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I hereby recommend that the thesis prepared under my supervision by Robert H. Wagner

entitled The Isolation from Liver and the Proof of Structure of 2-Phospho-4-hydroxy-4-carboxyadipic acid.

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

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THE ISOLATION FROM LIVER AND THE PROOF OF STRUCTURE OF 2-PHOSPHO-4-HYDROXY-4-CARBOXYADIPIC ACID

A dissertation submitted to the

Graduate School of Arts and Sciences
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1951

by

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A. B. DePauw University 1943

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INTRODUCTION

In the course of a study (1,2,3,4,5) of the normal distribution of the acid-soluble phosphorus compounds in the liver and the changes in distribution of these compounds under different physiological conditions, a previously unknown nitrogen-free phosphorus compound was isolated from liver by Rapoport (6) and subsequently reported in a paper by Rapoport and Nelson (4) in 1945.

This compound, which although extremely labile in warm excised liver is very resistant to acid hydrolysis, accounts for a significant portion (5-10%) of all the difficultly hydrolyzable phosphate in the acid-soluble-phosphate fraction of liver. Its presence in the mercuric acetate precipitate of trichloroacetic extracts of both liver and kidney was indicated by ratios of difficultly hydrolyzable phosphate per purine N greater than the value of 1:5 which would be expected from nucleotides alone.

Preliminary analytical work (6,7) indicated that the compound was a phosphate ester of a di- or tri-hydroxy dicarboxylic acid. The presence of considerable amounts of such a compound in liver and kidneys raised questions as to its possible significance in intermediary metabolism.

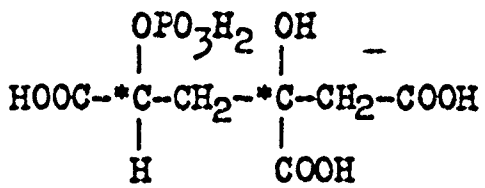
This research was undertaken with the following objectives:

1) To establish the structure of the compound.

2) To improve the tedious method of isolation and purification.

DESIGNATION OF STRUCTURE

As a result of the work presented in this paper, the structure of the new liver compound has been designated as 2-phospho-4-hydroxy-4-carboxyadipic acid,



In a preliminary report, however, by Rapoport and Wagner (8) evidence was presented which appeared to establish that the compound was α-phosphotrihydroxyglutaric acid. At that time, work was begun on the steric configuration of the compound. Four stereoisomeric forms of trihydroxyglutaric acid are possible and known. Two optically active antipodes are derived from d- and l-arabinose, respectively, and the two inactive forms from ribose and xylose. Only the i-ribotrihydroxyglutaric acid is known to give a lactone; the lactone is further characterized by its solubility in ethyl acetate (9).

Since there had been previous titrimetric evidence that a lactone could sometimes be formed from the phosphate-free compound, it appeared that preparation of the lactone in crystalline form with mixed melting points with the synthetic lactone would complete the proof of structure. Hydrolysis of the phosphate ester with hydrochloric acid

followed by removal of the interfering inorganic substances and extraction with ethyl acetate led to a product which when heated in solution required nearly twice as much alkali as when cold. Although partially crystallized products were obtained repeatedly, some gum formation always occurred. It was thought that the strong mineral acid was responsible for this difficulty. This supposition was supported by experiments on synthetic trihydroxyglutaric acid, which also became gummy and formed lactone crystals with difficulty after exposure to hot hydrochloric acid. However, although the use of gentle enzymatic hydrolysis with an alkaline phosphatase preparation* yielded a crystalline lactone, the product became gummy immediately upon exposure to air and it was not practical to take melting points.

Further, because of the recalcitrant behavior of the lactone, considerable doubt was cast on the conclusions drawn in the earlier proof of structure. Some of the earlier work, especially that of periodate oxidation, was repeated with conflicting findings.

As a result of a new investigation, the structure of 2-phospho-4-hydroxy-4-carboxyadipic acid was decided upon as most likely.

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* An alkaline phosphatase preparation from intestine was generously supplied to us by Dr. Gerhard Schmidt, Tufts College Medical School, Boston, Massachusetts.

ISOLATION

Since there were no methods for the determination of the unknown compound, the technique of isolation was based upon the elimination of other contaminants for which analytical methods existed and upon selective precipitation procedures.

Advantage was taken of the relative insolubility of the mercury and barium salts and the solubility of the silver salt. Final purification was achieved by repeated precipitation as the mercury salt.

The following were the steps in the initial isolation procedure: (1) The removal of the liver of a narcotized animal with rapid freezing in dry ice-ether mixture to minimize enzymatic breakdown, followed by extraction with trichloroacetic acid. (2) Precipitation of the trichloroacetic acid extract with mercuric acetate. The precipitate contained, in addition to the compound sought, nucleotides and glutathione. (3) Removal of mercury, preparation of the barium salts and their fractionation into soluble and insoluble fractions. The soluble fraction containing glutathione, adenylic acid, and a significant portion of the compound was discarded. To minimize losses the barium salts were prepared in a small volume. (4) Removal of the barium and glycogen. (5) Repeated fractionation with silver nitrate in 0.2 N HNO_3. The insoluble fractions containing

adenosine di- and triphosphate were discarded. All steps up to this point were carried out at 2-6° C. in order to minimize decomposition of the nucleotides. (6) Final fractionation involving a short hydrolysis at 100° C. in 1 N acid to hydrolyze remaining nucleotides. The compound, being hydrolized with difficulty, remained intact. (7) Removal of the inorganic P and repeated precipitations with mercuric acetate were carried out. After hydrolysis, ribose phosphate originating from the nucleotides was the main contaminant. The solubility of its mercury salt was the basis of the fractionation. By these procedures 0.3 to 0.8 mM (60 to 160 mg.) of compound of 85-90% purity could be isolated from a kilogram of liver.

Several changes have been made in the technique of isolation. The most important procedural change was made in step (5). This was the removal of about 98-99% of the nucleotides by a combination of 1) alcohol-acetone precipitation in a small volume, which removed some adenosine tri-, di-, and monophosphate (ATP, ADP, and AA) as well as a compound which we believe to be an isomeric ADP, 2) one or two-fold acid silver fractionation, and 3) repeated absorption of nucleotides on decolorizing charcoal. These modifications took less time and gave a purer product than the repeated acid-silver fractionations. The mixture was

brought to this step relatively free of ribose phosphate, thereby facilitating the removal of this contaminant by mercury fractionation.

Another improvement in the procedure was the removal of the inorganic P in two stages, 1) removal of the bulk of the inorganic P as ammonium phospho-molybdate, 2) quantitative removal of the remaining inorganic P as magnesium ammonium phosphate. This combination of steps largely eliminated the co-precipitation of the compound which occurs in the presence of large quantities of magnesium ammonium phosphate.

PROOF OF STRUCTURE

According to the evidence presented here the compound is one of the optical isomers of 2-phospho-4-hydroxy-4-carboxyadipic acid.

Elemental Analyses, Equivalent Weight, Molecular Weight, Titrations.

Elemental analysis of the barium salt (prepared by Dr. Rapoport) of the phosphate-free compound and the analysis of the silver salt, after correction for excess Ag as Ag_2O, were in good agreement with the formula: $C_7H_7O_8M_3$.

This evidence for the presence of three carboxyls per molecule was further sustained by titrations of the phosphate-containing compound which showed 4.46-5.0 equivalents of H^+ per mole of P.

Equivalent and molecular weights were close to the theoretical values calculated.

(Calculated from the Formula: $C_7H_{10}O_8$)	Calcd.	Found
Equivalent weight of the phosphate-free compound	74	71.1-78.5
Molecular weight of the phosphate-free lactone	204	201 ± 18
Equivalent weight of the phosphate-free lactone	68	68
Equivalent weight of the phosphate ester	60.4	66-59

Analytical Work.

A. The compound was easily oxidized with dilute permanganate solution at acid reaction, with the formation of 2 moles of carbon dioxide.

The phosphate group was hydrolyzed to the extent of about 4% under these conditions. The product gave a good test for a carbonyl group. This indicated the presence in the original compound of another hydroxy group besides the hydroxy esterified with the phosphate.

On further oxidation at 70-90° C., 1.3 moles of carbon dioxide were evolved and 45% of the P was hydrolyzed. These facts indicate that the phosphate is located on a carbon adjacent to a terminal carboxyl.

B. Oxidation of the phosphate-free compound with permanganate or dichromate at 100° C. in the presence of $HgSO_4$ and H_2SO_4 gave a characteristic precipitate which was collected and identified as the Deniges' acetone complex by various tests. This indicated the presence of an acetone nucleus in the compound.

C. Acid permanganate-bromine oxidation of the phosphate-free compound at 7° C. yielded 3.94 CO_2 per mole and pentabromoacetone, identified by several color tests and by mixed melting points. Under the same conditions citric acid gave pentabromoacetone and 2.8 CO_2.

The formation of pentabromoacetone is supposedly quite specific, from acetone dicarboxylic acid or from citric acid by way of acetone dicarboxylic acid (10,11); its formation from our compound indicates a structure which is capable of forming acetone dicarboxylic acid in the process of oxidation.

D. In the consideration of the various possible seven-carbon isomers having three carboxyls and two hydroxyl groups, various compounds of the form $R-\underset{\underset{OH}{|}}{\overset{\overset{COOH}{|}}{C}}-COOH$ and $R'-\underset{\underset{H}{|}}{\overset{\overset{COOH}{|}}{C}}-COOH$ were considered. On heating, such compounds readily split off CO_2. For example, desoxalic acid, $HOOC\cdot CHOH\cdot C(OH)(COOH)_2$ evolves CO_2 when its aqueous solution is evaporated (12).

A similar compound, corresponding more closely to the one which we are considering is α,γ-dihydroxypropane-α,α,γ-tricarboxylic acid, $HOOC\cdot CHOH\cdot CH_2\cdot C(OH)(COOH)_2$ (12), which loses CO_2 at 100° C.

Compounds of the form $R\cdot CH(COOH)_2$ resemble substituted malonic acids and readily eliminate CO_2 on heating (13), e.g., isobutane-α,α,β-tricarboxylic acid, $(CH_3)_2\underset{\underset{CH(COOH)_2}{|}}{C}-COOH$ loses CO_2 to form as-dimethylsuccinic acid (13).

When the phosphate-free compound was heated <u>in vacuo</u> to 160° C., it lost no CO_2, therefore these isomeric forms

have been ruled out as most unlikely.

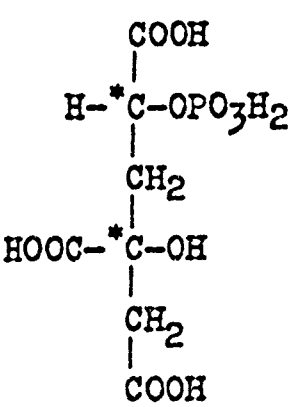
Conclusion.

The only form which appeared to fit with all the experimental evidence was 2,4-dihydroxy-4-carboxyadipic acid,

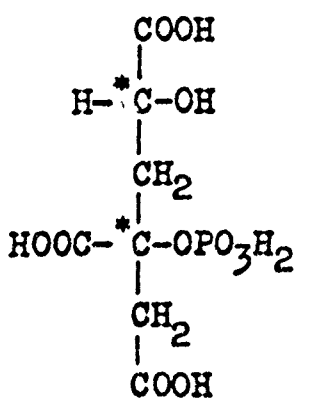
$$\underset{1}{HOOC}\cdot\overset{*}{\underset{2}{C}}(HOH)\cdot\underset{3}{CH_2}\cdot\overset{*}{\underset{4}{C}}(OH)(COOH)\cdot\underset{5}{CH_2}\cdot\underset{6}{COOH}$$

I

The position of the phosphate has been designated as on the 2-carbon, II, since it did not appear likely that III would yield on mild permanganate oxidation 2 moles of CO_2 and a product, containing the phosphate and a carbonyl group, which was relatively stable to further oxidation.



II



III

The elucidation of the steric configuration appeared to be a very long and difficult undertaking and was not attempted.

CHARACTERISTICS OF THE PHOSPHATE COMPOUND

1. The compound displays slight optical activity.

$[M]^{20^\circ}_{5461\ A^\circ} = -3.0^\circ \pm 0.4^\circ$

2. The phosphate is very stable to acid hydrolysis; 100% hydrolysis is approached with 6 N sulfuric acid at 100° C. for 55 hours. (See Graph #7.)

3. The compound gives a strong iodoform reaction, a behavior consistent with the presence of active hydrogen atoms.

CHARACTERISTICS OF THE PHOSPHATE-FREE COMPOUND

1) In the presence of ammonium molybdate, the optical rotation of the phosphate-free compound is enormously enhanced, a behavior typical of α-hydroxy acids (14).

2) Evidence for the existence of a mono-lactone is clear cut. There is some reason to believe that a di-lactone may be formed which is very unstable.

3) The phosphate-free compound gave a cherry-red color with the Fürth-Herrmann pyridine-acetic anhydride test (15), indicating a close relationship with citric acid.

Only citric acid has been known to give this cherry-red color test, previously. Fürth and Herrmann found that aconitic acid gives a violet-red color. Tricarballylic acid forms a blue-red color, while malic, fumaric, maleic, citraconic, and itaconic acids give a brown color. Tartaric acid forms an emerald green color and malonic acid gives a mahogany color which turns to a green fluorescing solution on dilution with acetic anhydride. Mucic, glutamic, succinic, aspartic, β-hydroxybutyric, pyruvic, glycolic, and oxalic acids, as well as glucose and glycerol give no color at all (15).

4) The ultraviolet absorption curve at a pH of 11.5 or 1.3 was similar to that which might be expected (16,17,18).

5) The phosphate-free compound gave no test for the formation of an oxime or a dinitrophenylhydrazone.

6) There was no bromine addition in acid solution. This showed the absence of a double bond.

DISCUSSION

From the work presented in this paper, it is evident that two objectives have been attained: the probable structure of the compound has been established as 2-phospho-4-hydroxy-4-carboxyadipic acid, and the method of isolation and purification has been greatly improved.

Limitations of time have prevented the study of some problems which have arisen:

1) The development of an analytical method for the determination of the compound in crude tissue extracts.

2) The determination of how the compound is broken down so readily by freshly excised liver and kidney tissues.

3) The elucidation of the role which the compound plays in intermediary metabolism.

From a preliminary experiment* in which C^{14} was injected into a rabbit as $NaHC^{14}O_3$ and the phosphate compound, glycogen, and a mixture of nucleotides were isolated from the liver of the rabbit, it was found that the phosphate compound had a high activity compared with the other two fractions. It is not known whether carbon dioxide fixation occurred with a six-carbon parent compound or whether a 2-, 3-, 4-,

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* A sample of $NaHC^{14}O_3$ was generously supplied to us by Dr. Harland G. Wood, Dept. of Biochemistry, Western Reserve University School of Medicine, Cleveland, Ohio, with the permission of the United States Atomic Energy Commission. The counts were performed for us by Dr. Wood.

5-carbon parent was formed by carbon dioxide fixation and followed by a condensation reaction (19). The possibility also exists that the compound is a breakdown product of a larger compound.

EXPERIMENTAL PROCEDURES

Analytical Methods

Method for Determination of Compound.

Since the compound was a difficultly hydrolyzable monophosphoric ester, the non-nucleotide, difficultly hydrolyzable phosphorus gave a measure of the maximum amount of compound that could be present in a given fraction, i.e., Compound = Total P - 15 minute hydrolyzable P - stable purine P.

After removal of the last traces of nucleotide, by acid hydrolysis and mercury fractionation, the compound was assayed as follows: Compound = Total P - 15 minute hydrolyzable P - pentose P.

Total Phosphorus.

After ashing, total P was determined colorimetrically in the Coleman Junior Spectrophotometer by a slightly modified method of Fiske and Subbarow (20).

Fifteen Minute Hydrolyzable P.

The P hydrolyzed in 1 N H_2SO_4 in fifteen minutes in a boiling water bath was determined colorimetrically as above.

Stable Purine P.

Stable purine P was determined roughly by reading the ultraviolet absorption of a dilute solution in a silica cuvette against a water blank. Readings were taken at wavelengths of 260 and 300 mμ in the Beckman Quartz Spectrophotometer. From the reading and the extinction coefficient of adenylic acid, the stable purine P was calculated on the basis of one stable P per purine.

The molecular extinction coefficient, at the peak wavelength of 260 mμ, was determined very carefully on the basis of ultraviolet absorption and sample weight, for a pure sample of myoadenylic acid*. $e_{260} = 14.78 \times 10^3$/mole/liter. The absorption at 300 mμ was, as nearly as could be determined, the same as water. For a sample of Bischoff and Co. adenylic acid, lot #418, $e = 14.25 \times 10^3$. These values compare with those reported in the literature: 15.8×10^3 (22); 12.9×10^3 (in 0.05 N HCl); 14.0×10^3 (in 0.05 N NaOH) (23).

It is of interest to note that the molecular extinction coefficient for myoadenylic acid in 0.0167 N HCl read against a blank of 0.0167 N HCl = 14.4×10^3/mole/liter. The myoadenylic acid in 0.0167 N NaOH read against a 0.0167

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* Prepared by Rapoport after the method of Kerr (21).

N NaOH blank had a molecular extinction coefficient of 14.9×10^3/mole/liter.

Pentose Phosphorus.

Since it was impossible, by virtue of the precipitation procedures, to have free pentose present in the mixtures, the measure of the pentose was actually a measure of the pentose phosphate present.

Pentose was determined by adapting the slightly modified (5) method of Mejbaum (24) to the Coleman Junior Spectrophotometer. Samples were read at 670 mμ instead of 660 mμ. Readings were also taken at 520 mμ. The reading at 670 should be at least twice as great as the reading at 520 if the color is pure. Adenylic acid was used as the pentose standard. It yields about 20% more color than arabinose (5).

Typical Isolation Experiment

Removal and Freezing of the Liver.

Since separation of the compound from ribose phosphate is very difficult, the procedure through the separation of the nucleotides was carried out in the cold room at 0-5° C., or, when that was not feasible, in ice-cooled containers, in order to prevent liberation of ribose phosphate by hydrolysis of the nucleotides.

The removal of the liver required two workers for convenience. A rabbit was deeply anesthetized by the injection of 2-3 cc. of veterinary Nembutal in an ear vein which had been prepared for the entry of a #22 needle by previous wetting of the ear with warm water and gentle scraping with a scalpel blade. The liver was rapidly removed and the attached gall bladder was quickly cut away. The liver was then cut into small pieces and dropped into a dry ice-ether mixture kept in large crocks. The livers (1050 g.) of eleven rabbits were used in this preparation. No attempt was made to exsanguinate the livers as it was necessary to work very rapidly at this point to minimize the enzymatic breakdown of the compound which occurs before freezing and deproteinization.

Trichloroacetic Acid (TCA) Extraction.

TCA extraction precipitates the liver proteins and leaves behind the lipids. Amino acids, sugars, glycogen, some polypeptides, and the acid-soluble phosphorus compounds are extracted.

One hundred-gram portions of the frozen liver were homogenized in a Waring Blendor with 400 cc. of 5% trichloroacetic acid. After the homogenate was centrifuged in 250 cc. centrifuge bottles, the supernate was decanted and filtered into a large measuring cylinder. The filtrate, measuring 3,890 cc., displayed an opalescence caused by the presence of glycogen, and a strong yellow color from the flavins.

TCA Wash of Precipitate.

The precipitate remaining from the centrifugations was mixed in a crock with an equal volume of 2.5% TCA and then centrifuged. This wash supernate was also decanted and filtered into a separate graduated cylinder and measured as part of the check of the volume recovery. The table below illustrates a satisfactory recovery.

Amounts used		Recovery	
1050	g. liver	3,890	cc. 5% TCA Filtrate
4200	cc. 5% TCA	965	cc. 2.5% TCA Wash Filtrate
820	cc. 2.5 TCA Wash	920	cc. TCA Precipitate
6070	cc. total used	5,775	cc. recovered

Mercury Precipitation.

This procedure precipitates the compound, flavins, some amino acids, probably tyrosine, tryptophan, and glutamic acid, nuclectides, and some of the glycogen. Alpha and β-phosphoglycerol, phosphorylated sugars, and some little investigated phosphorus compounds are left in the mercury supernate.

After the addition of cracked ice, the combined TCA filtrates were neutralized to pink with phenol red with 10 N NaOH (97 cc.). One hundred and fifty-six cc., four per cent by volume of the 5% TCA filtrate, of 20% mercuric acetate in 2% acetic acid was added, and the flocculant precipitate was left to settle overnight.

Removal of the Mercury.

After the supernate was siphoned off, the mercury precipitate was collected in two 250 cc. centrifuge bottles and the precipitate in each bottle was washed with 50 cc. of a 1-40 dilution of the acidic mercuric acetate reagent.

It should be emphasized at this point that to a large degree the success of the subsequent fractionation procedures depends upon keeping both the volumes and the salt concentrations as low as possible.

The precipitate in each bottle was broken up by adding 25 cc. of water and by very vigorous shaking of the rubber

stoppered bottle. Hydrogen sulfide was bubbled into the mixture for about an hour to precipitate the mercury as HgS. The supernatant fluid was collected by centrifuging and decanting. The HgS was treated with water and H_2S two more times for complete extraction, and after the combined extracts (260 cc.) were aerated to remove H_2S an aliquot was removed and frozen for subsequent analysis.

Analysis:

Total P	15 min. P	Difficultly Hydrolyzable P	Purine
11.6 mM	5.0 mM	6.6 mM	5.0 mM

The maximum amount of compound that could be present, then = 11.6 - 5.0 - 5.0 = 1.6 mM.

The maximum purity at this point would be $\frac{1.6}{6.6}$ = 24%.

Barium Precipitation.

This barium step precipitates a large part of the compound, the nucleotides, and some glycogen. Most of the glutathione, most of the adenylic acid, amino acids, and a significant portion of the compound are left in the supernate, but the labor required to recover the compound is prohibitive.

Ten per cent by volume (26 cc.) of 50% barium acetate was added. The mixture was neutralized to pink with phenolphthalein with 10 N NaOH (8 cc.) and finally 20% by volume

(52 cc.) of ethanol was added. The precipitate was allowed to settle overnight.

Reprecipitation of Barium Salts.

This step is performed to lower the salt concentration.

The barium precipitate was collected and partially redissolved by shaking with 6 cc. of 1 N HNO_3 and 10 cc. of water. The fraction was then reprecipitated by adding 0.5 cc. of 50% barium acetate, adding 10 N sodium hydroxide to pink with phenolphthalein, and 3.5 cc. of ethanol. After being allowed to settle for one-half hour, the precipitate was collected by prolonged centrifugation.

Removal of Barium and Glycogen.

The precipitate was dissolved by shaking with 6 cc. of 1 N nitric acid, 7 cc. of water, and 3.5 cc. of 10 N sulfuric acid. After a centrifugation, the supernate gave a negative test for Ba^{++}. The $BaSO_4$, Ba precipitate was extracted four more times with a mixture of 9 cc. of water, 2 cc. of 1 N nitric acid and a drop of 10 N sulfuric acid. When the fourth extraction was roughly analyzed for nucleotides to test the completeness of the extraction, 0.3 mM of nucleotides was found. As the fifth extract contained only 0.1 mM of purine, it was discarded.

Glycogen was then removed from the first four combined extracts (93 cc.) by the addition of ethanol (45 cc.) until the glycogen flocculated. The mixture was centrifuged quickly (4 minutes) and decanted. The glycogen was dissolved in 5 cc. of water and reprecipitated by the addition of 2.5 cc. of ethanol. The glycogen precipitate was discarded.

Preliminary Removal of Nucleotides.

At this point, the volume of the combined supernates from the glycogen precipitations was 98 cc. A yellow and white semi-crystalline precipitate had begun to form in the supernate. This material, which has been the subject of a separate investigation, was removed by the following steps: (1) Addition of 50 cc. more of absolute ethanol to make the solution 50% alcoholic, forty minutes' standing, and addition of another volume of ethanol to make the alcoholic concentration 66%. At about this point the precipitate flocculates. (2) After one-half hour standing, slow addition of one volume of acetone with only slight additional precipitation occurring. After longer standing, one more volume of acetone was added and the precipitate was allowed to settle overnight. (3) Collection of the precipitate by centrifuging and washing once with a minimal amount of a 1-2-1 mixture of water, ethanol, and acetone. The wash was added to the

supernate. The precipitate was washed with ether and stored in the deep freeze for further investigation. Analysis of this precipitate gave the following results:

Total P	9.55 mM
15 min. Hyd. P	5.06 mM
Diff. Hyd. P	4.49 mM
Purine	4.05 mM
Diff. Hyd. P - Purine	0.45 mM

$$\frac{\text{Diff. Hyd. P - Purine}}{\text{Diff. Hyd. P}} = 0.1$$

Acid-Silver Fractionation.

This process takes advantage of the relative insolubility of the silver salts of the nucleotides in nitric acid solution and the solubility of the compound under these conditions.

For the successful acid-silver fractionation of the supernate, the acetone and ethanol were removed by vacuum distillation using a dry ice-ether trap and the aqueous phase was reduced to 41 cc. The solution contained about 12 m.eq. nitric acid and 7 m.eq. sulfuric acid.

One-tenth volume of 80% silver nitrate (5 N) was added to the acidic solution; the white precipitate of nucleotides which formed immediately was allowed to settle for five minutes and was then collected by centrifugation. The silver precipitate (Ag_I) was washed with a little acidic silver nitrate containing 15% ethanol. The supernatant liquid was

neutralized to purple with bromocresol purple at pH 6 and precipitated by the addition of three volumes of alcohol. This fraction (Ag_{II}) was collected by centrifugation and washed with alcoholic acidic silver nitrate.

The Ag_I and Ag_{II} fractions were freed of silver with H_2S, aerated, and analyzed.

	Total P mM	15 min. P mM	Diff. Hyd.P mM	Purine mM	Diff. Hyd. P - Purine mM	Diff. Hyd. P - Purine / Diff. Hyd. P %
Ag_I	0.532	0.178	0.354	0.263	0.091	25.7
Ag_{II}	1.36	0.55	0.81	0.230	0.58	72

It is evident from the analyses that Ag_{II} contains relatively little nucleotides. The supernate from Ag_{II} contained little phosphorus, all of which was easily hydrolyzable, so it was discarded.

The Ag_I fraction may be subjected to one or more acid silver fractionations such as those just described to give a product of greater purity. In this preparation several corresponding fractions from earlier preparations (EP) were combined and purified in this manner and added to the Ag_{II} fraction of this preparation.

	Total P mM	15 min. P mM	Diff. Hyd.P mM	Purine mM		Diff. Hyd. P - Purine mM	Diff. Hyd. P - Purine / Diff. Hyd. P %
EP	6.07	4.90	1.17	0.50		0.67	57
Ag_{II}	1.36	0.55	0.81	0.23	-	0.58	72
	7.43	5.45	1.98	0.73		1.25	63

Charcoal Removal of Nucleotides.

The combined fractions had a volume of 87 cc. and contained 0.73 mM of purine. One-half gram of decolorizing charcoal (Darco) was added with stirring, the charcoal was removed by centrifugation and after the procedure was repeated with fresh charcoal the supernate was analyzed for purine. This procedure was repeated until the purine phosphorus was only 1% of the difficultly hydrolyzable phosphorus.

Initial value of purine	0.73 mM
After charcoal treatment #2	0.63
After charcoal treatment #5	0.30
After charcoal treatment #7	0.123
After charcoal treatment #10 and washing of the charcoal residues with water	0.013 mM

Earlier tests had shown that only a negligible loss of the unknown compound occurred with this charcoal treatment up to the point where only traces of purine remained. Removal of these traces with charcoal then became a costly process.

Removal of Inorganic Phosphorus.

Since there were very large amounts of inorganic phosphorus present in the supernatant liquid, initial removal

of about 80% of the inorganic phosphorus was achieved by careful precipitation as ammonium phosphomolybdate (unpublished method of E. Leva) avoiding an excess of ammonium molybdate.

To the 98 cc. of solution containing the desired compound, inorganic phosphorus, and very little nucleotide, was added 12 cc. concentrated nitric acid and some crystals of ammonium phosphomolybdate to aid crystallization. The mixture was warmed to 40° C. and enough (125 cc.) molybdate reagent (90 g. of $(NH_4)_2MoO_4$, 100 cc. of 6 N NH_4OH, 240 g. of NH_4NO_3 dissolved and diluted to one liter) was added slowly over a period of several hours to precipitate about 80% of the inorganic phosphorus. After the precipitate had formed and settled, it was filtered off and the precipitate was washed twice with 10 cc. of 5% ammonium nitrate.

The supernate, which was now rich in inorganic salts was neutralized to phenolphthalein with 10 cc. of 10 N sodium hydroxide. To remove the remaining inorganic phosphorus, 2 cc. of 50% magnesium acetate and 3 cc. of concentrated ammonium hydroxide were added. After the precipitate of $MgNH_4PO_4$ was allowed to crystallize overnight, it was

removed and washed, and the supernate was analyzed*:
TP = 0.735 mM, 15 min. P = 0.107 mM.

Collection of Compound as a Lead Salt.

The 250 cc. of supernate, containing the desired compound, ribose phosphate, a trace of nucleotides and salts, was neutralized to pH 6-7 with acetic acid using Acutint papers.

A lead precipitate was obtained by the addition of 9 cc. of 40% lead acetate and 3 volumes of alcohol. The lead precipitate was collected and washed five times with 10 cc. of 50% ethanol to remove as much of the soluble salts as possible.

Hydrolysis of Residual 15 Minute Hydrolyzable P and Removal of Lead.

Eleven cc. of 2 N HNO_3 was used to dissolve the bulky lead precipitate. This mixture was heated in a boiling water bath for thirty minutes to hydrolyze the remaining 15 minute hydrolyzable P. After cooling, the solution was diluted with 10 cc. of water and saturated with H_2S to

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* The apparent loss of non-purine difficultly hydrolyzable phosphorus incurred in the removal of the nucleotides and inorganic P was not too serious. Since the non-purine difficultly hydrolyzable P had been initially determined by difference, an error of a few per cent in the value of total and/or easily hydrolyzable P analysis could account for this apparent loss.

precipitate the lead. The lead sulfide was removed, shaken with 10 cc. of water and 1 cc. of 2 N HNO_3 and treated with H_2S. The lead sulfide was again separated by centrifugation and the procedure was repeated.

Removal of Inorganic Phosphorus.

After the H_2S was removed by aeration, the solution was neutralized to phenolphthalein with a little 10 N sodium hydroxide. The solution was diluted to about 100 cc. One cc. of 50% magnesium acetate and one cc. of concentrated ammonia were added. After the mixture was let stand overnight, the magnesium ammonium phosphate was removed.

Collection of Lead Salt.

Since it is essential to have a low volume of salt-poor solution for the mercury fractionation, a lead collection step was interposed at this point.

The desired compound was precipitated with lead acetate using 3 volumes of alcohol. The lead precipitate was collected, the lead was removed with H_2S, and the H_2S was removed by aeration. The volume of lead decomposate equaled 23 cc.

Analysis at this point showed 0.45 mM of phosphorus, of which 0.063 mM was ribose phosphate.

Mercury Fractionation.

This step is taken to remove the more soluble mercury salt of ribose phosphate.

The solution was neutralized to pink with phenol red with a little sodium hydroxide. Five cc. of 20% mercuric acetate in 2% acetic acid was added, and the gelatinous precipitate was let stand overnight.

The first precipitate was collected by centrifugation, washed with a little 1-40 diluted mercuric acetate reagent, thoroughly decomposed with H_2S, and aerated (Hg_I).

The supernatant fluid from the first mercury precipitate was again neutralized to pink with phenol red and precipitated with one volume of alcohol in the cold. This second precipitate was allowed to come to room temperature; it was then washed with 65% ethanol, collected, and treated as above (Hg_{II}).

The mercury was removed from the supernate of the second mercury precipitate with H_2S and the supernate was aerated. The organic phosphorus was collected as a lead precipitate. This was washed with ethanol and extracted with dilute sulfuric acid. This procedure was used instead of H_2S as it is an easier procedure to keep the volume low with a bulky precipitate.

The sulfuric acid extract (15 cc.) was neutralized to pink with phenol red. One cc. of the acetic acid-mercuric acetate reagent was added and the precipitate was allowed to settle. The precipitate was centrifuged off, to permit better observation, and washed.

The supernate was again neutralized to pink, one volume of ethanol was added, and the precipitate was collected after thirty minute standing and washed. To the supernatant fluid was added another volume of ethanol, more mercuric acetate and more alkali. This precipitate was also collected and washed.

A final precipitate was obtained by adding more alkali and more mercuric acetate*. The four mercury fractions just obtained were combined, decomposed with H_2S, and analyzed (combined Hg fraction).

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* The mercuric acetate precipitation is partially a co-precipitation with HgO. This back and forth addition of alkali and mercuric acetate reagent is the only way we have been able to recover our compound with mercury. This process does not, however, precipitate much ribose phosphate, so it is a very useful step.

Material Subjected to Hg Fractionation	Phosphorus mM 0.450	Pentose Phosphorus mM 0.063
Material Recovered by Fractionations:		
Hg_I	0.087	0.004
Hg_{II}	0.185	0.002
Combined	0.066	0.004
Total	0.338	0.010
Material left in Hg Supernate	0.112	0.053

This represents a good removal of pentose phosphate.

Since most of this material was to be used for the preparation of the phosphate-free compound by means of enzymatic hydrolysis, the ribose phosphate removal was not carried out any farther, because after enzymatic splitting the separation of inorganic phosphorus and of ribose per se presents no problem.

The subsequent history of this sample is also pertinent to other parts of the experimental portion of this thesis. It is described here to illustrate certain procedures used for specialized purposes.

The combined mercury decomposates were neutralized to a pH of 5.6 with sodium hydroxide, and the compound was

precipitated by the addition of 3 cc. of 40% lead acetate. After the precipitate had settled, further additions of 2.5 cc. portions of the lead reagent gave no further precipitation. The precipitation was then made quantitative by addition of the usual three volumes of alcohol and standing in the cold. After the precipitate was collected by centrifuging and washed twice with 75% ethanol and once with 95% ethanol, the precipitate was let stand in vacuo over KOH for several hours to remove any water or ethanol which might interfere with the next step.

The dry lead salt was ground in a graduated centrifuge tube; 1.00 cc. of 2 N sulfuric acid was added and mixed thoroughly. The centrifuge tube was stoppered, and the mixture was allowed to digest at room temperature overnight.

Addition of a capillary drop of 2 N sulfuric acid showed that the sulfate ion was present in excess; 0.20 cc. of water was added, plus a capillary drop of ethanol to reduce surface tension. After a thorough mixing, the precipitate of lead sulfate was centrifuged down. The supernate was used for phosphorus and polarimetric analysis (see page 68).

The contents of the polarimeter tube were combined with dilute sulfuric acid extracts of the lead sulfate. Hydrogen sulfide was bubbled in to remove traces of mercury which

were present and the H_2S was removed with nitrogen. The solution was made 0.1 N with nitric acid, and a few drops of 40% lead acetate were added to precipitate traces of $SO_4^=$ present. The lead sulfate was removed, the solution was neutralized to pH 5.3, and 2 volumes of CO_2-free alcohol were added.

After standing in the cold, the lead precipitate of the compound was collected in a tared centrifuge tube. The lead salt was washed four times with 5 cc. of 70% ethanol, once with 95% ethanol, and dried <u>in vacuo</u> overnight over KOH. The salt was dried to constant weight in high vacuum over P_2O_5, in a drying pistol at the temperature of refluxing acetone.

The 232 mg. of dry lead salt so obtained was ground with 3 cc. of water and one drop of ethanol, and treated with hydrogen sulfide. After centrifugation, the supernate was decanted into a tared glass-stoppered cylinder. The procedure was repeated 3 more times. The hydrogen sulfide was then removed with a stream of nitrogen. The volume of solution, calculated from the weight, was 17.6 cc. Part of this solution was used for an electrometric titration (see page 43) and part was used for the preparation of the phosphate-free compound (see following pages).

Enzymatic Hydrolysis of Phosphate Compound

Activity Test of Alkaline Phosphatase.

The substrate for the activity test of the preparation of Schmidt's alkaline intestinal phosphatase consisted of 0.85 g. of sodium barbital and 1.0 g. of sodium glycerophosphate per 200 cc. of solution.

For the test, two tubes, each containing 5 cc. of substrate and 0.3 cc. of 0.3 M magnesium chloride were prepared and preheated to 37° C. To one tube was added 0.01 cc. of a solution of alkaline phosphatase. Both tubes were incubated for 16 minutes at 37° C.

Five cc. of 7% TCA was then added to each tube to stop the reaction and the contents of each tube were analyzed for inorganic phosphorus.

The 0.01 cc. of enzyme solution was found to split off 200 µg. of P per 16 minutes. This activity checked that reported to us by Dr. Schmidt. The enzyme material was kept stored in the deep freeze. The same test repeated a year later gave the same activity.

Typical Preparation of Phosphate-free Compound by Enzymatic Hydrolysis.

A solution of 0.34 mM of the phosphate compound in 25 cc. was acidified with 4 cc. of 0.5 N acetic acid, so that

the solution was approximately 0.05 N with acetic acid. The solution was extracted a few times with an equal volume of ether to remove any fatty acids that might be present.

The volume was now 33 cc. with the optimal concentration of 0.01 mM organic P per cc. or about 0.3 mg. P per cc. The solution was neutralized to a pH of 9.1-9.3 with 3 N ammonium hydroxide. One-half cc. of 2.3 M magnesium acetate and 0.5 cc. of Schmidt's alkaline phosphatase were then added and the mixture was incubated at 37° C. After about ten minutes and some scratching of the tube with a glass rod to initiate crystallization, a fine white precipitate of magnesium ammonium phosphate began to form. After several hours incubation a little more magnesium acetate and enzyme were added without any further precipitation.

The incubation was continued for five more hours to insure completion of the hydrolysis. The precipitate was then filtered off and washed with dilute NH_4OH, and the filtrate was analyzed for phosphorus. Within the limits of sensitivity of the analysis, less than 0.6% P was left.

The supernatant liquid contained the desired phosphate-free compound, sulfate, acetate, magnesium and ammonium ions, a trace of ribose, and the enzyme.

The $SO_4^=$ and compound were precipitated as lead salts in the usual manner and washed thoroughly. The precipitate

was extracted with dilute nitric acid and the $SO_4^=$-free supernate reprecipitated as a lead salt.

This salt was again washed thoroughly with 75% ethanol and dried to constant weight. The salt is quite stable and may be kept at least several years in a desiccator.

Proof of Structure

Elemental Analysis of the Barium Salt of the Phosphate-free Compound.

The barium salt of the phosphate-free compound was prepared by Rapoport from a sample of compound which had been freed of the phosphate by long acid hydrolysis.

The elemental analysis was performed by William Saschek* with the following results:

6.440 mg. were dried at 100° C. in vacuo
0.275 mg. loss on drying
6.165 mg. dry weight gave:

1) H_2O 0.85 mg. . . . . H: 1.54%

2) CO_2 3.50 mg. (from combustion)
$+CO_2$ 1.00 mg. (calcd. from $BaCO_3$ ash)
Total CO_2 4.50 mg. . . . . C: 19.92%

3) Ba (calcd. from 4.475 mg. $BaCO_3$). .Ba: 50.52%
Ba (calcd. from 5.380 mg. $BaSO_4$). .Ba: 51.35%

4) O (calcd. by difference) . . . . . O: 28.02%

Found:	C, 19.9; H, 1.54; O, 28.0;	Ba, 50.5
Calcd. for $C_7H_7O_8Ba_{1.5}$:	C, 19.8; H, 1.65; O, 30.1;	Ba, 48.5

* William Saschek, Kent-Jones Laboratory, The University of Chicago, 5747 Ellis Avenue, Chicago 37, Illinois.

The Ba value from the $BaSO_4$ is 2% higher than the Ba value calculated from the $BaCO_3$ because of a little impurity probably present in the $BaCO_3$. The Ba value is thus slightly higher than the true value.

Preparation of the Silver Salt of the Phosphate-free Compound for Elemental Analysis.

One and six-tenths cc. of a solution of the phosphate-free compound, equivalent to 28.8 micromoles, and free of contaminating salts, was neutralized with the calculated amount of carbon dioxide-free 0.2 N sodium hydroxide. Silver nitrate equivalent to 250 micromoles was added with stirring. The mixture was cooled and 10 cc. CO_2-free absolute ethanol was added. The precipitate was collected and washed thoroughly with 80% ethanol. The last two washes gave a negative test for the silver ion. The silver salt was then dried *in vacuo* over phosphorus pentoxide. The salt was protected from light throughout the preparation, as the silver salt is light sensitive.

Elemental Analysis of the Silver Salt of the Phosphate-free Compound.

The elemental analysis was performed by William Saschek with the following results:

13.830 mg. gave: 1) 1.36 mg. H_2O . . . . . H: 1.10%
2) 5.92 mg. CO_2 . . . . . C: 11.68%
3) 9.41 mg. Ag (residue). Ag: 68.04%
4) 0 (calcd. by difference) O: 19.18%

The analysis expected was $C_7H_7O_8Ag_3$. The preparation of the sample was checked, and it was found that there was probably some Ag_2O precipitated along with the compound. The analysis was corrected on the basis of C_7Ag_3, with the excess Ag calculated as Ag_2O.

The result was as follows:

Found: C, 15.5; H, 1.47; O, 23.2; Ag, 59.6

Calcd. for $C_7H_7O_8Ag_3$: C, 15.5; H, 1.30; O, 23.6; Ag, 59.6

Titration of the Phosphate Compound.

I. A solution of the phosphate compound contained 84 micromoles of organic P and 28 micromoles of inorganic P. The solution was titrated with 0.622 N sodium hydroxide, with phenolphthalein as an indicator.

A total of 474 microequivalents of alkali were needed to neutralize the compound.

Calculations:
474 total microequivalents
− 56 microequivalents of inorganic P
418 microequivalents of compound

$$\frac{\text{418 microequivalents of compound}}{\text{84 micromoles organic P}} = 5.0 \text{ acid equivalents per phosphate.}$$

II. The 232 mg. of dry lead salt which had been freed of lead (see page 36) was now in solution in a volume of 17.6 cc.

Exactly 3 cc. of this solution was transferred to a

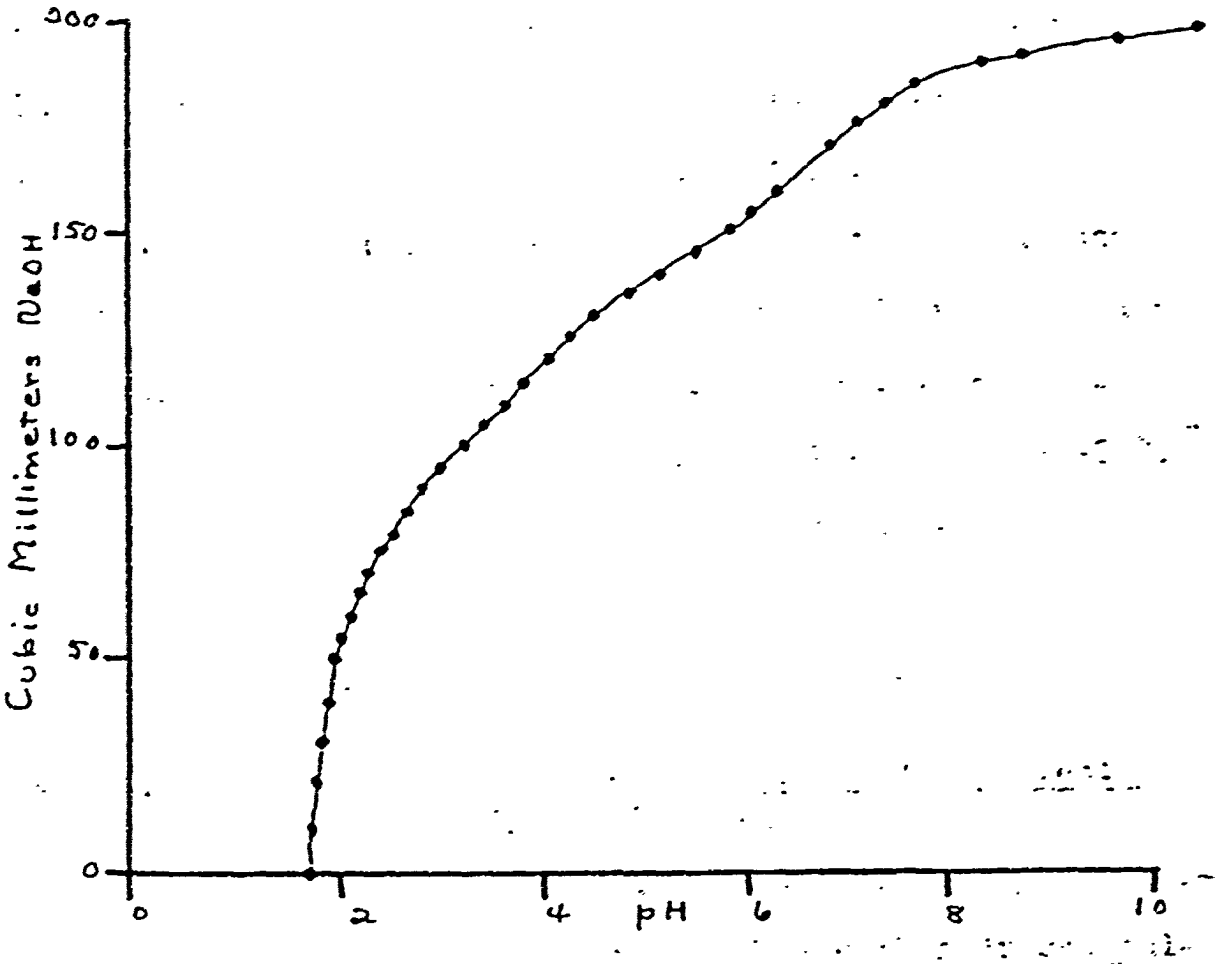
microbeaker and titrated electrometrically with a Cambridge pH meter reading ± 0.01 pH units. Carbon dioxide-free sodium hydroxide (0.849 N) was used which had been standardized against 0.100 N hydrochloric acid, prepared from constant boiling acid and checked with potassium biniodate titrations. The alkali was added from a 0.200 cc. Rehburg buret calibrated in cubic millimeters, and the mixture was stirred with a capillary stream of nitrogen. The end point was interpolated from a graph of pH versus cubic millimeters of alkali and found to be 187 cubic millimeters.

The inflection points are not clear and the pH curve is typical of polybasic acids.

The total titratable acidity of the 3.00 cc. of solution was 0.177 milliequivalents. Calculated for the 17.6 cc. of solution: 1.040 milliequivalents.

Total P analysis: 0.233 mM (triplicate)

$\frac{1.040}{0.233} = 4.46$ acid groups per phosphate.



Electrometric Titration of Phosphate Compound

Graph #1.

Equivalent Weight of the Phosphate Compound.

The lead sulfide obtained from the hydrogen sulfide decomposition of 232 mg. of dry lead salt of the phosphate compound (see page 36) was brought to a constant weight by drying in a high vacuum over phosphorus pentoxide, at the temperature of refluxing toluene (110° C.). The weight of lead sulfide equalled 181 mg., which is equivalent to 157 mg. Pb or 1.52 milliequivalents of Pb.

Since the total titratable acidity of the compound obtained from this lead salt was only 1.040 milliequivalents, it is very likely that the 0.48 meq. of extra lead was present in the form of a basic salt, X-Pb-OH or X-Pb-O/2.

Equivalent weights based on these figures:

66 corrected as OH basic lead salt, or

69 corrected as O/2 basic lead salt.

Equivalent Weight of Phosphate-free Compound and Electrometric Titration of Phosphate-free Compound.

A sample of 111.4 mg. of a lead salt of the phosphate-free compound was decomposed thoroughly with hydrogen sulfide and the hydrogen sulfide was removed with nitrogen. The lead sulfide was collected and dried to a constant weight. The volume of the solution of the compound was determined by calculation from its weight in a tared glass-stoppered cylinder.

A 3.00 cc. sample out of a total volume of 12.51 cc. was titrated electrometrically in the manner described in a previous experiment. As may be seen from the accompanying titration curve the pK's are not clearly defined. The calculated values, $pK_I = 2.50$, $pK_{II} = 3.80$, $pK_{III} = 5.50$, are reasonable for a tribasic acid.

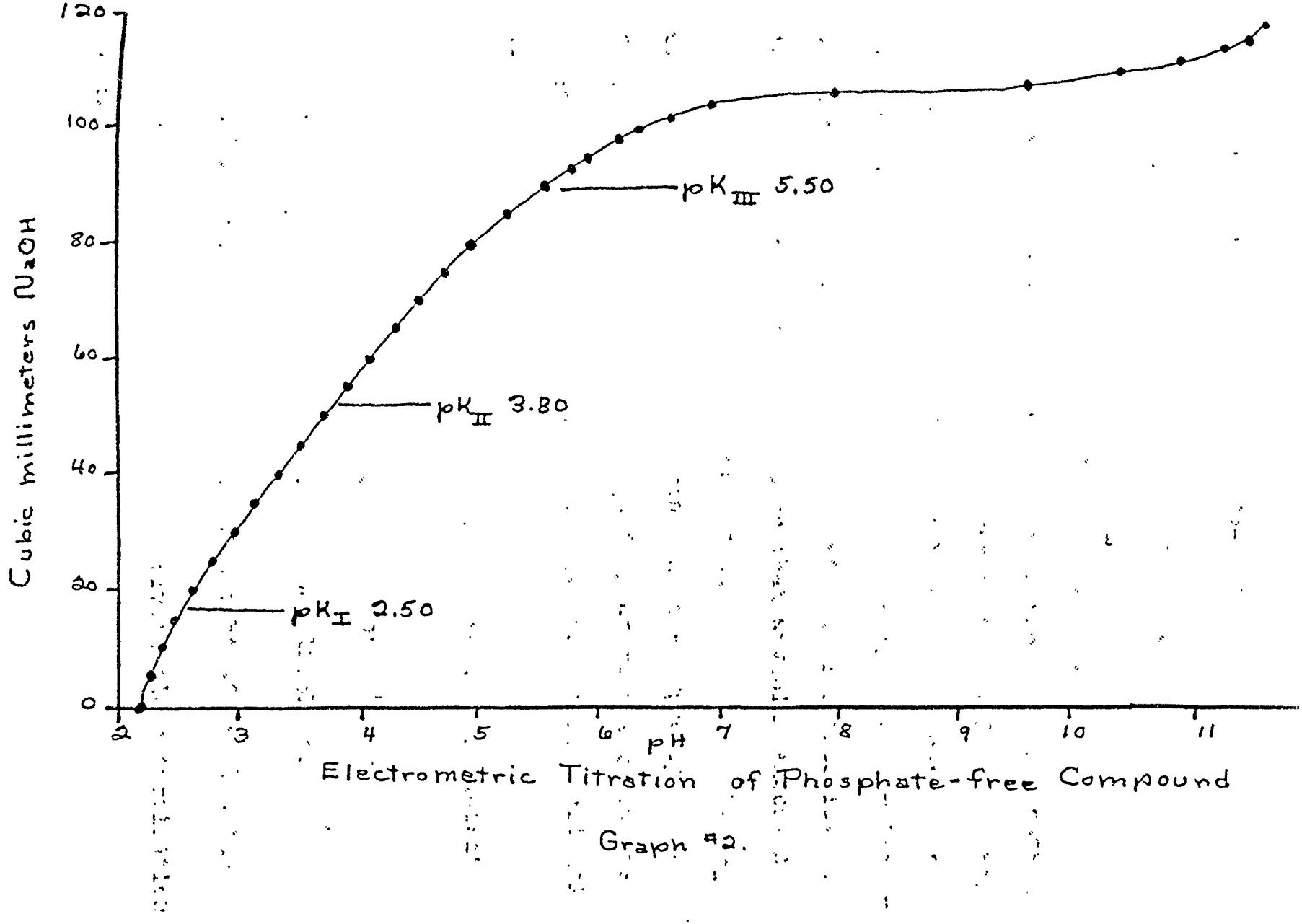
Results: Total acidity calculated from the 12.51 cc. = 0.420 meq.

Weight of Pb, calculated from the weight of PbS obtained = 76.3 mg. = 0.735 milliequivalents of Pb.

An excess of 0.315 milliequivalents of lead was indicated, present as the OH or O/2 basic lead salt of the compound.

Equivalent weights of phosphate-free compound based on these figures: 71.1, corrected as OH basic salt

78.5, corrected as O/2 basic salt.



Electrometric Titration of Phosphate-free Compound

Graph #2.

Equivalent Weight of the Phosphate-free Lactone.

1) Lactone Prepared from Phosphate Ester Hydrolyzed Enzymatically.

A sample of 9.28 cc. (determined by weight) of solution of the phosphate-free compound, out of the 12.51 cc. obtained in the preceeding experiment, was transferred to a tared platinum dish and evacuated overnight at 37° C. over phosphorus pentoxide. The next day the dish appeared completely dry with a pure white amorphous or crystalline material in the dish. This was the first time that anything except a gum had been obtained from numerous attempts to produce a crystalline lactone. The material, however, proved to be deliquescent. After ten days standing at room temperature in vacuo and protected from the light, the material weighed 21.2 mg.

This gave an opportunity to calculate the equivalent weight of the lactone:

12.51 cc. had a titratable acidity of 0.420 meq.

$\frac{12.51 \text{ cc.}}{9.28 \text{ cc.}} \times 21.2 \text{ mg.} = 28.6 \text{ mg. per } 12.51 \text{ cc.}$

$\frac{28.6 \text{ mg.}}{0.420 \text{ acidity}} = 68$, equivalent weight of the lactone.

This material was used for the cryoscopic determination of molecular weight.

2) Lactone Prepared by Acid Hydrolysis of Phosphate Ester.

A sample of the phosphate ester containing 1.03 millimoles of organic phosphorus including 2.2% ribose phosphate was heated in 2 N HCl in a boiling water bath for 48 hours.

The solution was decolorized with Darco charcoal, and after the liberated phosphorus and some sulfate which had been carried along in the preparation were precipitated with $BaCl_2$, the HCl and water were removed in vacuo. The residue was extracted repeatedly with hot anhydrous ethyl acetate and the combined extracts were filtered and evaporated. After several days drying in a vacuum desiccator 102 mg. of a dark brown semi-crystalline gum remained. Repeated ethyl acetate extractions of the gum and decolorizations yielded 62.2 mg. of an almost colorless semi-crystalline mass which showed a total uptake of 0.925 m. Eq. of alkali from which an equivalent weight of 67.5 is calculated. The titration, unfortunately, was not performed as a lactone titration.

Molecular Weight Determination of the Phosphate-free Compound.

1) Introduction.

It was necessary to establish, as closely as possible, the molecular weight of the phosphate-free compound. Because of the size of the sample available (20 mg., or approximately 0.1 millimole) and the desire to use this sample for later

work certain difficulties were encountered.

Because of the polar nature of the compound measurement of the boiling point elevation was not practical. Elevation of the melting point of camphor was ruled out because of our wish to recover the sample after the determination.

The Barger (25) method for the determination of molecular weight or osmolarity in a capillary tube was investigated thoroughly, but it was found that several days equilibration at 37° C. were required to show the difference between 0.016 M NaCl and 0.020 M NaCl. Our material, being particularly susceptible to mold growth, could not be easily investigated with this method.

Freezing point depression of a solution of the phosphate-free compound coupled with a measurement of the hydrogen ion concentration and calculation of the molecular weight (after the method of Warburg as used by Kiessling and Meyerhof (26)) was decided upon as the most direct method.

The only micro-freezing point apparatus at hand, which was one developed by C. D. West and S. Rapoport, required 3 cc. of solution. If the 20 mg. sample had been diluted to 3.0 cc., the resulting freezing point depression would have been about 90 milli-degrees. With an absolute error in the apparatus of about 10 milli-degrees, it was thought that an increase in accuracy could be obtained by scaling down the

required dilution of the sample.

Because of its large bulk and relatively large thermal lag, a Beckman thermometer could not be used, if the reduction of the sample volume required were to be accomplished. It was decided to use the type 14B Thermistor, manufactured by the Western Electric Company, as a small resistance thermometer. This Thermistor has a maximum diameter of 0.1 inches, it has a thermal time constant in water of only one second, and the remarkable temperature coefficient of about -0.04 ohms per ohm per degree centigrade. The use of these small elements as resistance thermometers has been described previously (27,28,29,30).

The procedure used was adopted from that of Richards and Campbell (28) to make use of equipment already on hand. Since the setup used is considerably more simple than that of Richards and Campbell, it is described in some detail.

2) Electrical Setup.

The Wheatstone bridge used is shown in Figure 1. The bridge arms R_1 and R_2 are the 500 ohm resistances of a Leeds and Northrup Wheatstone Bridge with the multiplier switch set at position 1. R_3 is a Leeds and Northrup Student Slide Wire with a fixed resistance of 450 ohms, and a variable resistance of 100 ohms, which could be read

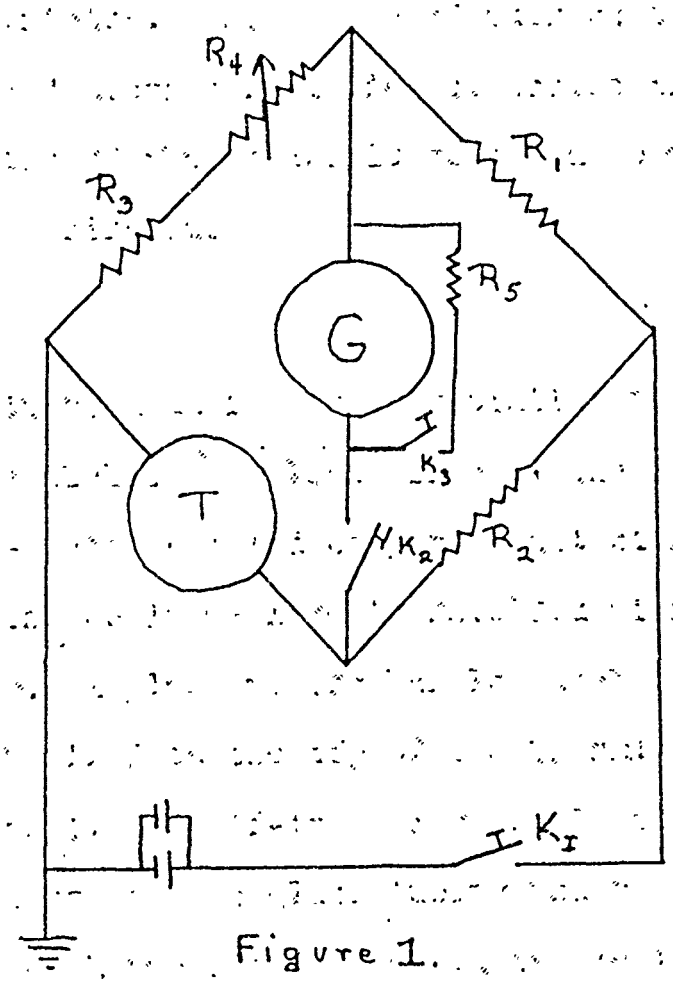


Figure 1.

directly to 0.1 ohm. R_4 is the three dial resistance box of the Leeds and Northrup Bridge reading to 11,100 in steps of 10 ohms. R_5 is a 100,000 ohm shunt for the protection of G, a Pfaltz and Bauer Multiple Mirror Galvanometer with a sensitivity of 1.9×10^{-9} amperes per millimeter deflection. "T" is the Thermistor. The source of current is the 2 volt cells of two lead storage batteries connected in parallel. The negative side of the bridge is grounded to maintain stability of the circuit.

3) Thermistor.

The Thermistor mounting is very similar to that used by Richards and Campbell. The lead wires of the Thermistor are soldered to 28-gauge enameled copper magnet wire. The wire to be used as the positive lead is shielded with polyethylene catheter tubing. A glass tubing handle which contained the lead wires is affixed to the top of the Thermistor with DeKhotinsky cement. A half-inch piece of Tygon tubing is attached to the top of the glass tubing and the leads are brought out through two small holes in the tubing. The handle is completed by attaching a glass tube close at the top of the Tygon joint. Two rubber spacers assure centering of the Thermistor in the sample tube and a rubber collar is fixed to allow the proper depth of immersion in the sample fluid.

4) Sample Tubes.

Sample tubes used are 10 mm. by 75 mm. pyrex tubes. They are fitted with spacers and a collar made from rubber tubing. The jacket tubes are 16 mm. by 100 mm. pyrex tubes. Lead sinkers are placed in the bottom of the jackets to keep the jackets in the freezing bath. The jackets are supported on the cover of the bath by larger rubber spacers.

5) Freezing Bath.

The freezing bath used was a large Dewar flask, which was fitted with a 1/2" wooden plate countersunk to fit the flask snugly. A hole was bored through the center of the plate to hold a brass sleeve which served as a bearing for the motor-driven stirrer. Other concentric holes were bored to receive the freezing point jackets and their tubes.

About 800 grams of Epson Salts ($MgSO_4 \cdot 7H_2O$) was mixed to a slush with ground ice and the Dewar flask was filled with this slush to within 1 1/2" to 2" of the top.

6) Procedure.

One-half cc. samples of the solutions to be determined were introduced into the sample tubes. The tubes were fitted into the lead weighted jackets, and the jackets fixed in the plate. The propeller was introduced and stirring was begun.

When the temperature of the bath reached -2° C., as determined with an accessory thermometer, the Thermistor was placed in a mercury-filled tube to determine the stability of the bath temperature. When the bath temperature became stable, with resistance measurements varying by only a few ohms per minute, freezing point determinations were begun. Current was kept flowing through the Thermistor throughout all the determinations to eliminate transient heating effects which would be caused by closing the circuit and which would necessitate additional equilibration.

Seeding with the Thermistor was not possible, in our hands, even when the external surface of the Thermistor had been roughened with emery paper. The solutions were seeded as follows: a Nichrome wire hook was equipped with a very small cotton plug which was wetted with water. The tiny drop of water was frozen and supercooled by placing it in a test tube which rested in an ice-salt freezing mixture. Seeding was accomplished by immersing the frozen seed in a sample tube for 1-2 seconds and then removing it. The wire was tapped sharply to remove excess solution and returned to the supercooling bath. The thermistor was removed from the tube last measured, wiped dry and introduced into the next sample to be determined. Since the transfer of the

Thermistor requires only 1-2 seconds and the thermal constant of the Thermistor is 25 seconds in air, the Thermistor is only heated a few degrees by the exposure to the air.

After the initial large drop in resistance following the seeding, the resistance drifts slowly upwards or stays constant for as much as five minutes. The final balances at this time are taken with maximum galvanometer sensitivity.

The sensitivity of the setup and the reproducibility of the electrical null point were tested as follows:

A four decade Leeds and Northrup resistance box was substituted for the Thermistor in the circuit, the box resistance was set at 5700 ohms, and the balance with the variable resistors, R_3 and R_4, was found to be 5601.6 ohms. At this time a setting of 5601.0 ohms gave a galvanometer reading of -1.8%, while a setting of 5602.0 ohms gave a reading of +0.8%, so it may be seen that the setup is capable of detecting a change in resistance of 0.1 ohm. Several days later the balance was found to be 5601.5 ohms. The stability of the battery voltage and of the resistance measurement was thus established.

7) Calibration.

The ice point was determined a few times to establish the reproducibility of the freezing points. This was followed by measurements of the freezing points of 0.050, 0.060, 0.070 N NaCl solutions.

			Average Value
Results: Ice Points	5613.4	ohms	
	5612.8	"	
	5612.1	"	
	5613.0	"	
	5612.6	"	5612.6 ohms
0.05 N NaCl	5662.3	"	
	5661.6	"	5662 ohms
0.06 N NaCl	5683.9	"	
	5673.9	"	
	5670	"	
	5670	"	
	5673.0	"	
	5669	"	
	5669	"	
	5669	"	5671 ohms
0.07 N NaCl	5680.1	"	
	5679.1	"	
	5680.9	"	
	5678	"	
	5682	"	
	5680	"	5680 ohms

The ΔR per milliosmol in the range 0.05 N to 0.07 N NaCl = -0.45 ohm per milliosmol, uncorrected for activity values of the sodium chloride. This figure was calculated merely to show the linearity of the method over this range of concentration.

8) Measurement of the Freezing Point of the Phosphate-free Lactone.

The 21.2 mg. of lactone (see page 48) was dissolved in 1.054 g. of water. One-half cc. of this solution was used for the freezing point determination. Concentration of the solution equalled $\frac{21.2}{1.054} = 20.1$ mg. per cc.

The resistance at the freezing point for the solution of the lactone was 5671 ohms. Since only one determination was made, the maximum uncertainty would be $\pm$ 4 ohms. This corresponds to a milliosmolar concentration of 120 $\pm$ 9, uncorrected for activity of the sodium chloride solutions used for the calibration curve.

The activity of 0.06 N NaCl is $\frac{3.506}{3.72}$ (31). The true milliosmolar concentration of the solution, then was

$$120 + 9 \times \frac{3.51}{3.72} = 113 + 9.$$

9) Measurement of Hydrogen Ion Concentration.

After the freezing point determination, the material was allowed to thaw. It was then diluted by a factor of 2.2 and the hydrogen ion concentration was determined at 4° C. in the Cambridge pH Meter as follows: Direct readings of the electromotive force of 0.05 M potassium acid phthalate and the unknown against the glass electrode were taken.

Results:

4^{o} C.	0.05 M potassium acid phthalate	85.5	milli-volts
4^{o} C.	Unknown	- 12.5	"
	Electromotive force	98.0	"

The known values were substituted in the formula:

$$Emf = \frac{RT}{nF} \ln \frac{C_2}{C_1}, \quad 0.098 = 0.000198406 \times T \log \frac{[H^+] \text{ unknown}}{[H^+] \text{ KH phthalate}}$$

From $[H^+]$ phthalate $= 1 \times 10^{-4}$, the hydrogen concentration of the unknown solution was 6×10^{-3}, this times the dilution factor of 2.2 equalled 13×10^{-3}, for the original freezing point solution.

10) Calculation of Molecular Weight of the Lactone.

113 ± 9 milliosmolar concentration of lactone solution

- 13 milliosmolar concentration of hydrogen ion

100 ± 9 milliosmolar concentration of lactone molecules.

Since the concentration = 20.1 mg. per cc,

Molecular weight of lactone $= \frac{20.1}{0.100 + 0.009} = 201 \pm 18.$

Oxidation of Phosphate-containing Compound with Acid Permanganate Performed by Rapoport and Van Fossen (7).

1) Titration.

One cc. of a solution of the phosphate compound, acidified to 1 N strength with sulfuric acid, and containing 7.5 micromoles of organic phosphorus was titrated in the cold with 0.01 N potassium permanganate. Thirty-two microequivalents, i.e., 4.27 equivalents per mole of P, were consumed. Analysis of the inorganic P indicated that only 4.3% of the compound was hydrolyzed under these conditions.

Like the original compound, the product of the oxidation gave a positive iodoform reaction. The oxidation product also gave a negative Benedict's test for a reducing group, a negative test with Deniges' reagent, and a positive test for a carbonyl group with 2,4-dinitrophenylhydrazine.

2) Measurement of the Carbon Dioxide Liberated.

These experiments were performed in a closed system in a current of carbon-dioxide-free nitrogen. The carbon dioxide evolved in the reaction was collected in two receivers placed in series and containing barium hydroxide of known normality. The carbon dioxide collected was determined either by titration with HCl or gravimetrically with consistent results.

In a given experiment 75 micromoles of the compound were oxidized in 1 N sulfuric acid. First, permanganate was added in the cold until a pink color persisted. A total of 400 micro-equivalents of permanganate were required, or 5.33 equivalents per mole. The carbon dioxide collected at this point amounted to 150.5 micromoles, i.e., 2.01 moles per mole of P. Addition of permanganate was continued, with warming of the reaction vessel to 90° C., until a total of 1,000 micro-equivalents had been added. Another 100 micromoles of CO_2, or 1.3 moles per mole of P were collected. Whereas less than 10% of the P was hydrolyzed with liberation of the first 2 moles of CO_2 in the cold, 45% was hydrolyzed during continued oxidation.

Deniges' Reaction (32) with Phosphate-free Compound Prepared Enzymatically.

a) To two drops of a solution of the phosphate-free compound containing 3 micromoles per cc. was added one drop of 10% mercuric sulfate in 4 N sulfuric acid and one drop of 5% potassium dichromate. On standing, the solution became turbid. When the solution was heated to 100° C. in a tube capped with a marble, a heavy precipitate formed which looked like the typical acetone complex.

b) To another two drops of the test solution was added one drop of 10% mercuric sulfate in 4 N sulfuric acid plus

one drop of 0.05 N potassium permanganate. A white precipitate formed which looked like the typical acetone complex.

c) A few drops of citric acid containing 1 mg. per cc. was treated as in b) with the same results.

The precipitate from a) was collected on a filter stick and washed with water. Five cc. of borate-HCl buffer and the filter stick were added to a distilling flask. Four and one-half cc. of distillate was collected in an ice-cooled receiver and the following procedure was employed to test for acetone:

Behre's salicylaldehyde test - One cc. of distillate was tested for acetone with the potassium hydroxide, ethanol, salicylaldehyde test (33) and compared with a blank run and a standard acetone test. The distillate gave an orange-red color the same as that produced with acetone.

Friedemann-Haugen test (34) - To the rest of the distillate was added one cc. of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl. The mixture was extracted for ten minutes with 2 cc. of carbon tetrachloride. The carbon tetrachloride extract was colored yellow, so it was washed two times with 2 cc. of water. One drop of the solution gave a red color with sodium hydroxide and water.

Formation of Pentabromoacetone.

Test of phosphate-free compound for the formation of pentabromoacetone (10,11).

To 0.1 cc. of a solution of the compound containing 3 micromoles per cc. was added one drop of potassium bromide-potassium bromate solution and one drop of 18 N sulfuric acid, four drops of water, and two drops of 5% potassium permanganate. After ten minutes standing at room temperature, the mixture was cooled to 0° C. and decolorized with 3% hydrogen peroxide. White crystals which were left had a greasy appearance characteristic of pentabromoacetone.

The precipitate, like pentabromoacetone, was soluble in petroleum ether. Dilute sodium sulfide added to the petroleum ether extract gave a yellow color, a confirmatory test for pentabromoacetone (35).

Preparation of Pentabromoacetone from Phosphate-free Compound.

To 4.2 cc. of solution containing 14 micromoles of compound was added 2 cc. of water, 0.2 cc. of 1 M potassium bromide, 1 cc. of 10 N sulfuric acid, and 5% potassium permanganate dropwise until a slight excess was present. The mixture was let stand for 10 minutes at room temperature, cooled to 0° C. and decolorized slowly with 3% ice-cold hydrogen peroxide. The mixture, containing the white crystals of pentabromoacetone, was let stand on ice overnight.

The precipitate was collected in a tared tube in the cold and washed 3 times with 1 cc. portions of ice-cold water. The precipitate was dried over potassium hydroxide. The yield was 3.0 mg. of pentabromoacetone.

The melting point determinations were made on micro-slides in an uncalibrated Fisher-Johns melting point apparatus which was preheated to about 60° C.

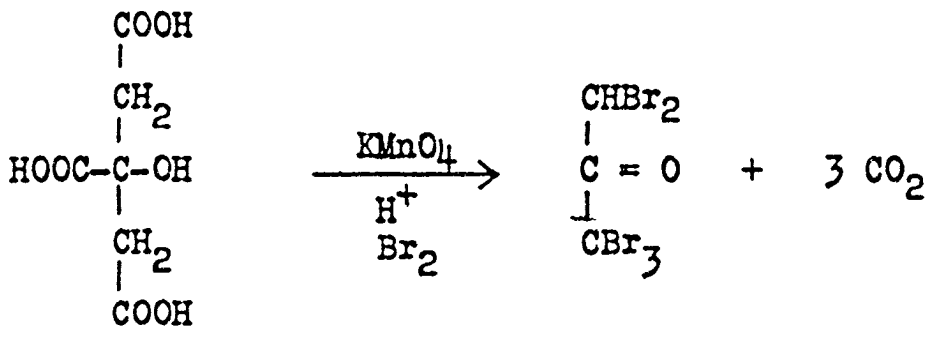
Pentabromoacetone was prepared from citric acid for purposes of comparison.

Results:

Material Tested	Sintering Point °C.	Melting Point °C.
Standard	67	70.0 - 70.5
Standard	67	69.5 - 70.0
Standard	67	70.0 - 70.5
Standard	67	69.5 - 70.0
Unknown	66	70.0 - 70.5
Mixture	67.5	70.0 - 71.0
Unknown	66	70.0 - 70.5
Mixture	66	69.5 - 70.0

Gasometric Measurement of Carbon-dioxide Evolution with Permanganate-bromine Oxidation.

a) Using citric acid as a model substance, the conditions were explored which would yield 3 moles of carbon dioxide per mole of citric acid in the Warburg apparatus.



Acid, bromine, and permanganate concentrations and temperature were varied. The following procedure was found suitable.

The Warburg bath was kept at 7° C. Twenty-cc. Warburg vessels with a single side arm were used. Into the main vessel was introduced 1 cc. 10 N sulfuric acid, 0.1 cc. of 1 M potassium bromide, 0.4 cc. of 0.4 M potassium permanganate and water to make the final volume, after tipping of the side arm, 2.6 cc. In the side arm was introduced 0.1 to 0.3 cc. of 0.025 M citric acid standard (2.5-7.5 micromoles). After equilibration at 7° C., the citric acid was tipped in and a time curve of the gas evolution was taken. After 40 minutes, the carbon dioxide evolution per mole of citric acid was close to 3.0 for samples ranging from 2.5 to 7.5 micromoles of citric acid.

b) The same procedure was used for a sample of the phosphate-free compound, the total titratable acidity of which was determined on a different aliquot as follows: To 0.5 cc. of solution was added 1 micro drop of phenolphthalein. The solution was titrated to a definite pink

with 0.216 N sodium hydroxide, using nitrogen stirring and a Rehburg buret. A total of 87 cubic millimeters of sodium hydroxide was used. Since this sample has been prepared by solution of a lactone preparation, the titrated mixture was heated at 100° C. and more alkali was added until a stable pink was obtained. The total alkali consumption of 95 cubic millimeters gave a calculated 41.1 micro-equivalents of acid per cc. On the basis of a tribasic acid the 0.1 cc. sample used for the Warburg measurement was equal to 1.37 micromoles of compound.

The first determination gave about 3.7 moles of carbon dioxide per mole after 2 hours and 4.0 after about 3 hours.

With the idea that the lactone ring might be hindering the rate of carbon dioxide formation, another sample was treated with alkali, heated to 100° C. for 100 minutes to cleave the lactone and then acidified with sulfuric acid before the oxidation experiment. After 86 minutes, 3.7 moles of CO_2 per mole had been given off. The value stabilized at 4.1 after two hours.

Lack of Carbon-dioxide Evolution on Heating of Phosphate-free Compound.

One-tenth cc. of solution of the phosphate-free compound equivalent to 1.4 micromoles of compound was evaporated to dryness at room temperature in the side arm of a Thunberg tube.

One cc. of saturated barium hydroxide was added to the main tube of the Thunberg tube and the tube was evacuated. The side arm was heated, while the barium hydroxide arm was kept cool. At 100° C. no carbon dioxide was evolved. At 130° C. the material browned, but at 160° C. there was still no barium carbonate formation detectable.

The residue was dissolved, removed, and titrated using a Rehburg buret and nitrogen stirring. The total acidity of the residue was only about 60% of the theoretical. A reduction of some sort is presumed to have occurred.

A blank test with one micromole of sodium carbonate mixed in an evacuated Thunberg tube with 1 cc. of barium hydroxide gave a heavy turbidity.

Characteristics of the Phosphate Compound

Optical Rotation of Phosphate-containing Compound.

A concentrated solution of the compound had been obtained by dissolving a lead salt with sulfuric acid, (see isolation experiment). The volume of the solution was 1.20 cc.

After an aliquot of the solution was analyzed for phosphorus to determine the concentration of the compound, the rotation was read to the nearest thousandth of a degree in a Rudolph Half-Shade Polarimeter at 20° C., using the mercury vapor line of 5461 A°. The solution was contained in a micro-cuvette with a volume of 0.6 cc. and a length of 1 dm. Air blanks had been shown previously to give the same readings as water blanks.

Results: P analyses (duplicate) = 264 μM P/cc.
= 0.264 M/l.

Air blank (av. of 11 readings) = + 0.090°

Compound (av. of 15 readings) = + 0.011°

From the formulas: $[\alpha] = \frac{100\alpha}{bc}$ and $[M] = \frac{M}{100}[\alpha]$

Where $[\alpha]$ is specific rotation, α is observed rotation, b is depth of solution in decimeters, c is concentration in grams per 100 cc., $[M]$ is molecular rotation power, and M is molecular weight (36), the formula is derived that

$$[M] = \frac{10\,\alpha}{\text{moles per liter}}$$

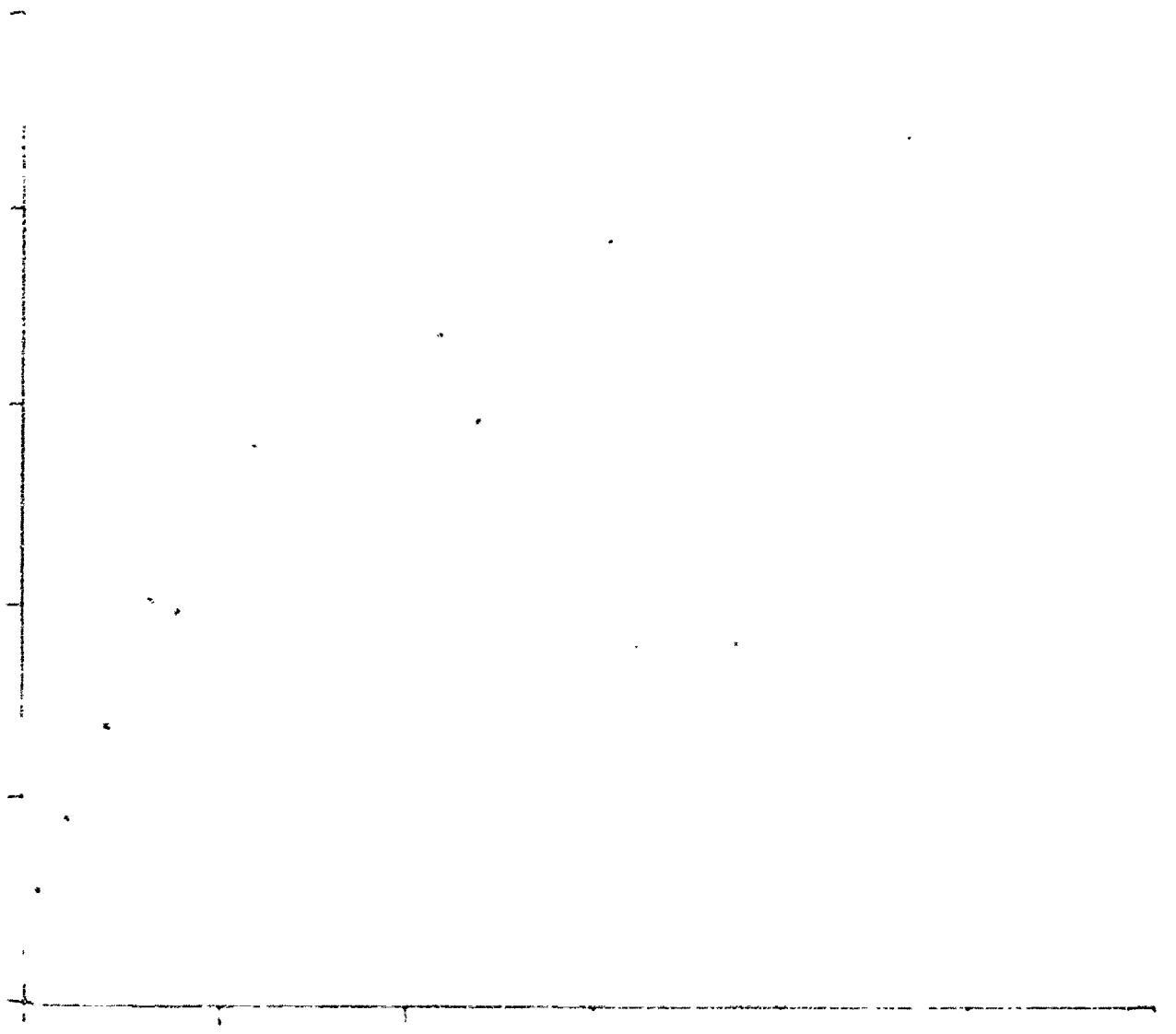
$$[M]^{20^\circ}_{5461\ A^\circ} = -3.0^\circ \pm 0.4^\circ$$

Acid Hydrolysis Curve.

The hydrolysis curves of the phosphate compound in 1 N and 6 N sulfuric acid are presented in Graph #3.

Iodoform Reaction.

In a series of experiments performed by Rapoport and Van Fossen, the phosphate ester was found to give a positive haloform reaction with hypoiodite. About six equivalents of iodine were consumed per mole and iodoform was isolated as a product of the reaction. This behavior is consistent with the presence of active hydrogen atoms.



Characteristics of the Phosphate-free Compound

Enhancement of Optical Rotation of Phosphate-free Compound with Ammonium Molybdate.

In a previous experiment, the molecular rotation of the phosphate compound was determined and found to be only $-3.0^{\circ} \pm 0.4^{\circ}$.

Since very little phosphate-free compound was available, its rotation was not measured; however, since the phosphate-free compound has the same asymmetric carbon atoms and differs from the parent compound only in the substitution of an OH group for an OPO_3H_2 group, it was assumed that its rotation would also be very small.

A 0.55 cc. sample of solution, with a titratable acidity of 10.06 micro-equivalents per cc. and calculated concentration of 3.35 millimoles per liter, was mixed with 0.30 cc. of 25% ammonium molybdate and read in a micro-cuvette against a water blank. The rotation (average of 10 readings) was $+ 0.050^{\circ} \pm 0.01^{\circ}$.

Calculations:

$$\alpha = 0.050 \times \frac{0.85}{0.55} \text{ (dil. factor)} = + 0.077^{\circ} \pm 0.015^{\circ}$$

$$[M]^{20^{\circ}}_{5461\ A^{\circ}} = \frac{10\ \alpha}{\text{moles/liter}} = \frac{10 \times 0.077}{0.00335} = + 230^{\circ} \pm 46^{\circ}$$

Lactone Formation.

1) An aqueous solution of a small preparation of the lactone, prepared by acid hydrolysis and ethyl acetate extraction, titrated at room temperature 48.6 micro-equivalents of sodium hydroxide and 38.7 micro-equivalents more when heated.

2) Another aqueous solution of the lactone prepared by acid hydrolysis followed by continuous ether extraction titrated 193 micro-equivalents in the cold and 65 micro-equivalents more when heated to 100° C.

3) In a titration of another preparation the end points were not clear, but there was some evidence for the presence of a di-lactone.

Fürth-Herrmann Pyridine-acetic Anhydric Color Test (15) in the Phosphate-free Compound (Prepared Enzymatically).

Two drops of a solution of compound equivalent to about 60 micrograms was evaporated to dryness in a porcelain dish. One-half cc. of acetic anhydride and 3 cc. of anhydrous pyridine were added. On warming the mixture on the steam bath, a faint but definite cherry-red color became evident. The intensity of the color was between that produced in similar tests with 25 and 50 micrograms of citric acid.

Ultraviolet Absorption Curve.

A solution of the phosphat ·](e compound, prepared enzymatically, which had been n . ta l zed to a pH of 11.5 and contained 3.35 micromoles p(:)c . , was read in a Beckman Quartz Spectrophotometer a gst a water blank.

After this curve was read, l c:. concentrated hydrochloric acid was added and t · : e sulting mixture, which had a calculated pH of 1.3, w u also read against a water blank.

APPENDIX

THE ISOLATION FROM LIVER AND THE PRELIMINARY STUDY OF A NUCLEOTIDE WITH UNUSUAL SOLUBILITY PROPERTIES

Introduction

In the course of the purification of 2-phospho-4-hydroxy-4-carboxyadipic acid from dog or rabbit liver, following the steps involving the removal of barium and glycogen, it was observed that 50% ethanolic solutions which were concentrated with respect to nucleotides gave a light yellow semi-crystalline precipitate on standing in the cold (see page 25).

In the crude precipitates, molar ratios of adenine, 1.0; 15 minute hydrolyzable phosphorus, 1.1; difficultly hydrolyzable phosphorus, 1.1; and total phosphorus, 2.2 were typical. These molar ratios would be 1,1,1,2 for ADP or diadenosine tetraphosphate.

The solutions in which precipitation occurred were 0.1 N with respect to nitric acid. Since this precipitation behavior is not characteristic of AA, ADP, or ATP and is more reminiscent of the behavior of the diadenosine tetraphosphate of Kiessling and Meyerhoff (26) which has been prepared from yeast, the material was studied at some length.

The initial yield of crude precipitate was increased greatly by keeping the volume very low during the removal of barium and glycogen (see page 24), and by the addition of a total of 2 volumes of alcohol and 3 volumes of acetone to the aqueous solution. From the analysis (given on page 26)

of a precipitate obtained in this manner the molar ratios, adenine, 1.00; 15 minute hydrolyzable phosphorus, 1.25; difficultly hydrolyzable phosphorus, 1.11; and total phosphorus, 2.36, were obtained.

From studying various purification procedures, the assumption was made that the equivalent weight of the material per adenine group could approach 427, the molecular weight of ADP, as a limit.

All operations were carried out in the cold at 2-6° C. in order to minimize hydrolysis of the nucleotides.

Purification Procedures

Acid Silver Fractionation.

A precipitate of the nucleotides was obtained in the manner described on page 25. The precipitate was dissolved in the minimal amount of water (16 cc.); and some water-insoluble material was centrifuged off. The supernatant contained 1.7 mM of adenine, 1.3 mM of 15 minute hydrolyzable P, 1.5 mM of difficultly hydrolyzable P, and 2.8 mM of total P.

A total of 6 cc. of saturated picric acid was added to the solution of nucleotides in small portions until precipitation was complete. The picrate precipitate was collected, washed with water, and discarded. The supernate and washes were extracted with ether to remove the picric acid.

A precipitate of the nucleotides was obtained by the addition of 1 volume of alcohol and 1 volume of acetone. After standing for two days, the precipitate was collected, dissolved in water and reprecipitated with alcohol and acetone. The precipitate, which was collected, washed with a water-ethanol-acetone mixture and dried in vacuo over potassium hydroxide, had a dry weight of 911 mg.

A sample of 447 mg. of the above precipitate was dissolved in 7 cc. of water. Six-tenths cc. of 5 N silver nitrate was added and a heavy white precipitate formed,

which was not increased by further additions of silver. The silver precipitate was collected, decomposed with hydrogen sulfide, and the hydrogen sulfide was removed.

The solution was again subjected to the procedure described above. To the resulting solution of nucleotides (18 cc.) was added 2 volumes of alcohol and 2 volumes of acetone. The precipitate obtained in this relatively dilute solution weighed only 100 mg. and analyzed approximately as adenylic acid, so it was discarded.

After the supernate from the preceding step was diluted with water to make it 50% aqueous, a silver precipitation was used to collect the remaining nucleotides. The solution obtained after the removal of silver from the precipitate was lyophilized. The 206 mg. was dissolved in 5.0 cc. of water and an aliquot was analyzed. The molar ratios obtained were adenine, 1.00; 15 minute hydrolyzable P, 1.01; difficultly hydrolyzable P, 0.96; total P, 1.97, but the equivalent weight per adenine group was 583, so it was believed that a non-purine impurity still remained.

The 5 cc. of solution was acidified to 0.02 N with nitric acid. Fifty microequivalents of silver nitrate were added and the resulting small silver precipitate was discarded.

Seven hundred and fifty microequivalents of silver nitrate were added to the supernate. The silver precipitate was collected, washed with alcohol, dried and weighed.

Analysis of Silver Salt	Molar Ratios
Adenine	1.00
15 min. hydrolyzable P	0.84
Difficultly hydrolyzable P	0.98
Total P	1.79
Ag	1.72

Molecular weight of the silver salt calculated on the basis of one adenine group per mole equalled 695. The molecular weight of the disilver salt of ADP would be 641.

Purification by Means of Anion Exchange.

Since the procedure previously described had not given as pure a product as was desired, other attempts at purification were made. Fractional precipitation of 0.1 N nitric acid solution with alcohol and acetone gave very little purification, so it was decided to try anion exchange chromatography.

The method used was adapted from the methods described by Carter and Cohn (37) and Cohn (38) for the separation of cytidylic, uridylic, adenylic, and guanylic acids. At the time this work was undertaken in November 1949, the separa-

tion of AA, ADP, and ATP by ion exchange had not yet been described. The separation of the nucleotides was achieved by increasing the acidity of the eluting fluid in a stepwise manner. This procedure is inferior to that recently reported by Cohn and Carter (39) in which the hydrogen ion concentration of the eluant is kept constant and the salt concentration is increased, but it served in this preliminary work.

1) Anion Exchange Procedure, Illustrating a Separation of Adenylic Acid and Adenosine Triphosphate.

Dowex 1* (250-500 mesh) washed free of fines by repeated decantations was slurried into a glass tube fitted with a flat glass-wool plug and a fine glass tip at the base. The resin column was 9 cm. long and had a cross-section of 0.65 square cm.

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* Samples of the various Dowex resins were generously supplied to us by Dr. W. C. Bauman, The Dow Chemical Co., Midland, Michigan.

Two cc. of a solution containing Bischoff My-B-Den adenylic acid (ev = 115)* and Rohm and Haas tetrasodium salt of adenosine triphosphate (ev = 89) was allowed to pass slowly through the column.

The column was eluted with 0.003 N hydrochloric acid. Twenty cc. portions of eluate were collected, mixed and analyzed spectrophotometrically for adenine. When elution appeared to be practically complete, the eluting fluid was changed to 0.009 N hydrochloric acid. The process was repeated for 0.03 N and 0.10 N hydrochloric acid (Graph #1). The rate of elution was about 1 cc. per minute. A total recovery (calculated on the basis of absorption measurements) was obtained. The 0.10 N acid eluate analyzed 63% easily hydrolyzable P, evidencing fairly pure ATP.

2) Purification of ADP

A sample of 98.1 mg. of Sigma barium salt of adenosine diphosphate was freed of barium and put into solution with sulfuric and nitric acid. The solution was neutralized to pH 6 and adsorbed on a column similar to that described

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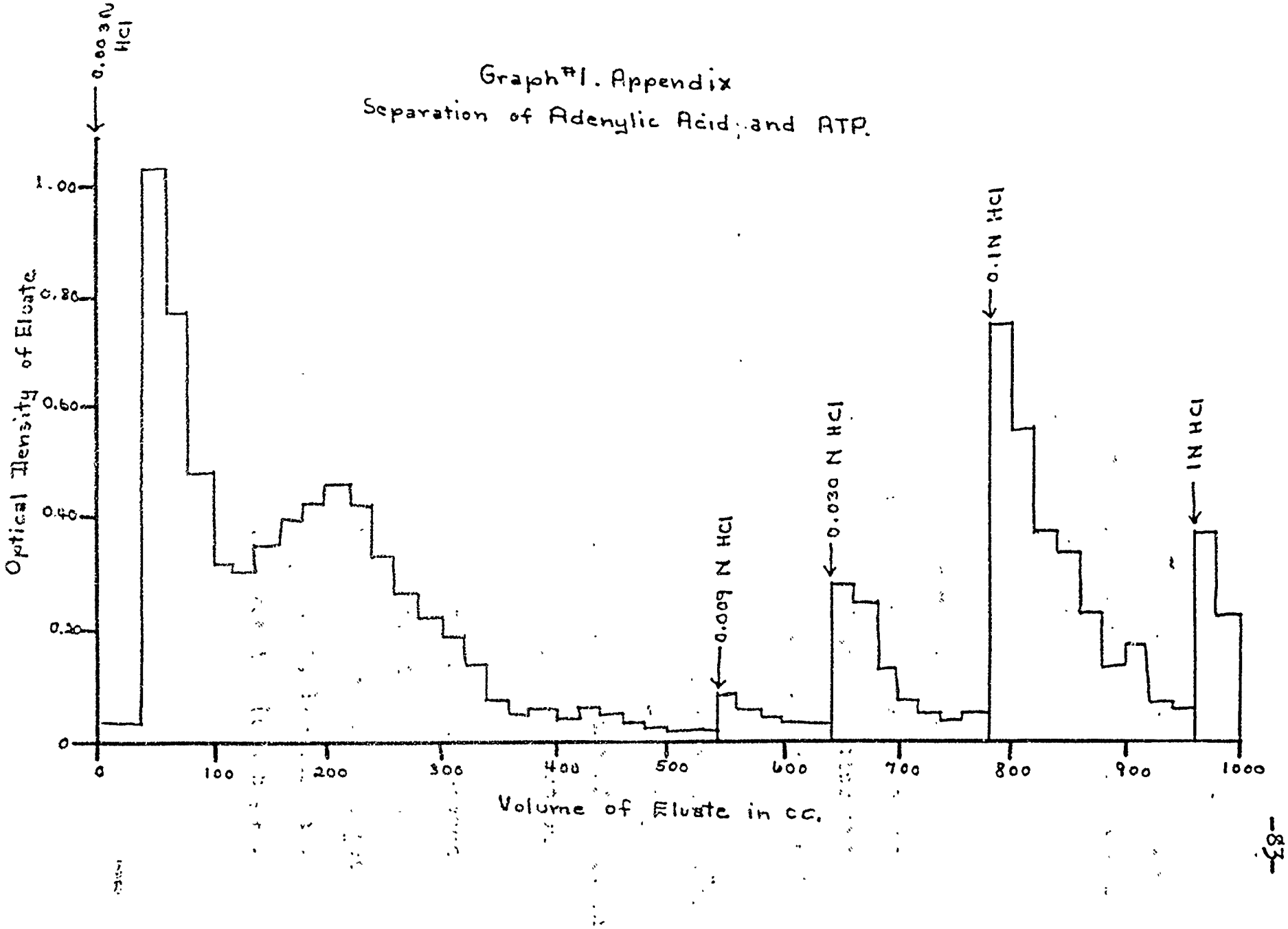
* (ev) represents the product of the volume of the solution and the extinction of the solution. It provides a convenient number with which to express the total amount of material present, since

$$\frac{ev \times 100}{\text{molecular extinction coefficient}} = \text{micromoles of material in the solution.}$$

Graph #1. Appendix

Separation of Adenylic Acid and ATP.



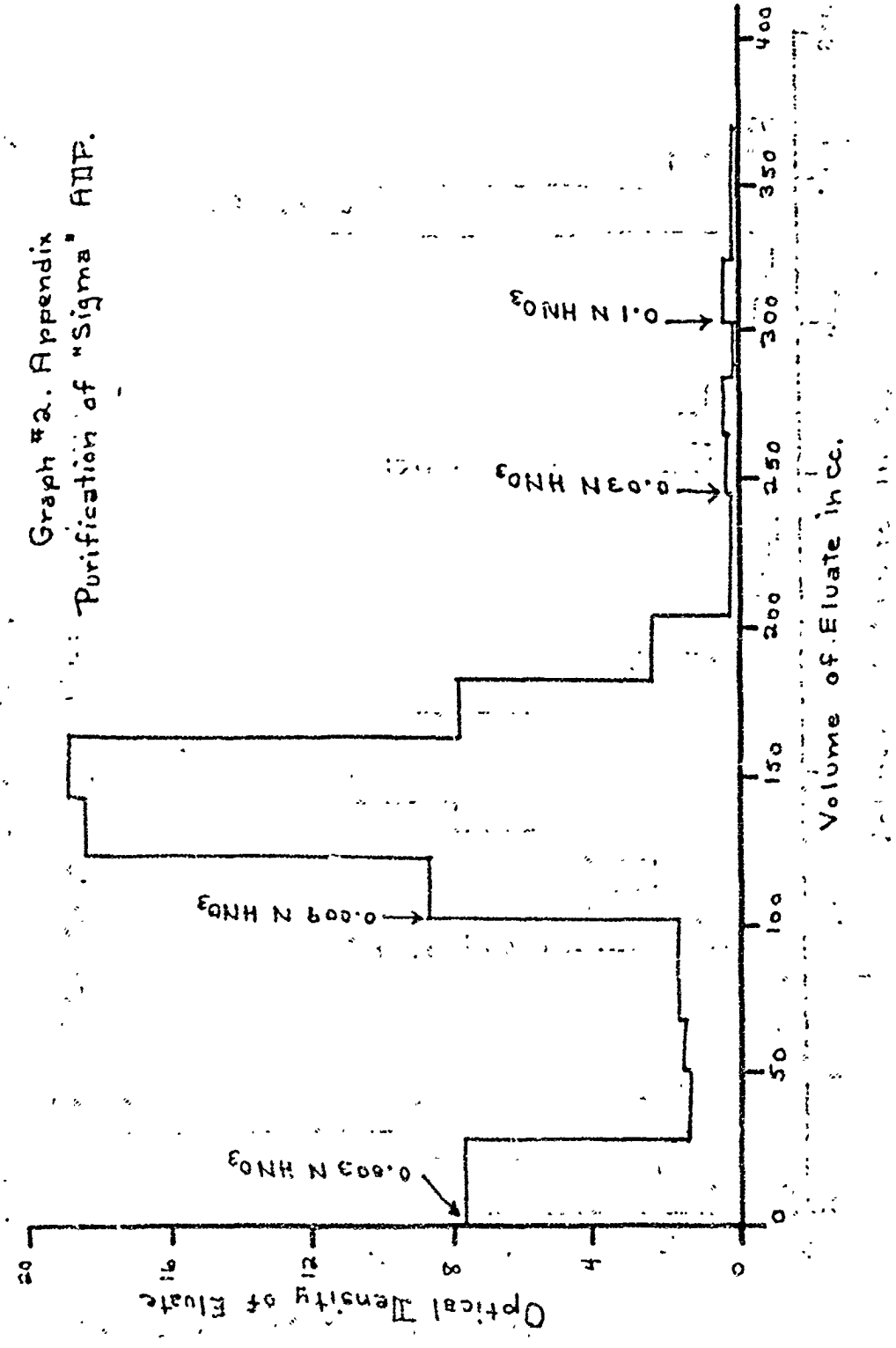
See Graph #2. Total recovery was only 65%. Twenty-three per cent of the eluate was in the 0.003 N nitric acid fraction (AA). The 0.009 N nitric acid eluate was collected as a lead salt.

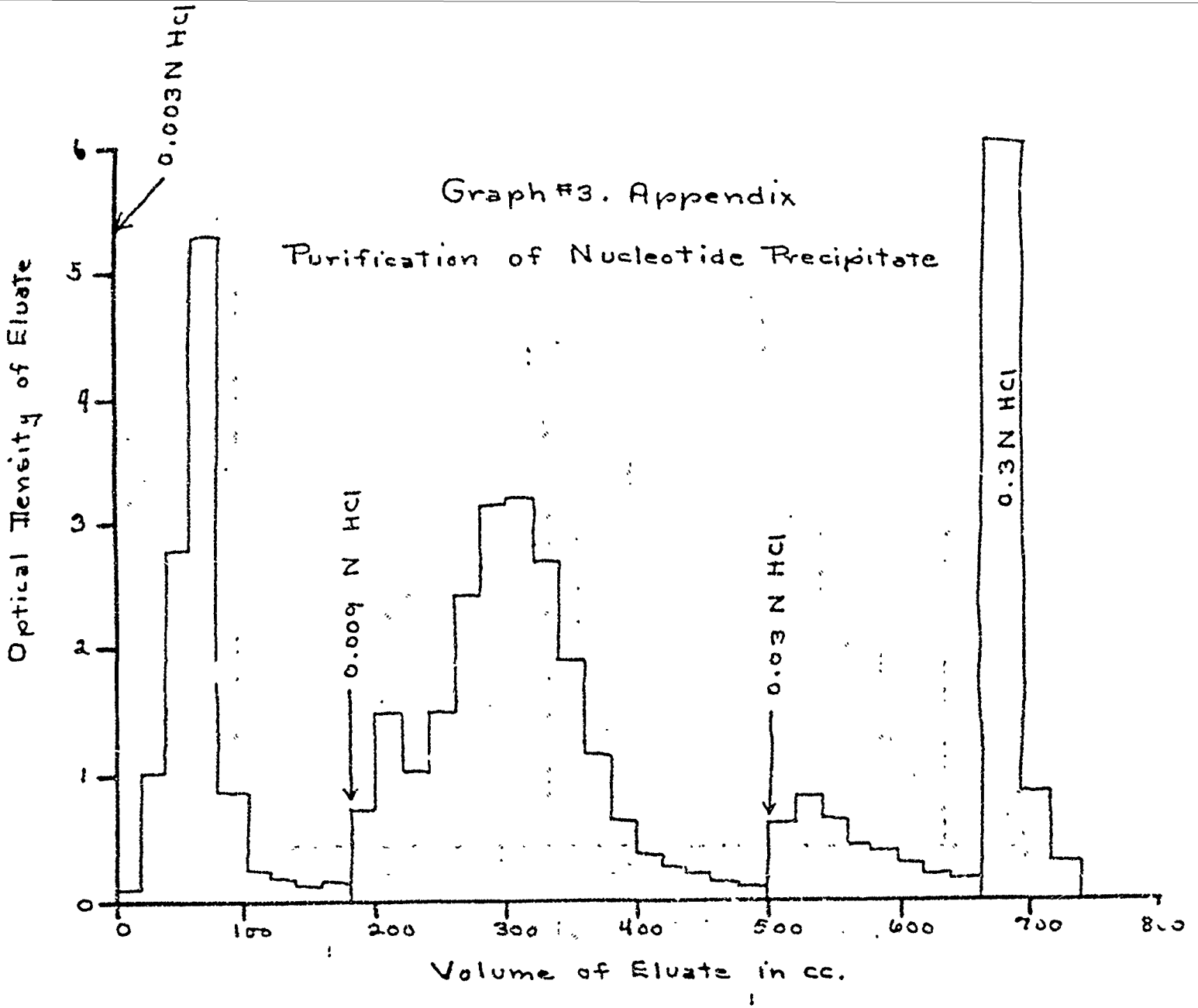
3) Purification of the Nucleotide Precipitate.

A sample of the crude precipitate of the nucleotides weighing 100.5 mg. was dissolved in 10 cc. of water and adsorbed on a Dowex 1 column which was 2 cm. high and had a cross-section of 1.3 square cm.

The material was eluted with varying concentrations of hydrochloric acid. A total recovery of 91% was obtained. Of the material recovered, 23% was eluted in the AA fraction, 47% in the "ADP fraction," and 30% in the ATP fraction (Graph #3).

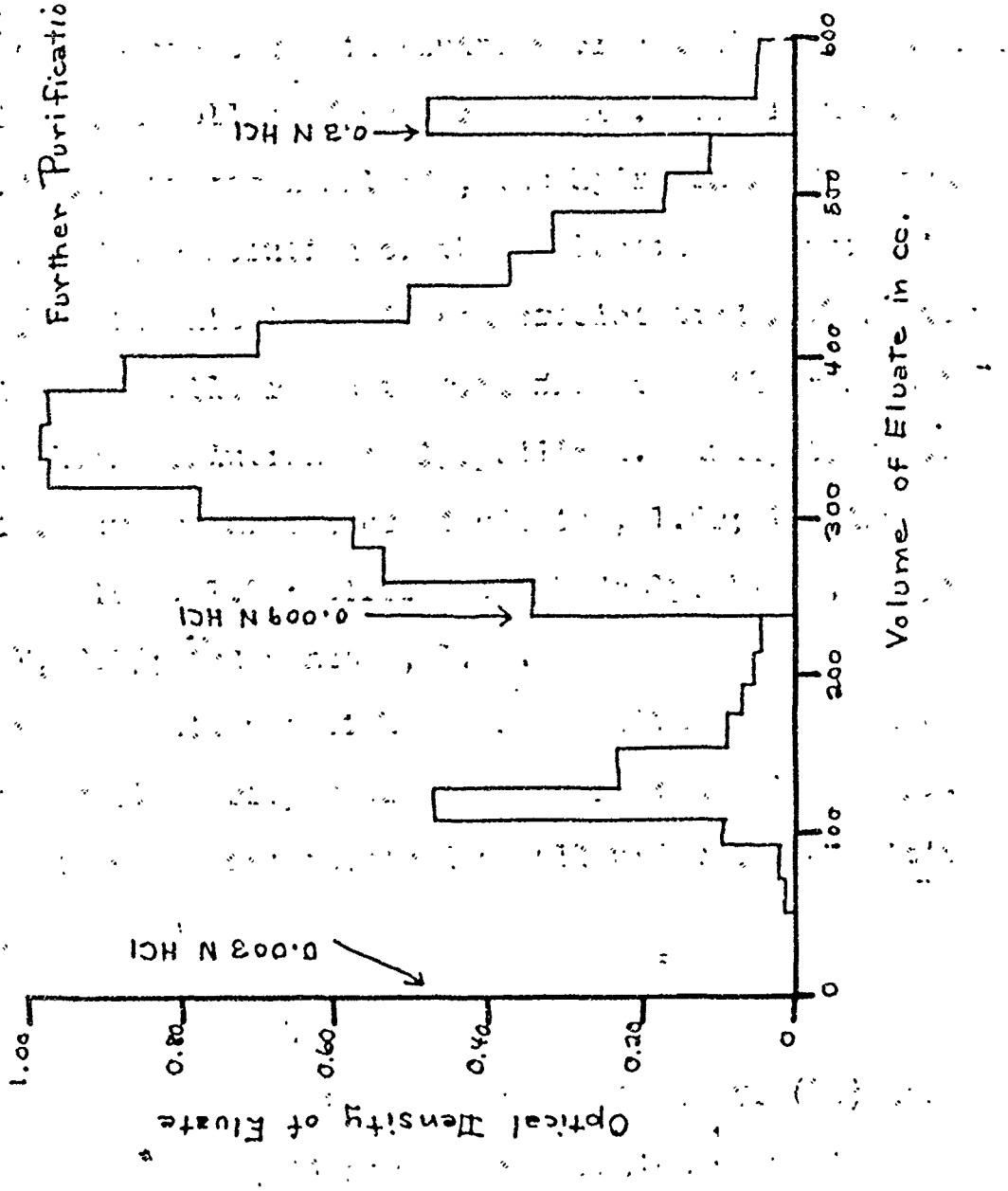
The "ADP fractions" eluted from the column between 260 and 340 cc. were combined, neutralized to pH 6.6, adsorbed on a new column, and eluted as before. The total recovery was 98%. Of the material eluted, 11% was in the AA fraction, and 6% was in the ATP fraction (Graph #4).





Graph #4 Appendix

Further Purification of Nucleotide



Attempts to Differentiate Between ADP and the Nucleotide

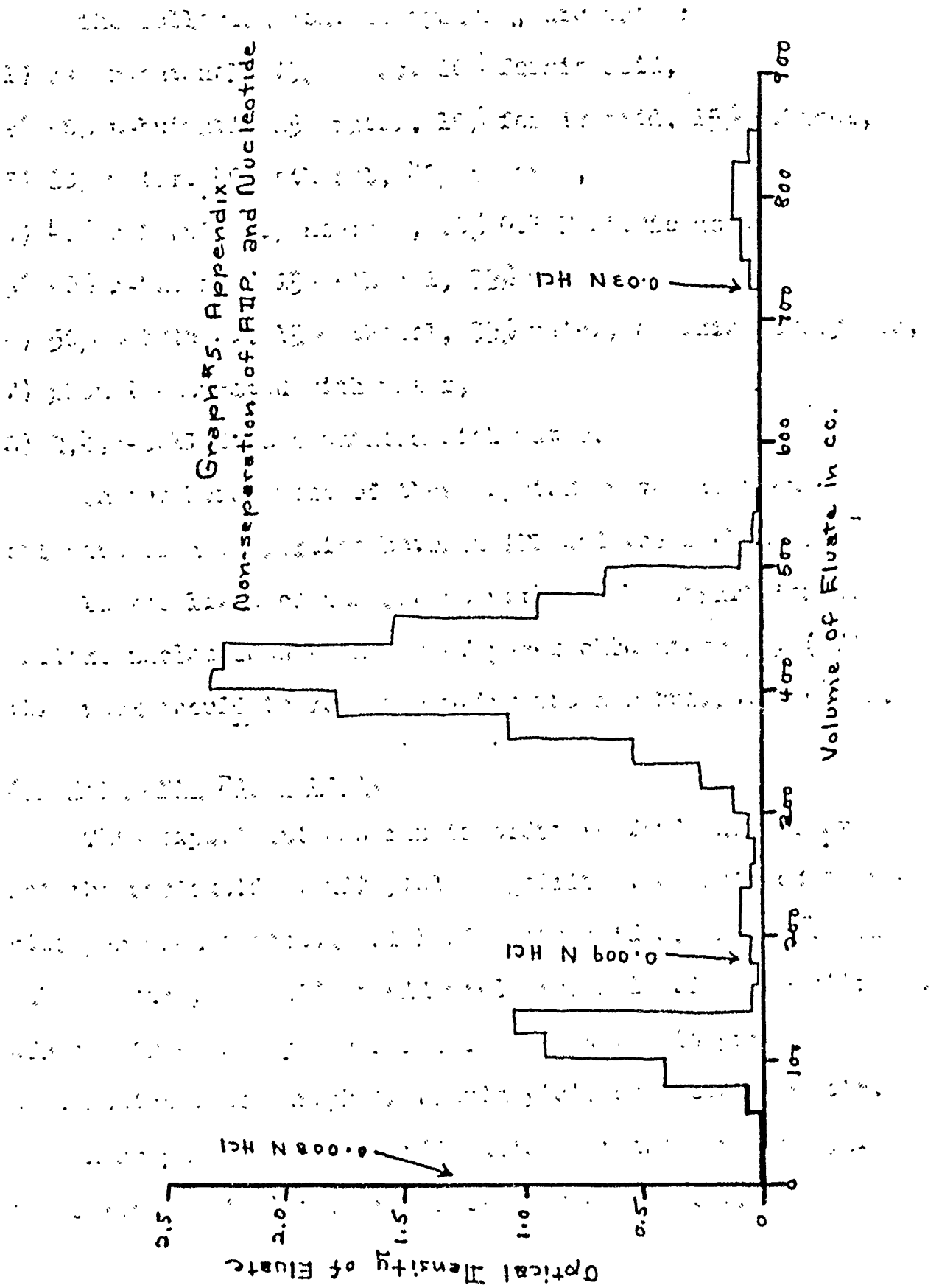
Anion Exchange Method Applied to a Mixture of A[illegible] and the Nucleotide.

A sample of the unknown material was adsorbed on a Dowex column and the acid eluate corresponding to the "ADP fraction" was collected as a lead salt. The lead was removed with hydrogen sulfide, the hydrogen sulfide was removed and the solution of the nucleotide was acidified to 0.05 N with nitric acid, and precipitated with silver nitrate. The silver was removed from the silver salt and the resulting solution was lyophilized. Analysis of the material gave molar ratios of adenine, 1.00; 15 minute hydrolyzable P, 1.09; difficultly hydrolyzable P, 1.03; total P, 2.07; and pentose, 1.03.

Some of this material was mixed with a solution of ADP from a Sigma Preparation of the Barium salt of ADP. This mixture was not resolved at all under the conditions used (Graph #5).

Paper Chromatography.

The methods used by Vischer and Chargaff (40) and Markham and Smith (41) for the separation of various purines and pyrimidines were adapted for our use.



The following solvent systems were tried:

1) 77% n-butanol, 13% water, 10% formic acid,

2) 50% n-butanol, 25% water, 10% formic acid, 15% ethanol,

3) 20% water, 40% ethanol, 40% acetone,

4) 40% ethanol, 40% acetone, 20% 0.1 N nitric acid,

5) 50% n-butanol, 15% ethanol, 35% water,

6) 50% n-butanol, 15% ethanol, 35% water, ammonia atmosphere,

7) phenol saturated with water,

8) 2,4,6-collidine saturated with water.

In our hands none of these systems gave clear-cut evidence of a separation between ADP and the nucleotide.

In the light of the recent work on the separation of various nucleotides by means of paper chromatography (37) this work should be repeated using the new solvent systems.

Adenylic Acid Desaminase.

This experiment was run in order to find whether or not the nucleotide would yield significant amounts of amino-nitrogen when incubated with adenylic acid desaminase. One mole of adenylic acid should yield one mole of amino-nitrogen, ATP or ADP should yield none, if the enzyme is pure. Diadenosine tetraphosphate should yield one mole per mole.

A preparation of adenylic acid desaminase was prepared after Lohmann (42) from the back muscle of a rat.

On incubation with the enzyme, adenylic acid gave close to the theoretical yield. ATP and the nucleotide gave very little amino-nitrogen.

Molecular Weight Determination.

The silver was removed from a sample of the silver salt of the nucleotide (see section on acid silver fractionation) and the solution of nucleotide was lyophilized.

A cryoscopic determination coupled with a measurement of the hydrogen ion concentration gave a molecular weight of 470 ± 50. This result is strong evidence against the identity of the nucleotide with diadenosine tetraphosphate (MW = 836).

Precipitation Behavior of the Nucleotide and of ADP.

Barium-free solutions of ADP were prepared from the Sigma barium salt. With concentrations as high as 24 mg. of ADP/cc., no turbidity was produced by the addition of two volumes of ethanol. A faint turbidity finally appeared when a volume of acetone was added to the solution which was 66% ethanolic. A white gum was obtained when another volume of acetone was added.

Solutions of the nucleotide prepared by acid silver fractionation or by anion exchange and silver fractionation in concentrations of 10 mg./cc. were partially precipitated

by one volume of alcohol.

Summary of the Work on the Nucleotide

A nucleotide which is easily precipitated by alcohol from a concentrated solution has been isolated from dog and rabbit liver.

The nucleotide has a molecular weight of 470 ± 50. Molar ratios of adenine, 1.0; 15. min. hydrolyzable P, 1.0; difficultly hydrolyzable P, 1.0; and ribose, 1.0 are indicated. The material is not significantly attacked by adenylic acid desaminase. In our hands, mixtures of the nucleotide and ADP have not been separated by anion exchange or by paper chromatography. The nucleotide is precipitated from solution much more readily than ADP.

On the basis of these findings, it is suggested that the nucleotide is an isomeric ADP.

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