for the reaction of ethyl acetate may be caused by a restricted configuration in which there is hydrogen bonding between the β-hydrogens in the ester grouping and the carbonyl oxygen in the activated state. The same picture applies to the *n*-propyl ester, but to a lesser degree since only two such β-hydrogen atoms are available here. This type of H-bonding is, in all probability, found in the isopropyl ester in both reactant and activated state and would therefore not be reflected in the entropy term.

It should be noted that at all temperatures at which these studies were made ethylene glycol is definitely the best catalyst of the three considered, when comparison is made on the basis of equivalent hydroxyl concentration. Water is somewhat lower in catalytic activity and methanol is almost a power of ten lower than either of the other two catalysts.[6] It is apparent, as seen in Fig. 1, that, because of the temperature coefficients, at a higher temperature water might very well become a better catalyst than ethylene glycol.

Separating the various factors affecting catalytic activity of the three catalysts studied presents a difficult problem. The fact that the ammonolysis reaction as catalyzed by water gives the smallest decrease in the entropy of activation is not surprising in view of the small and unhindered water molecule. The high activation energy for water catalysis may very well be caused by the many and complex ways in which water and ammonia can be associated. Catalysis by ethylene glycol gives a low activation energy, possibly because of the adjacent hydroxyl groups, both of which may be attached to the same ammonia molecule. The fact that the total energy barrier for the reaction catalyzed by methanol is the same as that for ethylene glycol may possibly indicate that not one but two molecules of methanol bond with one molecule of ammonia to form the amide ion. This would also account for the relative order of decreasing entropies of activation.

Because of the necessary modifications of experimental procedure for ammonolysis of the lactates and the relatively large experimental error, quantitative deduction from the data obtained in these reactions is difficult. The reaction is self-catalyzed *via* the hydroxyl group contained in the lactate ester. The presence of ethylene glycol may be expected to lower the potential energy barrier, because of the increased availability of the hydroxyl groups, but evidently it causes a larger decrease in the total energy barrier by a large decrease in the entropy of activation. The latter may be caused by hydrogen bonding between the second hydroxyl group of the glycol and the hydroxyl group of the lactate. This in turn causes a more rigid complex in the activated state than would be true in the uncatalyzed reaction.

It is to be noted that comparison of the uncatalyzed reaction of methyl lactate with ammonia may be made with the reaction of methyl acetate with ammonia in the presence of ethylene glycol on the basis of either velocity constants or energies of activation, *i.e.*, potential energy factors. This is justified in view of the equality, within experimental error, of the entropies of activation of these two reactions. Such an interpretation has been made in the earlier work.[2a,b]

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(6) R. Baltzly, I. M. Berger and A. A. Rothstein (THIS JOURNAL, **72**, 4149 (1950)) have described methanol as the best catalyst for the aminolysis of a series of esters. These workers, however, used methanol as both solvent and catalyst for their investigation and a fair comparison of catalysts cannot be made under these circumstances.

[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

The Separation of Sugar Phosphates by Ion Exchange with the Use of the Borate Complex[1]

BY JOSEPH X. KHYM AND WALDO E. COHN

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Borate complexing is used to effect separations of the commonly encountered monophosphorylated sugars by anion exchange in an alkaline chloride system. Phosphoglyceric acid, fructose diphosphate and the adenosine polyphosphates are separated from the sugar monophosphates and from each other by simple *p*H and ionic strength adjustment. Using a sequence of eluting solutions of varying borate concentration, chloride concentration and *p*H, each member of both groups of substances can be isolated from all of the others in a single continuous process.

Introduction

Except for the almost complete separation of ribose-5-phosphate from ribulose-5-phosphate as achieved by Horecker and Smyrniotis,[2] a direct separation of monophosphorylated sugars by ion-exchange methods has not yet been reported. The complete separation of fructose-6-phosphate from fructose-1,6-diphosphate by anion exchange has been demonstrated by Benson, *et al.*[3] In this instance the presence of two phosphate groups in fructose-1,6-diphosphate accounts for the high degree of separation obtained. However, the similarity of the structural configurations and the nearly identical dissociation constants[4] of the commonly occurring sugar monophosphates are two factors which make unlikely their complete separation by simple ion exchange.

For purposes of identification of phosphate esters in complex mixtures, the techniques of paper chromatography have been more successful. Bandurski and Axelrod[5] used this technique to identify

(1) Work performed under contract No. W-7405-eng-26 for the Atomic Energy Commission.

(2) B. L. Horecker and P. Z. Smyrniotis, *Arch. Biochem.*, **29**, 232 (1950).

(3) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Hass and W. Stepka, THIS JOURNAL, **72**, 1710 (1950).

(4) W. D. Kumler and J. J. Eiler, *ibid.*, **65**, 2355 (1943).

(5) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

phosphate esters found in plants. Their system of two-dimensional chromatography resolves more components than the unidimensional method of Hanes and Isherwood.[6] The use of boric acid in organic solvent systems to develop papergrams has been used by Cohen and Scott[7] to distinguish between the pentose phosphates. Dulberg, *et al.*,[8] partially hydrolyzed the hexose phosphates in 1 *N* hydrochloric acid, then removed the unhydrolyzed hexose phosphates with anion-exchange columns, and characterized by paper chromatography the free sugars which passed through the column. This process is similar to the method of McCready and Hassid[9] who isolated glucose-1-phosphate from sugar mixtures by using anion exchangers. A combination of paper chromatography, ion exchange and radioautography has been used by Benson, *et al.*,[3] to characterize the sugar phosphates and related substances.

Most of these methods have been developed primarily for the qualitative identification of phosphate esters. The object of the present work was to investigate a direct ion-exchange method for the quantitative separation, identification, and recovery of each component from mixtures of glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, fructose-1,6-diphosphate, free hexose, inorganic phosphate, 2-phosphoglyceric acid (2-PGA) and the adenosine phosphates AMP, ADP, ATP.[10]

Preliminary Considerations

From the results of Cohn and Carter[11] as well as those of Benson, *et al.*,[3] it could be anticipated that the separation of the dibasic acids (ADP, 2-PGA) from the monobasic ones (AMP, fructose phosphate, etc.) would require only simple *p*H and ionic strength adjustments, whereas the separations within each group might require the additional factor of borate complex formation. The latter assumption was easily verified; the sugar monophosphates failed to separate appreciably in alkaline, neutral or acidic elution systems without borate present, whereas those dibasic acids here considered separated well without the use of this agent.

Since borate ion exists only in alkaline solutions where phosphate esters are doubly ionized and hence more strongly bound to anion exchangers, the use of borate as the replacing ion as well as the complex-forming ion involves higher concentrations than are compatible with easy recovery of the separated esters. Hence, we have investigated the possibility of using a chloride system for the separation with only sufficient borate for complexing. (Jaenicke and Dahl[12] have used boric acid in the ion-exchange separation of ribosides by sodium chloride solutions.) It appears that low (10^{-2} to 10^{-5} *M*) concentrations of borate, in ammonium hydroxide–ammonium chloride buffers, are sufficient to form complexes, and therefore affect the ion-exchange affinities of the sugar monophosphates. Thus the recovery of the esters from the eluting solution is more easily accomplished than from the strong borate solutions previously used for neutral sugar separations.[13]

Experimental

Apparatus and Methods.—Small conventional analytical columns were used throughout these experiments. An automatic sample changer was adjusted to collect serial samples of any desired volume. Free hexose, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate were assayed according to the anthrone method[14,15] and ribose-5-phosphate by the orcinol method.[16] Although these methods were originally intended for the determination of the free sugars, they can be used effectively for the determination of the respective phosphates. Inorganic and total phosphorus were assayed by the method of Fiske and SubbaRow.[17] All the colorimetric determinations were made with Coleman or Evelyn colorimeters while AMP, ADP and ATP were determined by ultraviolet absorption with the Beckman model DU spectrophotometer.

Ion Exchangers.—All separations were performed with 200–400 mesh strong base anion exchanger (Dowex-1). The exchanger was washed free of fines by decantation, slurried into the columns, and converted to the chloride form by washing with 1 *N* hydrochloric acid. A strong acid exchanger (Dowex-50) was used to convert the potassium and barium salts of test materials to acids. These solutions were then neutralized with ammonium hydroxide and stored in the cold.

Test Materials.—The hexose monophosphates, fructose-1,6-diphosphate, 2-PGA, inorganic phosphate (K_2HPO_4), AMP, ADP and ATP were obtained from commercial sources. In order to convert them to free acids, Dowex-50 in the hydrogen form was added batchwise to stock solutions of these compounds and then removed by filtration. A modification which greatly simplifies the method of Marmur, Schlenk and Overland[18] was used to prepare ribose-5-phosphate. Adenosine-5-phosphate, 400 mg., was dissolved in 10 ml. of 0.1 *N* sulfuric acid and heated at 100° for four hours. Barium hydroxide was then added to remove the sulfate ion and inorganic phosphate; then, Dowex-50 in the hydrogen form was added batchwise to remove barium ions, adenine and adenylic acid and additional treatments with Dowex-50 yielded a product of high purity. The ribose-5-phosphate can be isolated as the solid barium salt, if desired, by adding 3 volumes of alcohol.

No distinction is made between AMP (adenosine-5-phosphate), adenylic acid *a*[19] or adenylic acid *b*[19] in the procedures to be described; all three are eluted in the same position. Only adenosine-5-phosphate is considered in the work presented here.

Procedure.—After the columns in the chloride form were water washed to remove excess hydrochloric acid, the test material (approximate amounts of the components are given in Table I) was absorbed from 25 ml. or less of dilute ammoniacal solution at about *p*H 8.5. Free sugars which did not adsorb on the resin were washed free of the exchanger with 100 ml. of 0.001 *M* ammonium hydroxide. Next a succession of different eluting agents, in a given order, was passed through the column to selectively desorb the remaining components of the test material.

Identification.—Individual column runs were carried out on each substance in order to establish the elution order and to determine the presence of any impurities. The impuri-

(6) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(7) S. S. Cohen and D. D. M. Scott, *Science*, **111**, 543 (1950).

(8) J. Dulberg, W. G. Roesoler and T. H. Sanders, *J. Biol. Chem.*, **194**, 199 (1952).

(9) R. M. McCready and W. Z. Hassid, THIS JOURNAL, **66**, 560 (1944).

(10) AMP, adenosinemonophosphate; ADP, adenosinediphosphate; ATP, adenosinetriphosphate.

(11) W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1951).

(12) L. Jaenicke and K. V. Dahl, *Naturwissenschaften*, **39**, 87 (1952).

(13) J. X. Khym and L. P. Zill, THIS JOURNAL, **74**, 2090 (1952).

(14) R. Dreywood, *Ind. Eng. Chem., Anal. Ed.*, **18**, 499 (1946).

(15) D. L. Norris, *Science*, **107**, 254 (1948).

(16) A. H. Brown, *Arch. Biochem.*, **11**, 269 (1946).

(17) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(18) J. Marmur, F. Schlenk and R. N. Overland, *Arch. Biochem. Biophys.*, **34**, 209 (1951).

(19) W. E. Cohn, THIS JOURNAL, **72**, 1471 (1950).

TABLE I

ANALYTICAL DATA FOR SEPARATION DEMONSTRATED IN FIG. 2

Compound	Assay method	Wave length used, mμ	Approx. amount added,[a] ~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	U.V. absorption	260	8	95
ADP	U.V. absorption	260	5	102
ATP	U.V. absorption	260	6	100

[a] 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.

ties that were discovered by the use of assay methods previously described were, with one exception, identical with the components under investigation. By using a large amount of each test substance and making individual column analyses, the impurities were determined quantitatively. The purity of 2-PGA was found to be only 71% and that of ADP, 88%. The purity of all the other materials studied ranged from 90–100%. Anthrone-reacting impurities found in fructose-6-phosphate were free hexose and glucose-1-phosphate. Inorganic phosphate was detected in 2-PGA and in all the sugar phosphates; three of the sugar phosphates were found to contain considerable amounts of 2-PGA. An unidentified component eluted from the exchanger with 1 *N* hydrochloric acid and assayed by total phosphorus was found in 2-PGA. Individual analyses of AMP, ADP and ATP showed that these compounds were cross contaminated.[11] Accordingly, impurities in the initial test materials were considered during the calculation of the percentage of recoveries, which were essentially quantitative.

Further identification was desirable in some cases. In the case of the sugar phosphates, with the exception of ribose-5-phosphate, the fractions obtained by the procedure illustrated in Fig. 2 were concentrated *in vacuo* to about 5 ml. and the barium salts isolated. After removal of barium with Dowex-50, the sugar phosphates were identified by paper chromatography, using the method of Bandurski and Axelrod.[5] Ribose-5-phosphate was characterized by elution position, reaction to orcinol reagent, its negative behavior to the anthrone reagent, and its non-absorption in the ultraviolet. All these tests were applied in the region in which ribose-5-phosphate was eluted.

The adenosine derivatives, AMP, ADP and ATP, have been characterized and their elution order established by the anion-exchange method of Cohn and Carter[11] using a chloride system. Further identification was made on pooled fractions by chemical analysis[20] (adenine:acid labile P:total P).

Inorganic phosphate was characterized chiefly on the basis of elution position and its colorimetric determination. 2-PGA was characterized by total phosphorus, its negative reaction to all the other assay methods, as well as its ion-exchange behavior.

Results

The ion-exchange behavior of the sugar phosphates in ammonium hydroxide–ammonium chloride buffers with varying concentrations of borate

(20) A. L. Dounce, *et al.*, *J. Biol. Chem.*, **174**, 361 (1948).

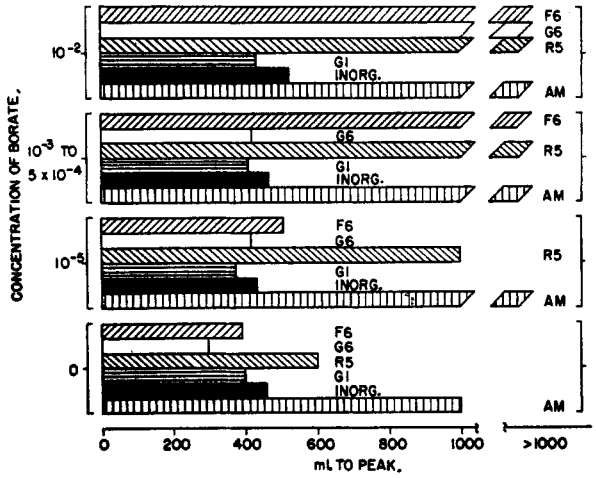

Fig. 1.—Average volume to peak of monophosphate elution curves as a function of borate concentration at *p*H 8.3 in 0.025 *M* NH_4Cl + 0.0025 *M* NH_4OH solution: column, Dowex-1, 200–400 mesh, 0.86 cm. × 12 sq. cm.; F6, fructose-6-phosphate, etc.

is shown in Fig. 1. It is to be noted that, whereas no differences of appreciable magnitude are to be found in the absence of borate ion, only very small amounts of the latter are required to change radically the affinities and hence the potential separabilities of the various esters. Ribose-5-phosphate is affected markedly by 10^{-5} *M* borate, followed by fructose-6-phosphate at 10^{-4} *M*, and glucose-6-phosphate at 10^{-2} *M*. Hence, an elution sequence in the order 10^{-2} *M*, 10^{-4} *M*, 10^{-5} *M* and 0 *M* borate should separate glucose-1-, glucose-6-, fructose-6- and ribose-5-phosphate in that order. Such a separation, conducted in the presence of polyacidic sugar esters, is shown in Fig. 2.

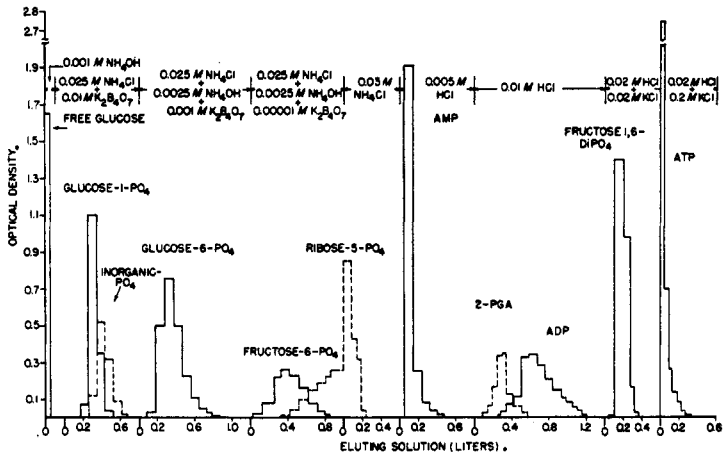

Fig. 2.—Ion-exchange separation of sugar phosphates, inorganic phosphate, adenosine phosphates and phosphoglyceric acid (amounts of each are given in Table I): exchanger, Dowex-1, *ca.* 300 mesh, 0.86 sq. cm. × 12 cm., chloride form; rate, 3.5 ml./min.

As already noted by Benson, *et al.*,[3] and by Cohn and Carter,[11] polyphosphorylated (or polyacidic) derivatives differ so markedly from the monophosphorylated ones in ion-exchange behavior that such devices as complex formation are not necessary for the separation of the two groups. The typical

analysis shown in Fig. 2 indicates that the latter compounds also differ sufficiently among themselves to permit simple *p*H and ionic strength adjustments to accomplish the separation of each component (*cf.* Carter and Cohn[11]).

Discussion

Since the elution position of glucose-1-phosphate is unaffected by moderate amounts of borate ion, it would appear that this compound does not form a borate complex. This observation is in agreement with the principles discussed in the studies on the ion-exchange behavior of the sugar–borate complexes and with the conductivity studies of sugar derivatives carried out in solutions of boric acid by MacPherson and Percival[21] which indicate that the presence of a phosphate group on carbon atom number one in glucose eliminates the possibility of a strongly ionized borate complex being formed.

From the behavior of neutral sugars in borate solutions, it was concluded[13] that the ion-exchange affinities of borate diols formed from furanose structures were greater than those formed from pyranose structures. A separation of these two borate diols of one given sugar was not obtained, presumably due to the equilibrium which exists between the two forms. In the present studies, this conclusion is confirmed. Glucose-6-phosphate can form a borate complex from either its pyranose or furanose structure, while the ring form of fructose-6-phosphate and ribose-5-phosphate can only be of the furanose type. These latter two sugar phosphates exhibit a very strong affinity for the exchanger when only small amounts of borate ion (about 0.0005 *M*) are included in the ammonium chloride–ammonium hydroxide eluting solutions. The affinity of glucose-6-phosphate remains unchanged unless the borate ion concentration is increased at least twentyfold (about 0.01 *M*). It is most probable, as assumed for the sugars, that an equilibrium exists between the two forms of the borate complex of glucose-6-phosphate, which allows less time for the borate complex of the furanose form to make contact with the exchanger. Therefore, if interconversion is possible in a sugar phosphate, less affinity for the exchanger is to be expected than where only a furanose ring form can exist.

The elution order of the adenosine derivatives has already been established.[11] After removal of the monophosphorylated sugars from the exchanger, the elution system reported here differs only slightly in that ADP is eluted at a lower chloride ion concentration allowing elution of fructose-1,6-diphosphate between ADP and ATP. The presence of two ionizable groups in fructose-1-diphosphate and 2-PGA easily explains the strong affinity of these compounds for the exchanger.

Acknowledgment.—The authors wish to express appreciation to Dr. G. R. Noggle for performing the paper chromatographic analyses on the sugar phosphates isolated by the ion-exchange columns during these experiments.

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[CONTRIBUTION NO. 292 FROM THE CHEMICAL DEPARTMENT, EXPERIMENTAL STATION, E. I. DU PONT DE NEMOURS AND COMPANY]

Alicyclic Diamines. Preparation of Bis-(4-aminocyclohexyl)-methane

BY A. E. BARKDOLL, D. C. ENGLAND, H. W. GRAY, W. KIRK, JR., AND G. M. WHITMAN

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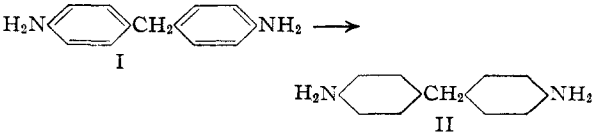
While the hydrogenation of simple aromatic monoamines to the corresponding alicyclic amines can be accomplished with common catalysts such as Raney nickel, the hydrogenation of more complex aromatic diamines such as bis-(4-aminophenyl)-methane (I) to bis-(4-aminocyclohexyl)-methane (II) presents a more difficult problem. Accordingly, the application of a number of catalysts to the hydrogenation of I to II has been studied, with the finding that ruthenium dioxide is a very effective catalyst for this hydrogenation. Also, German reports of the activity of a mixture of cobaltic oxide–calcium oxide–sodium carbonate as a catalyst for the hydrogenation of I to II have been confirmed, and some observations have been made on the role played by the various components of this catalyst.

Numerous catalysts and techniques[1] have been described for the hydrogenation of relatively simple monocyclic aromatic amines to the corresponding alicyclic amines. The use of certain of these catalysts which require an acid medium for successful operation is accompanied by a corrosion problem in metal equipment, and necessitates recovery of the product from the acid medium. Also, the hydrogenation of aromatic amines in glacial acetic acid solution with platinum oxide catalyst, as exemplified by the reduction of benzidine,[2] frequently yields ammonia and condensation products, in addition to the desired product. Other catalysts, such as Raney cobalt, when applied to relatively complex diamines such as bis-(4-aminophenyl)-methane (I) are reported to be ineffective.[3]



Recently, two excellent catalysts for the hy-

(1) P. Sabatier and J. B. Senderens, *Compt. rend.*, **138**, 457 (1904); R. Willstätter and D. Hatt, *Ber.*, **45**, 1471 (1912); A. Skita and W. Berendt, *ibid.*, **52**, 1519 (1919); G. S. Hiers and R. Adams, *ibid.*, **59**, 162 (1926); H. Heckel and R. Adams, THIS JOURNAL, **47**, 1712 (1925); G. S. Hiers and R. Adams, *ibid.*, **49**, 1099 (1927); H. Adkins and H. I. Cramer, *ibid.*, **52**, 4349 (1930).

(2) F. Balas and P. Ševčenko, *Collection Czechoslov. Chem. Commun.*, **3**, 171 (1931); *C. A.*, **26**, 2990 (1931).

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