trough. An aquarium or other cover is used to prevent evapo-

ration while water flows through the cut-out spot and collects in a small cen-

trifuge tube touching the point of the paper. Elution of sugars and amino

acids is complete after 50-100 µl. are collected while the phosphate esters

may require 1-200 µl. for almost complete elution. The solution is readily

evaporated if necessary by forcing a nitrogen stream at its surface.

Method for Phosphatase Hydrolysis

To the eluted phosphate ester is added 100-200 µg. of "Polidase-S"

(Schwarz Laboratories, Inc., Mt. Vernon, N.Y.) and the solution covered by

a layer of toluene. It is incubated 1 to 3 days at 35° and rechromatographed

on Whatman No. 1 filter paper in phenol-water followed by butanol-propionic

acid-water.[6] Alternately, a large-scale method may be used where an equivalent

amount of phosphatase is sprayed onto both sides of the paper wedges and incu-

bated in a humid atmosphere for several days. The free sugars, now readily

elutable, are transferred directly to the origin of a chromatogram by simul-

taneous elution in a covered chamber above the sheet and drying with a warm air

stream under the sheet (Figure 1).[7]

It is to be noted that C^{14} radioactivity counted directly on Whatman No. 1

paper with a thin-window G-M tube is one third as great as one obtained by count-

ing the same sample in the same area on an aluminum plate.

CHROMATOGRAPHIC SOLVENTS

The solvent chosen should be determined by the nature of the mixture to

be separated and the impurities present. There appears to be no ideal formu-

lation for universal separation. Both acid and basic solvents have been suc-

cessfully used. There seems to be no particular advantage to those miscible

with water or those of the single-phase type. All of them use an aqueous com-

ponent, except a novel formamide solvent developed by Mortimer.[3]

Preequilibration of the paper in the solvent vapor is advisable in most

instances and generally requires 2-4 hours. The advisability of preequilibra-

tion is dependent upon solvent composition.

Ascending Chromatography of Phosphate Esters[4]

The concentrated sample of decationized (Dowex 50) phosphates is dried in

an 0.5 cm. diameter origin on a 28 cm. square of Schleicher and Schuell No. 589

blue ribbon filter paper. The paper is then stapled into a cylindrical form,

avoiding contact of the adjacent edges. A cylindrical jar 15 cm. in diameter

and 30 cm. tall, containing 100 ml. of the acid solvent and covered by a

glass plate serves as the chamber. The cylindrical paper with a stainless

wire through it is temporarily held to the glass cover by a strong permanent

magnet during the 2-hour equilibration period. The magnet is then removed and

the solvent development begun. After this the paper is dried and re-equilibrated

and developed in the alkaline solvent.

Acid Solvent. - Methanol 80 volumes, formic acid (88%) 15 volumes and

water, 5 volumes. Develops 28 cm. in 6 to 6.5 hours at 2° C.

Basic Solvent. - Methanol 60 volumes, ammonium hydroxide (sp. gr. 0.9015)

10 volumes and water 30 volumes. Develops 28 cm. in 12-15 hours at 2° C.

Separation of the glycolytic intermediates is shown in Figure 2. This

technique has found application in several laboratories. It was used for sepa-

ration of phosphorylated compounds formed by incorporation of radiophosphate

by pea seed meal and extracts after preliminary barium precipitation.[8]

Picric Acid Solvent (Modified Hanes-Isherwood solvent)

Picric acid is sufficiently strong to repress the ionization of phosphate

groups. The esters then have good R_f values and separate well in a solvent

containing less water than required to move ionic phosphates. Disadvantages

of the picric acid solvent are that it is sensitive to the degree of hydration

of the paper, the picric acid is non-volatile and must be removed before further

chemical operations, the yellow picric acid front moves slower than the butanol

front and monophosphates move as rapidly as the diphosphorylated compounds (i.e.

F-6-P vs. FDP).

Procedure: A solution of 2 g. picric acid in 80 ml. t-butanol and 20 g. water is used for descending chromatography on oxalic acid-washed Whatman No. 4 paper without preequilibration. Development of 55 cm. requires 20 hours whereupon inorganic phosphate has moved 35 cm.

Phenol Solvent

Phenol is distilled at atmospheric pressure into tared bottles and 0.39 parts of decationized water added to give a single phase. The solvent is stored in the dark at 5° C. to prevent oxidation. The impurities in the phenol distort the chromatography of hydroxy acids and sugar phosphates. Although the R_f values of sugar phosphates in this solvent are low the solvent may be allowed to flow past the serrated edge of the paper to achieve improved separations. Organic dyes (croceine scarlet, ponceau 4R, tropeolin 000) placed near the origin may be used to observe the rate of solvent flow.

R_f VALUES AND POSITION CONSTANTS OF PHOSPHATE ESTERS

The measurement and comparison of absolute R_f values is hardly practical for the identification of compounds in complex systems. Variations in paper hydration, atmosphere saturation and temperature all affect the absolute R_f values obtained. Only rarely, however, do they affect the relative positions of phosphorylated compounds. It is always better to compare the position of an unknown compound to that of a similar authentic material rather than to depend on R_f measurements. For this reason, Mortimer[3] has used the term "position constant" in which the position of a substance is defined relative to that of inorganic phosphate. It might be better to compare even more similar compounds, however, in order to establish more reliable comparisons.

Table I includes published data for position constants of a variety of compounds and conditions. It must be borne in mind that these values are useful but not unequivocal in identifying an unknown. Successful cochromatography

does not necessarily demonstrate identity of the two substances. Chemical evidence is always invaluable in identifying an unknown. One or more observation of chemical transformation is more likely to provide conclusive identification than would chromatography in several solvent systems. When the chemical properties of the suspected compound are known it is often possible to use a chromatographic identification for the product of the chemical transformation.

CHROMATOGRAPHIC SEPARATION OF 2- AND 3-PGA ON MOLYBDATE PAPER (R. W. Cowgill)

Several methods for separation of 2- and 3-PGA have been reported in the literature and found by other workers to have limitations. The method of Cowgill (unpublished method) is based upon the well-known interaction of 3-PGA and molybdate and has proved very successful.

Procedure: The mixture of acids (25-50 μg. each) is chromatographed on Whatman No. 52 or S and S 602 ED paper which has previously been wet with 0.5% sodium molybdate solution and dried. Whatman No. 1 paper may be used although the spots are not as well formed as with the denser papers. The solvent is 1 part 88-90% formic acid, 29 parts water and 70 parts 95% ethanol freshly prepared before use. After 20 hours descending development the paper is dried. 2-PGA is found to have traveled 2.5 times as far as the more strongly complexed 3-PGA and 0.48 times as far as inorganic phosphate.

Application of Borate Complex Formation

Certain mixtures of sugar phosphates are readily separable by virtue of their ability to complex with borate ion (cf. use of borate in column chromatography). The highly dissociated complexes, being more hydrophilic, have lower R_f values. Separation of the ribose phosphates in borate solvent has been reported by Cohen and Scott.[9] Extension of this method using dilute borate buffers should be helpful in separating mixtures where differences in borate complexing is indicated.

<u>Method</u>: A solution of 0.64% boric acid in 80% ethanol is used for descending development on Schleicher and Schuell No. 597 paper. Arabinose-5-P (R_f 0.25), xylose-5-P (R_f 0.25), ribose-3-P (R_f 0.19) and glyceraldehyde-3-P (R_f 0.19) migrate readily while ribose-5-P and hexose phosphates failed to move.

EXCHANGE RESIN CHROMATOGRAPHY OF SUGAR PHOSPHATES

INTRODUCTION

Strong base anion exchange resins have been effectively used in separating large quantities of phosphate esters, with good recovery and little hydrolysis of acid or alkali-labile compounds. Separation is effected by taking advantage of differences in affinity of the anionic phosphate and carboxyl groups for the resin in which the following equilibrium is established.

$$\left[\text{Resin-}\overset{+}{\text{N}}\text{R}_3\text{Cl}^-\right] + \text{Sugar-OPO}_3\text{H}^- \rightleftharpoons \left[\text{Resin-}\overset{+}{\text{N}}\text{R}_3\overset{-}{\text{H}}\text{O}_3\text{PO-sugar}\right] + \text{Cl}^-$$
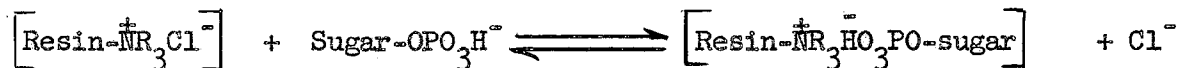
The equilibrium is, of course, dependent upon pH of the medium and acid strength of the weak acid involved.

One may compare the affinities of a variety of compounds for a unit mass of resin and predict relative elution rates. These can be determined by analysis of an equilibrated mixture of resin and adsorbate[10] where

$$\frac{\text{Fraction of solute on resin}}{\text{Fraction of solute in solution}} = \text{Constant}$$

The mobility of the sugar phosphate differs from that of chloride or other anion and as the eluting solution flows over the column the phosphate ester will be steadily displaced. Our understanding of the affinities of sugar phosphates for resins has been clarified by Khym, Doherty and Cohn[11] to a point where it may be possible to predict elution characteristics of known compounds. In fact, exchange resin elution characteristics have confirmed structure assignments for unknown esters.

Factors Affecting Acidity of Phosphate Groups

The sugar phosphates vary in affinity for anion resins for several reasons. The differences in acidity of sugar monophosphates arises by virtue

of possible internal hydrogen bonding of the phosphate group with adjacent

hydroxyl groups.[11,12] When there is a possibility of hydrogen bonding between

a phosphate -OH group and the ring oxygen (as in G-1-P or R-5-P and R-1-P)

the degree of dissociation of the phosphate hydrogen will be diminished,

especially when a 6-membered ring rather than a seven-membered ring results.

Those structures in which the phosphate oxygen can form a hydrogen bond with

an adjacent -OH group lead to increased dissociation. With two adjacent -OH

groups the acid strengthening effect is still more pronounced (i.e. ribo-

pryanose-3-P).
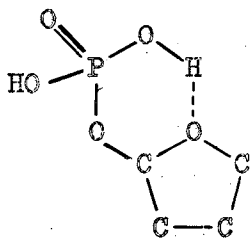
"Acid weakening" H-bonding   "Acid strengthening" H-bonding
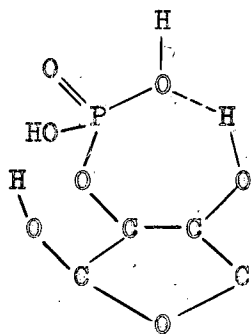
These predictions were borne out by ion exchange separations of the ribose

phosphates.[11]

The diphosphates are more strongly adsorbed on the anion resin. In order

to be moved on the column, both anionic groups must be displaced simultaneously

by the eluting agent. Therefore, a stronger or more concentrated eluting agent

is required to move the diphosphates. Phosphorylated carboxylic acids likewise

are difficultly eluted from the resin at pH values where the carboxyl group is

dissociated. They would be only difficultly separable from the diphosphates

were it not possible to repress the dissociation of the carboxyl group by

including a stronger acid in the eluting solution. In acid solution, then,

PGA or 6-P-G behave as if they were simple monophosphate esters.

Borate Complexing

The formation of borate complexes (reviewed by Böeseken[13]) by cis-hydroxyl groups was applied successfully by Khym and Cohn[14] for separation of monophosphate esters. Borate ion exists only in alkaline solutions where phosphate esters are completely dissociated and hence more strongly bound by the anion resin. Elution with borate ion alone involves excessive concentrations of eluant. While the borate form of an anion resin can be used,[15] the interference of the excess borate presents an unnecessary difficulty when phosphates are to be recovered. Low concentrations (10^{-2} to 10^{-5} $\underline{M}$) of borate are effective in complex formation[14] in sodium sulfate or ammonium chloride-ammonium hydroxide eluting solutions and hence affect the ion exchange affinities of the sugar phosphates. Furanose forms like F-6-P and R-5-P, on the other hand, exhibit very strong affinity for the exchanger with but small concentrations of borate ion (0.0005 $\underline{M}$). The dilute borate solution also allows a more effective recovery of the phosphate ester.[14]

Three generalizations concerning the reactions of borate with sugars have been set forth:[11] (1) cis-α-glycols are normally strongly complexed; (2) pyranose ring systems are not complexed compared to furanose systems; (3) the stronger the complex, the greater the ionization and affinity for anion exchangers. The efficacy of borate in separating a given group of phosphates may be deduced from these principles and is demonstrated in the accompanying examples. The pyranose structures of ribopyranose-2-P, ribopyranose-3-P and R-4-P are not rigid-ring forms and their vicinal hydroxyl groups are spread apart and are less readily complexed.[16,17] The strength of the borate complex is generally a function of the number of possible forms. When a cis-α-glycol system occurs only in an α or β form of the sugar, the borate complex is accordingly weaker, i.e. ribose-3-P.

Recovery of separated phosphates after borate elution. - Khym, Doherty and Cohn[11] removed borate from column eluates in the following manner: The eluate, containing sugar phosphate, sulfate and borate, is absorbed on a small anion column (IRA-400-acetate, 8 cm.^{2} x 4 cm.) after removal of sodium ion with cation resin and sulfate with excess barium acetate. The IRA-400-acetate column adsorbs sugar phosphates and inorganic phosphate but not borate because at pH 8.0-8.5 the phosphates are divalent and strongly bound while the high acetate concentration displaces the small amount of borate. The phosphates are then eluted with 125 ml. 1 $\underline{M}$ ammonium acetate, decationized with Dowex-50-H$^+$ and the acetic acid removed by evaporation in vacuo.

SEPARATION METHODS

Separation of G-1-P, G-6-P, F-6-P, R-5-P, F-1,6-diP, P$_i$, 2-PGA and the Adenosine Phosphates by Dilute Borate Elution (Khym and Cohn[14])

Separation of the monophosphates in this mixture requires the additional factor of borate complex formation. Adjustment of pH allows separation of the dibasic acids (ADP, 2-PGA) from the monobasic ones.

Ion Exchanger. - A 200-400 mesh trimethylammonium polystyrene resin (Dowex-1) is freed of fines by decantation of a stirred suspension or by passing a slow stream of water through a vertical column of resin suspension until the finer particles have passed out in the overflow. The resin is slurried into an 0.86 cm.^{2} x 12 cm. column. The resin is converted to the chloride form by washing with 1 $\underline{N}$ HCl and washed thoroughly with water to remove excess acid.

Preparation of Phosphates. - Dowex-50 in the acid form is prepared by acidifying a batch in a Büchner funnel with excess 1 $\underline{N}$ HCl and thoroughly washing during one to two hours with distilled water. Excess water is removed by suction and the damp resin may be stored for use. Solutions of phosphate

salts are treated with several batches of the acid Dowex-50 which is removed
by filtration or centrifugation. The usual capacity of Dowex-50 is 5-10 milli-
equivalents per gram of resin but an excess is advisable.

Procedure: The solution of acidic esters in dilute ammonia (pH 8.5) is
passed through the column at a rate of 3.5 ml./min. Free sugars are removed
with 100 ml. of 0.001 $\underline{M}$ ammonium hydroxide. A succession of eluting agents,
in the order described by the elution curve of Figure 3 is passed through
the column to selectively desorb the components of the mixture. Assay of
the effluant samples is described by the data of Table II.

Separation of the Ribose Phosphates

A classical example of the efficacy of the anion resin in separating
similar structures was reported by Khym, Doherty and Cohn.[11] They explain
the elution succession of the isomeric ribose monophosphates on the basis of
acidity of the phosphate groups in the five positions and the stability of
borate complexes of R-1-P, R-5-P and α and β forms of R-3-P and R-5-P.

Method: A pH 8.5 ammoniacal solution of 3-10 mg. each of the ribose
esters is absorbed on an 0.86 cm.^{2} x 12 cm. 300-mesh Dowex-1 sulfate column
and washed with water to remove non-anioniac materials. The column is eluted
successively with the solutions described in Figure 4 ca. at 3.5 ml./min. and
eluates analyzed by the orcinol method.[18]

It is seen that R-2-P and R-4-P are unaffected by Borate, that R-3-P
and R-5-P form complexes with R-5-P the more strongly affected. R-1-P is
strongly complexed by borate, but its phosphate acidity is diminished by
the hydrogen bonding effect.

Separation of Polybasic Esters, PGA, P-Glycolic Acid, FDP and Ribulose Diphos-
phate[15]

Principle. - Addition of dilute strong acid to the eluting agent represses
dissociation of the carboxylic acids and consequently their affinity for the
anion resin.

Materials chromatographed: a.   9.5 mg. barium 3-phosphoglycerate.

                              b.   10 mg. barium fructose diphosphate.

                              c.   250,000 cpm. ribulose diphosphate

                                   eluted from a stripe chromatogram of

                                   labeled photosynthetic products from

                                   Scenedesmus.

Eluting agent: 0.15 $\underline{N}$ NaCl + 0.05 $\underline{N}$ HCl

Eluting rate: 0.11 ml./mm.

Fraction volume: 0.4 ml. calculated from elution rate and time

                              interval of sample changer.

Procedure: A 0.6 cm. diameter x 28 cm. column of 200-400 mesh Dowex-2

anion resin (from which fines have been removed by decantation) is prepared

by pouring a slurry of the resin in distilled water into the column. After

the resin has settled evenly and most of the water has passed through the

column, more suspended resin is added. It should be emphasized that the

water level be never allowed to go below the resin level. It is not satisfac-

tory to apply suction to the bottom of the column as uneven packing and air

pockets may result.

The column is washed with excess 1 $\underline{N}$ HCl and then with distilled water

until the acidity is no longer detectable.

A solution of the two barium salts and the labeled ribulose diphosphate

in 50 ml. water was passed onto the column and thoroughly washed to remove the

cations. The eluting agent was passed through the column from a motor-driven

greased syringe through a syringe needle and rubber serum bottle stopper sealing

the top of the column.

The results of this separation are described by the curve of Figure 5a.

Radioactivity is determined by preparing "infinitely thin" plates on aluminum

discs from each fraction and counting under a thin-window GM tube. Phosphorus analysis of each fraction gave the block curve. It is seen that the added PGA and a certain amount of the radioactivity coincide and that there is a considerable peak of P-glycolic acid (identified by co-chromatography on paper) lying between the FDP and PGA peaks. Ribulose diphosphate is more strongly adsorbed on the resin than FDP.

Another experiment with no added carrier phosphates gave an almost identical elution curve (Figure 5b). There is apparently little distortion of the radioactivity elution curve by the added carriers. The quantity of labeled ribulose diphosphate added in this experiment was ca. 0.1 micromole. The amounts of PGA and P-glycolic, therefore, were much less. Even at this low concentration, very little irreversible adsorption is observed.

Isolation of Sedoheptulose Phosphate (Horecker, Smyrniotis and Klenow[19])

The mixture obtained from the action of transketolase upon 703 micromoles of R-5-P containing 235 micromoles of sedoheptulose-7-phosphate and residual pentose phosphates was chromatographed on a Dowex-1 (formate) column. The elution order, sedoheptulose-7-P: R-5-P: ribulose-5-P, may be explained by the acid weakening effect of the ring oxygen adjacent to the 7-phosphate group, the same effect in R-5-P and the acid strengthening effect of the adjacent -OH group of ribulose-5-P.

Procedure: A 13 cm. x 2.5 cm. diameter column of 300 mesh Dowex-1 (10% cross linked) is converted to the formate form by passing 2 $\underline{M}$ sodium formate through it until the effluate gives a negligible silver chloride precipitate upon addition of silver ion. After washing the column with water the equilibration mixture was placed on the column and the column again washed with 50 ml. water. The mixture was eluted with 0.2 $\underline{N}$ formic acid containing 0.03 $\underline{N}$

sodium formate at the rate of 2 ml./min. and 25 ml. effluate fractions collected. Heptulose assay by the orcinol method on 0.2 ml. samples showed that sedoheptulose phosphate emerged between fractions 24 and 29 while the pentose phosphates followed in fractions 35 to 50 which exhibited two peaks. The first at fraction 38 contained R-5-P while the last at fraction 46 contained ribulose-5-phosphate. The sedoheptulose-7-P in fractions 24-29 was adjusted to pH 6.2 with 5.0 ml. of 4 N NaOH and 21.0 ml. of saturated barium hydroxide solution. The precipitate obtained upon addition of four volumes of ethanol was collected, washed with 10 ml. of 80% ethanol and dried in vacuo. The yield was 117 mg. of barium sedoheptulose-7-P (73% pure).

"Gradient Elution" Technique

Elution peaks obtained in column chromatography often have satisfactorily sharp fronts but suffer from trailing. When this leads to overlapping of peaks it may be alleviated by an innovation in elution technique developed by Busch, Hurlbert and Potter[20] for separation of carboxylic acids on the anion resin.

To obtain a gradual increase in acidity, concentrated acid from a reservoir was passed into a mixing flask filled with distilled water and mixed constantly by a magnetic stirrer. The solution from the mixing flask passed into the 1 cm. diameter chromatographic column. This technique avoids the discontinuity of changing eluant solutions and produces sharp symmetrical elution peaks.

Method for Sedoheptulose Mono- and Diphosphates (Horecker, et al.[21]). - A transaldolase reaction mixture prepared from 250 micromoles sedoheptulose-7-P[32] and HDP is placed on a 11.5 cm. x 3.1 cm.[2] Dowex-1-formate column and eluted with 0.2 N formic acid containing 0.5 N sodium formate after it is passed through a stirred 400 ml. water reservoir at 1 ml./min. Fractions of 8 ml. were collected and analyzed with the orcinol reaction. Fractions 15-25 contained sedoheptulose-7-P and fractions 75-84 contained sedoheptulose di-P with very sharp peaks at 20 and 77 and negligible trailing.

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Table I-a

R_f VALUES AND POSITION CONSTANTS FOR
PHOSPHATE ESTERS IN CHROMATOGRAPHIC SOLVENTS

Solvent	Ethyl acetate 3, Acetic acid 3, Water 1		Methyl cellosolve 7, Methylethylketone 2, 3 N NH_4OH 3		Ethylacetate 1, Formamide 2, Pyridine 1	
Temperature	4°		26°		26°	
Paper	Whatman No. 1		Whatman No. 1		Whatman No. 1	
Reference	Mortimer 1952		Mortimer 1952		Mortimer 1952	
	a	b	a	b	a	b
Orthophosphate	33	100	21	100	50	100
Phosphoglycoladehyde	. .	. .	. .	. .	. .	. .
Phosphoglycolic acid	. .	. .	. .	. .	. .	. .
Glycerol-1-phosphate	26	79	39	192	54	114
Glyceraldehyde-3-phosphate	7	22	19	90	. .	. .
Dihydroxyacetone phosphate	. .	. .	. .	. .	. .	. .
2-Phosphoglyceric acid	27	81	41	200	23	47
3-Phosphoglyceric acid	23	71	22	116	28	57
2,3-Diphosphoglyceric acid	11	35	7	36	15	30
Phosphopyruvic acid	. .	. .	. .	. .	. .	. .
Phosphoerythronic acid	. .	. .	. .	. .	. .	. .
Ribose-1-phosphate	15	45	40	197	50	110
Ribose-5-phosphate	. .	. .	. .	. .	. .	. .
Ribulose-5-phosphate	. .	. .	. .	. .	. .	. .
Ribulose-1,5-diphosphate	. .	. .	. .	. .	. .	. .
Glucose-1-phosphate	14	37	36	170	44	89
Glucose-6-phosphate	12	29	29	140	50	100
Uridine diphosphate glucose	. .	. .	. .	. .	. .	. .
Glucose-1,2-cyclic phosphate	. .	. .	. .	. .	. .	. .
Glucose-1,6-diphosphate	. .	. .	. .	. .	. .	. .
Fructose-1-phosphate	. .	. .	. .	. .	. .	. .
Fructose-6-phosphate	17	48	36	171	54	108
Fructose-1,6-diphosphate	8	25	8	37	13	26
Galactose-1,2-cyclic phosphate	. .	. .	. .	. .	. .	. .
Mannose-6-phosphate	. .	. .	. .	. .	. .	. .
Mannoheptulose phosphate	. .	. .	. .	. .	. .	. .
Sedoheptulose-7-phosphate	. .	. .	. .	. .	. .	. .
Sedoheptulose diphosphate	. .	. .	. .	. .	. .	. .
Sucrose phosphate	. .	. .	. .	. .	. .	. .

(a) Numbers give R_f values in % of solvent travelled.
(b) Numbers are P-constants relative to orthophosphate.

Table I-b

Solvent	t-Butanol 80 Picric acid 2 g. Water 20	i-propylether 90, 90% formic acid 60	Phenol 72 g. Water 28	
Temperature	22°	20°	22°	
Paper	Whatman No. 1	Whatman No. 1	Whatman No. 4	
Reference	Wilson 1954	Hanes and Isherwood 1949		
	b	b	a	b
Orthophosphate	100	100	22	100
Phosphoglycolaldehyde	..	..	..	~170
Phosphoglycolic acid	106	..	23	102
Glycerol-1-phosphate	..	..	..	..
Glyceraldehyde-3-phosphate	..	..	..	..
Dihydroxyacetone phosphate	..	..	..	170
2-Phosphoglyceric acid	..	..	..	...
3-Phosphoglyceric acid	85	59	22	100
2,3-Diphosphoglyceric acid	..	..	..	..
Phosphopyruvic acid	120	..	26	110
Phosphoerythronic acid	..	..	..	74
Ribose-1-phosphate	..	..	..	..
Ribose-5-phosphate	..	..	..	139
Ribulose-5-phosphate	..	..	..	147
Ribulose-1,5-diphosphate	58	..	8	26
Glucose-1-phosphate	..	18	..	..
Glucose-6-phosphate	40	18	27	113
Uridine diphosphate glucose	dec.	..	26	111
Glucose-1,2-cyclic phosphate	54	..	..	170
Glucose-1,6-diphosphate	..	..	..	26
Fructose-1-phosphate	..	..	..	135
Fructose-6-phosphate	61	28	29	125
Fructose-1,6-diphosphate	..	15	..	26
Galactose-1,2-cyclic phosphate	45	..	..	170
Mannose-6-phosphate	52	..	29	125
Mannoheptulose phosphate	..	..	..	113
Sedoheptulose-7-phosphate	52	..	27	113
Sedoheptulose diphosphate	..	..	..	26
Sucrose phosphate	32	..	..	113

Table I-c

Solvent	Butanol 100, Propionic acid 50 Water 70	Methanol 80 88% Formic acid 15 Water 5		Methanol 60 28% NH$_4$OH 10 H$_2$O 30	
Temperature	22°	2°		2°	
Paper	Whatman No. 1	S and S No. 589		S and S No. 589	
Reference		Bandurski, Axelrod 1951		Bandurski, Axelrod 1951	
	b	a	b	a	b
Orthophosphate	100	63	100	28	100
Phosphoglycolaldehyde	~73	..	..	..	..
Phosphoglycolic acid	75	..	..	..	..
Glycerol-1-phosphate	..	..	..	..	..
Glyceraldehyde-3-phosphate	..	..	..	..	..
Dihydroxyacetone phosphate	59	..	..	..	..
2-Phosphoglyceric acid	..	46	13	18	64
3-Phosphoglyceric acid	65	50	79	35	125
2,3-Diphosphoglyceric acid	..	..	..	..	..
Phosphopyruvic acid	92	52	82	46	165
Phosphoerythronic acid	51	..	..	..	..
Ribose-1-phosphate	..	..	..	..	..
Ribose-5-phosphate	49	..	..	..	..
Ribulose-5-phosphate	53	..	..	..	..
Ribulose-1,5-diphosphate	22	..	..	..	..
Glucose-1-phosphate	..	27	43	60	215
Glucose-6-phosphate	40	38	60	48	170
Uridine diphosphate glucose	15	..	..	..	..
Glucose-1,2-cyclic phosphate	49	..	..	..	..
Glucose-1,6-diphosphate	22	..	..	..	..
Fructose-1-phosphate	46	..	..	..	..
Fructose-6-phosphate	46	34	54	44	156
Fructose-1,6-diphosphate	22	40	63	24	86
Galactose-1,2-cyclic phosphate	49	..	..	..	..
Mannose-6-phosphate	46	..	..	..	..
Mannoheptulose phosphate	40	..	..	..	..
Sedoheptulose-7-phosphate	40	..	..	..	..
Sedoheptulose diphosphate	22	..	..	..	..
Sucrose phosphate	40	..	..	..	..

Table II[a]

Analytical Data for Separation Demonstrated in
Figure 3

Compound	Assay method	Wave length used mμ.	Approx. amount added[b] mg.	Recovered %
Glucose	Anthrone	620	5	101
Glucose-1-PO_4	Anthrone	620	10	99
Glucose-6-PO_4	Anthrone	620	10	93
Fructose-6-PO_4	Anthrone	620	5	92
Fructose-1,6-$DiPO_4$	Anthrone	620	10	95
Inorg. $PO_4(K_2HPO_4)$	Phosphate	660	2	105
2-PGA	Phosphate	660	4	95
Ribose-5-PO_4	Orcinol	660	5	90
AMP	UV absorption	260	8	95
ADP	UV absorption	260	5	102
ATP	UV absorption	260	6	100

(a)   J. X. Khym and W. E. Cohn, J. Am. Chem. Soc., 75, 1153 (1953).

(b)   The mg. quantities given for the sugar phosphate represent the free sugar
content of these substances. The quantities given for inorganic phosphate
and 2-PGA are calculated as total phosphorus present. The amount of each
adenosine derivative was calculated from extinction coefficients.
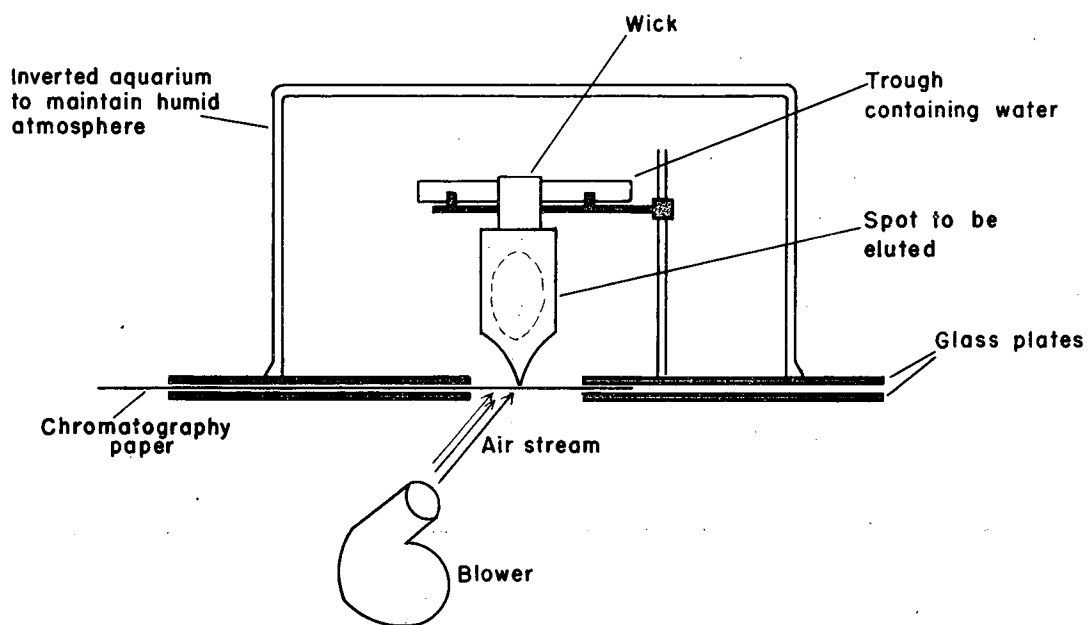
Fig. 1. Apparatus for transferring hydrolyzed chromatographed spot to
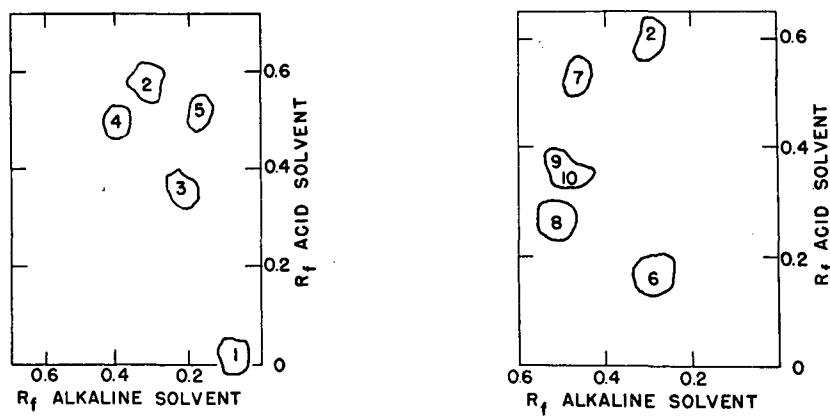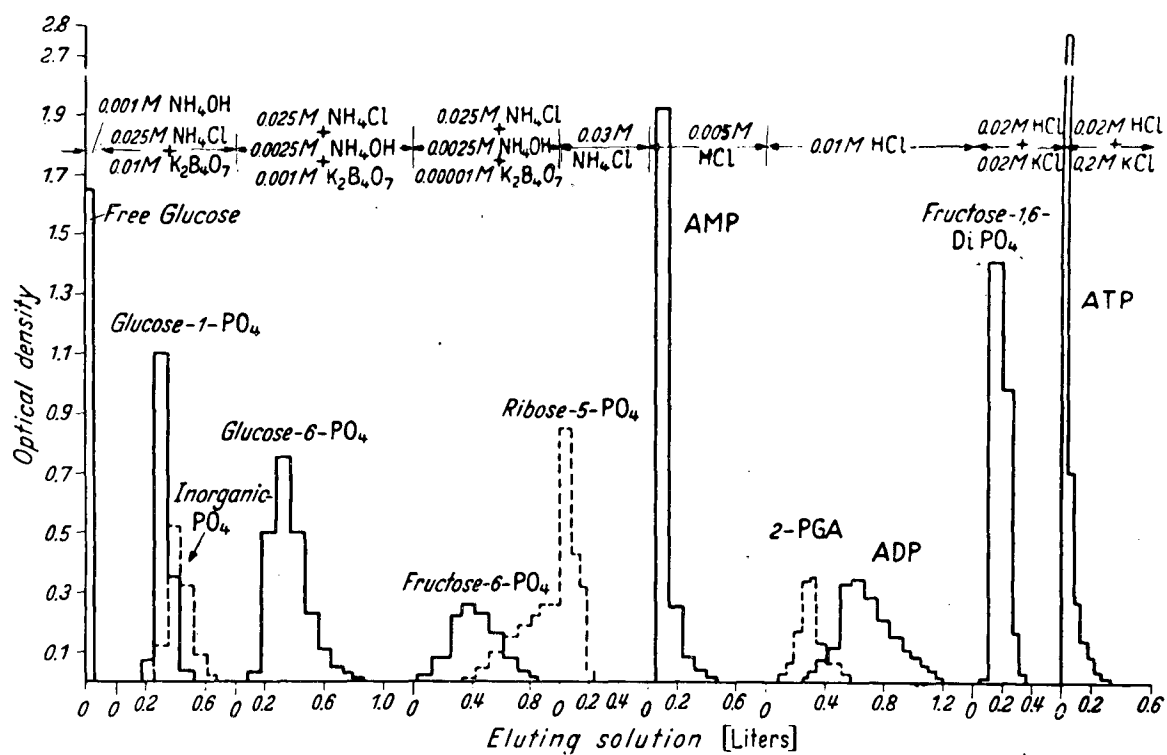   origin of a second sheet after phosphatase hydrolysis.[7]

Fig. 2. Two dimensional chromatograms of glycolytic intermediates.[4]

 A. "Barium insoluble fraction": 1, ATP; 2, orthophosphate; 3, FDP; 4, 3-PGA; 5, 2-PGA.

 B. "Barium soluble fraction": 2, orthophosphate; 6, adenosine-3-P; 7, P-pyruvate; 8, G-1-P; 9, F-6-P; 10, G-6-P.

Fig. 3.   Ion exchange separation of sugar phosphates, inorganic phosphate,
          adenosine phosphate and phosphoglyceric acid in the amounts given
          in Table II.

          Exchanger, Dowex-1 chloride, 300 mesh, 0.86 cm.^{2} x 10 cm.
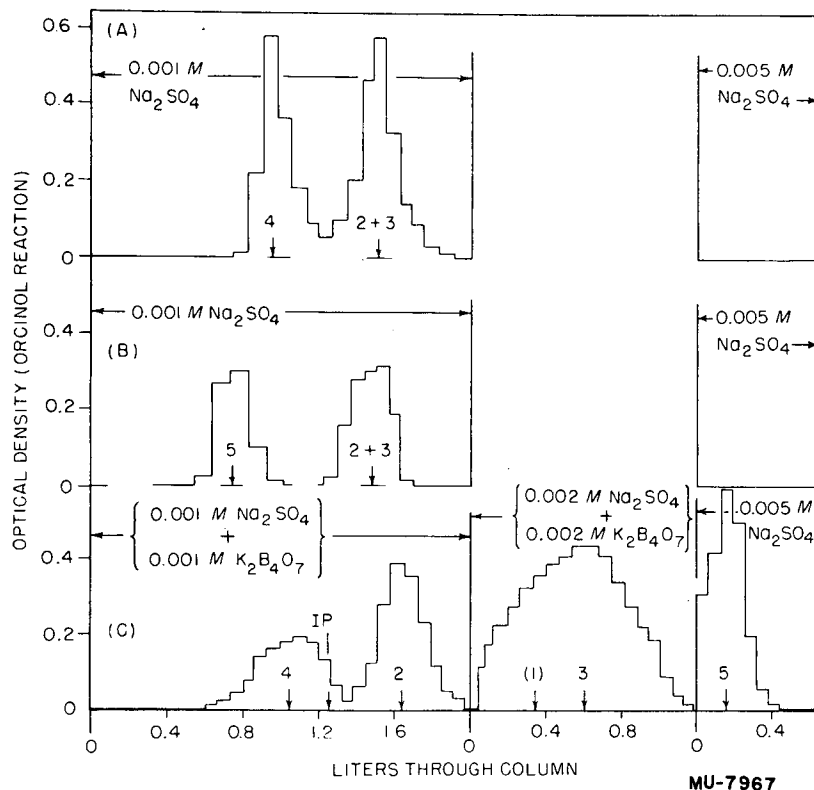   Flow rate 3.5 ml./min.[14]

Fig. 4. Ion exchange separation of ribose phosphates in the presence
and absence of borate (Khym, Doherty and Cohn[11]).

    A.   Hydrolysate of 60 mg. commercial adenylic acids heated
        with 6 ml. Dowex-50-H$^+$ in 6 ml. H_2O for 3 hours at 100°
        to yield ribose-2-, -3-, and -4-phosphates eluted with
        2 liters of 0.001 $\underline{M}$ Na_2SO_4 followed by 700 ml. of 0.005
        $\underline{M}$ Na_2SO_4.

    B.   8 mg. of ribose-5-phosphate plus 4 mg. each of ribose-2-
        and -3- phosphates eluted as in (A).

    C.   3-10 mg. each of ribose-2-, -3-, -4-, and -5- phosphates,
        eluted successively with 2 liters of 0.001 $\underline{M}$ Na_2SO_4 plus
        0.001 $\underline{M}$ $K_2B_4O_7$, 1.1 liters of 0.002 $\underline{M}$ Na_2SO_4 plus 0.002 $\underline{M}$
        $K_2B_4O_7$ and 0.005 $\underline{M}$ Na_2SO_4. (The probable position of the
        ribose-1-P peak, from a separate experiment is shown in
        parenthesis.)
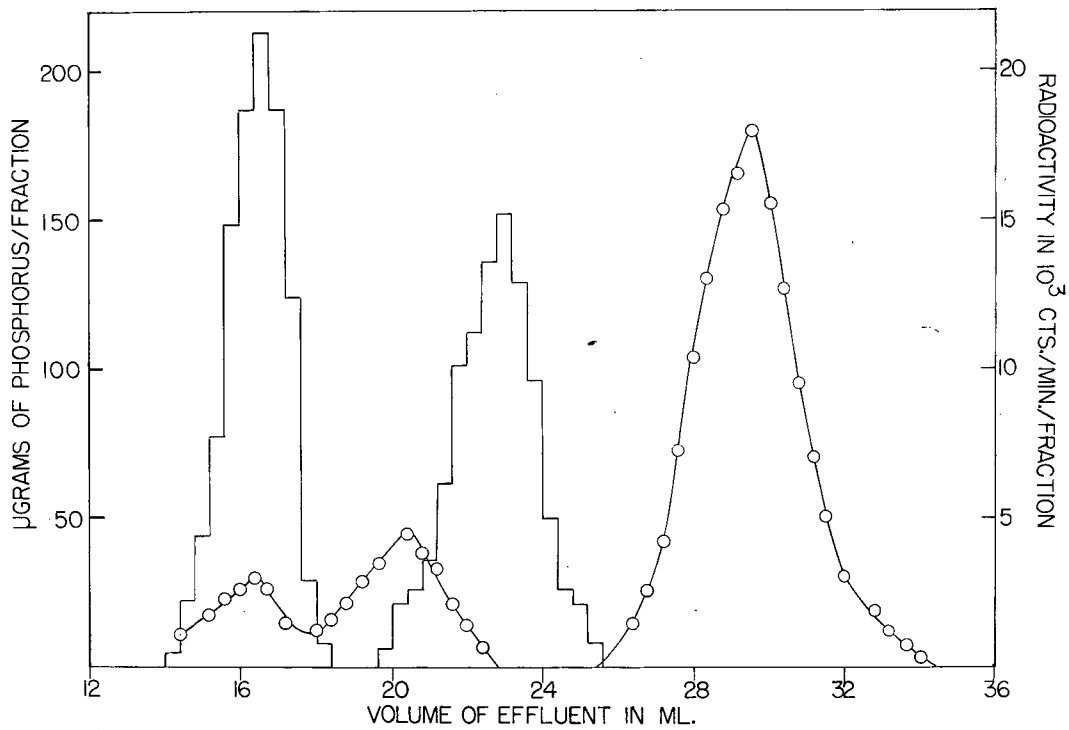
Fig. 5a.   Acid elution of phosphoglyceric acid, fructose diphosphate
           and radioactive ribulose diphosphate from a Dowex-2 chloride
           column (28 cm. x 0.6 cm.). Smooth curve denotes C^{14}. Block
           curve represents phosphorus analyses.

ELUTION OF DIPHOSPHATE RADIOACTIVITY

VOLUME OF EFFLUENT IN ML.
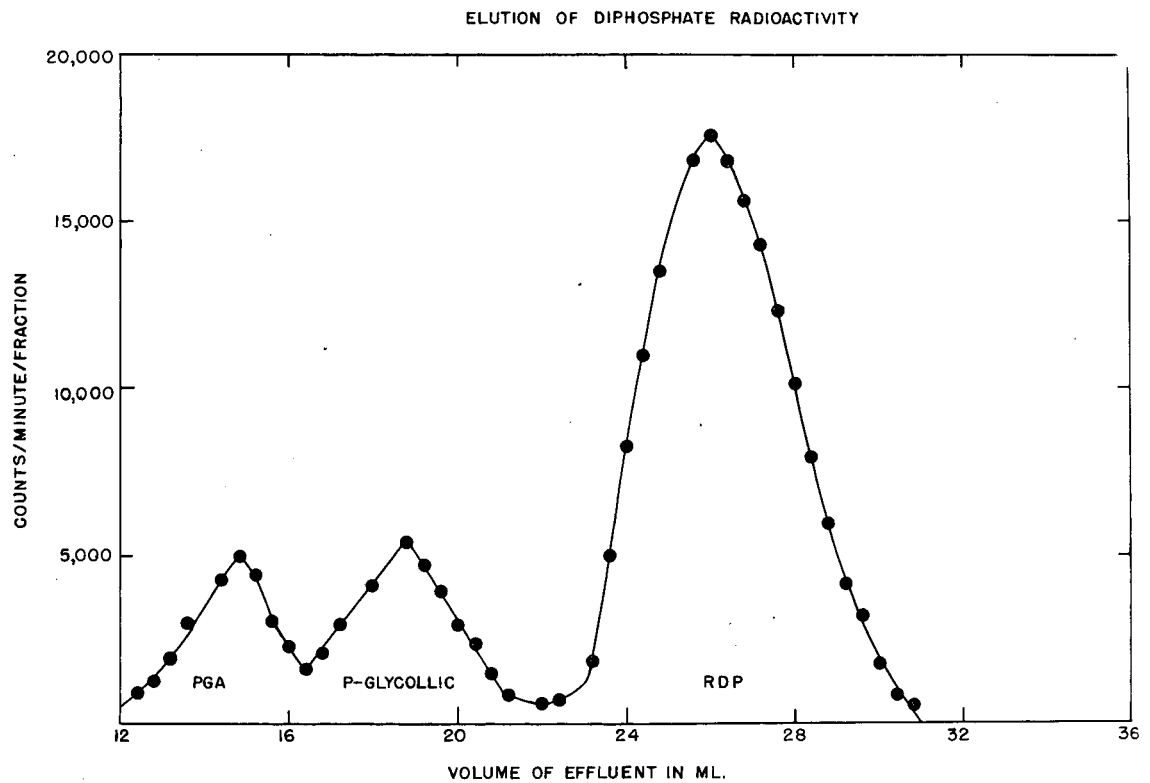
MU 4224

Fig. 5b.   Acid elution of radioactive ribulose diphosphate from a
Dowex-2 chloride column (28 x 0.6 cm.).[15]   Eluting agent:
0.15 $\underline{N}$ NaCl   0.05 $\underline{N}$ HCl; eluting rate 0.11 ml./min.;
fraction volume: 0.04 ml.