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

# Identification of the Purine Nucleotides *a* and *b* as the 2′- and 3′-Phosphoribosides, Respectively[1]

BY JOSEPH X. KHYM AND WALDO E. COHN

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The isomeric adenylic acids derived from the alkaline hydrolysis of ribonucleic acids and known as *a* and *b* have been identified as adenosine-2′- and -3′-phosphates, respectively, by measuring the amounts of each of the ribose phosphates produced from either nucleotide upon cleavage of the N-glycoside linkage. Hydrolysis to adenine and ribose phosphate was effected with a polystyrenesulfonic acid ion-exchange resin which absorbed the nucleotides but not the produced ribose phosphates. Post hydrolytic isomerization of the ribose phosphates was thus reduced to insignificance during the remainder of the brief hydrolysis period. Assay of the ribose phosphates produced was achieved by ion-exchange chromatography in the presence of borate ion, which differentially complexes all five of the ribose phosphates and permits the separation and assay of each. Periods of hydrolysis were short and were comparable to the time required for the starting nucleotide to produce the equilibrium amount of its isomeric form, thus allowing the ribose phosphate derived from the former to predominate. In this manner, each ribose phosphate is shown to be the daughter of one adenylic acid. Similar results were attained with the guanylic acids. Evidence is presented to indicate that the discrepancies in the earlier attempts to determine the structure of these nucleotides by similar degradative procedures arose from acid-catalyzed phosphomigration during the procedures.

## Introduction

Prior to 1949, the ribonucleotides derived from ribonucleic acids (RNA) were generally assumed to be the 3′-phosphates of the four known nucleosides.[2] This concept rested on the preparation of optically inactive ribitol phosphate by reduction of the ribose phosphate[2a] derived by acid hydrolysis of xanthylic acid[3] (from guanylic acid isolated from yeast RNA[4]). This inactivity was regarded as proof[2] that the phosphate group was at the 3-position in the ribitol phosphate and, therefore, at the 3′-position in the parent nucleotide.

Uncertainty regarding this assignment was introduced by certain observations which followed the discovery, in 1949, of two isomeric adenylic acids[5] (and, subsequently, isomeric pairs of guanylic,[5b] cytidylic[6] and uridylic[6a] acids), termed *a* and *b*, in alkaline hydrolyzates of yeast RNA. These observations were (1) the ready acid-catalyzed interconversion of the isomers[6a,7] (which cast doubt on the reliability, with respect to phosphate position, of any degradative steps requiring acid) and (2) the discovery that the supposed synthetic 2′-isomers were actually 5′-isomers,[7,8] leading to the re-formulation of the intermediate benzylidene adenosine as 2′:3′ instead of 3′:5′ and thus invalidating the prior synthetic identifications of the 2′- and 3′-nucleotide isomers.[9]

The formulation of each pair of *a* and *b* nucleotides as nucleoside-2′- and 3′-phosphates, but not necessarily respectively, was based on the following observations: (1) the acid-catalyzed conversion of either one into a roughly equal (40–60) mixture of both (most easily observed in the acid-stable pyrimidine nucleotides[6a,7]); (2) the elimination of the 5′-isomer from consideration, by comparison of ion-exchange and enzymic behavior of the isomers with synthetic material of unquestioned authenticity, and of amino group isomerism by the demonstration[10] of continued isomerism in deaminated adenylic acids; (3) the production of both isomers simultaneously by the phosphorylation of $N_6$,5′-dityrityladenosine or of 5′-trityladenosine and subsequent removal of trityl groups[11]; (4) the stability of both isomers to periodate[7] and their non-reactivity with copper ion[10a] (indicating the absence of a free *cis*-glycol system); (5) the alkaline hydrolysis of adenosine benzyl hydrogen phosphate *a* (and, independently, of *b*) to a mixture of adenylic acids *a* and *b*,[11] indicating phosphomigration during alkaline hydrolysis, a direct parallel to the method by which the isomers are derived from RNA[12]; (6) the synthesis[13] and the isolation from enzymic digests[14] of the cyclic-2′:3′-phosphates of adenosine, cytidine and uridine and the demonstration that these hydrolyze in alkali to give quantitatively the same mixtures of *a* and *b* isomers found in RNA hydrolyzates; (7) identical rates of acid hydrolysis of the phosphate group in the purine isomers,[10] differing from the 5′ resistance to acid hydrolysis,[2f,15] indicating either similarity in structure or, as now known common intermediates[14]; (8) the lack of chemical[7,16a] or enzymic[16b,c] evidence

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

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(9) (a) J. M. Gulland and H. Smith, *ibid.*, 338 (1947); 1527 (1948); (b) A. M. Michelson and A. R. Todd, *ibid.*, 2476 (1949).

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(12) D. M. Brown and A. R. Todd, *ibid.*, 52 (1952).

(13) D. M. Brown, D. I. Magrath and A. R. Todd, *ibid.*, 2708 (1952).

(14) R. Markham and J. D. Smith, *Nature*, **168**, 406 (1951); *Biochem. J.*, **52**, 552, 558, 565 (1952).

(15) (a) H. G. Albaum and W. W. Umbreit, *J. Biol. Chem.*, **167**, 369 (1947); (b) P. A. Levene and E. T. Stiller, *ibid.*, **104**, 299 (1934).

(16) (a) J. Davoll, B. Lythgoe and A. R. Todd, *J. Chem. Soc.*, 833 (1946); V. M. Clark, A. R. Todd and J. Zussman, *ibid.*, 2952 (1951); G. A. Howard, G. W. Kenner, B. Lythgoe and A. R. Todd, *ibid.*, 855, 861 (1946); (b) H. S. Loring, H. W. Bortner, L. W. Levy and M. L. Hammell, *J. Biol. Chem.*, **196**, 807 (1952); H. S. Loring, M. L. Hammell, L. W. Levy and H. W. Bortner, *ibid.*, **196**, 821 (1952); P. Reichard, Y. Takenaka and H. S. Loring, *ibid.*, **198**, 599 (1952); (c) A. Kornberg and W. E. Pricer, Jr., *ibid.*, **186**, 557 (1950).

for $\alpha,\beta$-furanoside isomerism, suggested as a possible alternative explanation of the isomerism.[5c,10,17] Although not all of these observations were made on each pair of nucleotides, the early assumption, based on ion-exchange behavior, that all *a* isomers were derivatives of one ribose phosphate structure and all *b*'s of the other was strengthened by the derivation of uridylic acid *b* from cytidylic acid *b*[18] and by the similarities in the susceptibilities of all *a*'s and all *b*'s to a *b*-specific phosphatase.[19a]

The certainty, from these and related experiments, that the *a* and *b* nucleotides were the -2'- and -3'-phosphates of the nucleosides, but not necessarily respectively (a certainty originally heightened by the supposed 2',3'-structure of RNA[2e]), preceded by only a short time the discovery of the 5'-phosphates as products of the enzymic hydrolysis of RNA.[19] This discovery led to the postulate[19c,12] and subsequent confirmation[14,20,21] of a 2',5'- and/or 3',5'-phosphodiester structure for RNA in place of the older 2',3'-structure. This postulate had indeed been advanced thrice[22] before (at least once[22a] on evidence comparable to the newer[19] evidence) but so strongly entrenched was the older hypothesis of a 2',3'-structure, although unsupported by any direct evidence, that it was withdrawn by the proposer.[23] The 2',5'- and/or 3',5'-structure, when viewed in the light of older observations on the migration of phosphate[24] in glycols, indicated at once a mechanism of the production of the 2'- and 3'-isomers in the alkaline hydrolysis of RNA.[12-14] However, the same ease of interconversion in acid and of simultaneous production of isomers in acid or alkali which strengthened the belief that they were the nucleoside-2'- and -3'-phosphates served, as just mentioned, to invalidate the conclusions drawn from the classical degradative and synthetic methods of determining the structure of either.

In the absence of unambiguous degradative or synthetic methods, measurements of the physical and chemical properties of the cytidylic and the adenylic acid isomers supported the original insecurely based conclusions that the *a* isomers were the 2'-nucleotides and the *b*'s the 3'-nucleotides, exactly as first postulated on questionable evidence. Differences in the ultraviolet absorption characteristics,[6] solubilities[6b] and melting points[6b] of the pyrimidine nucleotide isomers were noted at the time of their discovery and subsequently,[16b,25] as were differences in the *pK* values of the phosphate groups,[10a,25] rotations,[10a,16b] melting points[10a,11,16b] and solubilities[10a,16b] of the adenylic acid isomers. That the *b* isomers of these two nucleotides have the greater separation of charge, which is consistent with a 3'-phosphate structure, is indicated by their greater densities[25] and the lower *pK* values[25] of the amino groups. A similar conclusion has been reached from alkaline spectrophotometric measurements[26] which relate the cytidylic acid *a* to deoxycytidylic acid in that neither has a free 2'-hydroxyl.

## Preliminary Considerations

The degradative approach to the problem requires (1) a method to remove the base component and leave the ribose phosphate under conditions in which incomplete isomerization or destruction, either of the parent isomer or of the derived ribose phosphate isomer, occurs, and (2) a method to assay quantitatively each of the produced isomeric ribose phosphates. Enzymic N-glycosidolysis, which should take place at a *p*H ineffective in producing isomerization, failed for lack of an enzyme preparation[27] of appropriate specificity. The purine–ribose linkage is easily split by acid[2a,b] which, however, simultaneously catalyzes the same phosphomigration which makes impossible an interpretation of the older experiments. This problem was solved by the use of a sulfonic acid ion exchanger which absorbs the nucleotides and thus subjects them to a high acidity for the hydrolysis, and also removes the hydrolyzed adenine (or guanine) from solution. More important for the problem at hand, the catalyst ejects the (anionic) ribose phosphates, once formed, into a solution of their own *p*H where they isomerize much more slowly than do the resin-absorbed nucleotides. The amounts of the ribose phosphate isomers found thus reflect the amounts of the adenylic acid isomers from which they were formed rather than an equilibrium mixture.

It soon became apparent that the rate of isomerization of the nucleotides under the influence of the acid resin was comparable to the rate of hydrolysis[2b] so that, even with negligible post hydrolytic isomerization, both forms of ribose phosphate would always be encountered in substantial amounts. This made imperative the development of a method for the identification and assay of each ribose phosphate in a mixture. This need was met by a modification of our method for the separation of the sugar phosphates by ion exchange in the presence of borate[28] and earlier experiments.[29] Ribose-1-phosphate was not expected to be present because of its acid-lability.[30] Nevertheless, the positions, in the elution sequence, of the 1- and 5-isomers were determined by the preparation and testing of each separately, then

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(28) J. X. Khym and W. E. Cohn, THIS JOURNAL, **75**, 1153 (1953).

(29) J. X. Khym and L. P. Zill, *ibid.*, **74**, 2090 (1952).

(30) H. M. Kalckar, *J. Biol. Chem.*, **167**, 477 (1947).

with the others. The positions of the 2- and 3-isomers were determined by large-scale separations and preparations of each followed by chemical determinations of structure.[31] The circumstances attending the appearance of a fifth isomer permitted a tentative designation of it as ribose-4-phosphate. Thus each possible isomer was identifiable as a separate peak in the elution sequence, the order being 4, 2, 3, 1 and 5.

With a hydrolysis procedure to produce ribose phosphates with minimal isomerization and an analytical procedure for determining the amount of each ribose phosphate isomer produced, it became possible to associate ribose-2-phosphate with adenylic acid *a* and ribose-3-phosphate with adenylic acid *b*. The procedures and the results are described in this paper.

## Experimental

**Nucleotides.**—The adenylic and guanylic acids *a* and *b* used were prepared by ion-exchange chromatography[5b] and by crystallization.[32] The 5'-adenylic acid (muscle adenylic acid) was procured commercially.

**Ion-exchange Resins.**—The sulfonic acid resin used as a catalyst to hydrolyze the purine nucleotides to their ribose phosphates (and to remove barium from barium ribose phosphates) was Dowex-50,[33] 100–200 mesh, $H^+$ form, previously washed by decantation with strong base, acid and water. The chromatographic separations of the ribose phosphates (and of the residual nucleotides) utilized columns of a quaternary ammonium polystyrene resin (Dowex-1[33]) in the chloride or sulfate forms.

**Hydrolytic Procedure.**—Although in large-scale preparations of the ribose phosphates from their parent nucleotides a ratio of water (ml.): wet Dowex-50 (ml.): nucleotide (g.) of 10:10:1 was employed, it was found more convenient, on the smaller scale utilized in the analytical and investigative experiments to use a ratio of 1:1:0.01. The water and resin, in a wide-mouth test-tube or beaker, were brought to 100° in a boiling water-bath with mechanical stirring. The nucleotide was then added promptly and the heating and stirring continued for a predetermined period. At the end of this period, the boiling water-bath was replaced by an ice-bath. The stirring was discontinued when temperature equilibration seemed assured and the resin was filtered off through a sintered disc. The purine base and nucleotide retained on the resin were removed by stirring for one hour in excess sodium hydroxide (3 meq. of sodium hydroxide per ml. wet resin), which was neutralized (after filtration and washing) with ammonium chloride and diluted to 0.01 *M* chloride before ion-exchange analysis. The filtrate containing the ribose phosphates (plus a fraction of the unhydrolyzed adenylic acid), ribose, and inorganic phosphate was absorbed at *p*H 8.0 on an ion-exchange column and analyzed for the various substances present.[5b,10] Ribose-1-phosphate was prepared for us by Dr. Volkin, using the method of Friedkin.[34]

**Analytical Procedures.**—Ribose, either free or in combination with phosphate, was assayed by the conventional orcinol colorimetric procedure.[35] Phosphate was estimated as inorganic phosphate by standard procedures.[36] Adenylic acid and adenine were estimated by ultraviolet spectrophotometry at 260 mμ, utilizing extinction coefficients of 14,200 and 12,700, respectively, at *p*H 2.[37]

**Ion-exchange Separations.**—The filtrate from the resin-catalyzed hydrolysis, containing free ribose and inorganic phosphate (usually in negligible amounts), all of the produced isomeric ribose phosphates, and some unhydrolyzed nucleotides (both isomers) was neutralized to *p*H 8 and passed through a column of Dowex-1 chloride approximately 1 sq. cm. × 10 cm. in size. Ribose did not absorb and was completely removed with water. Elution and separation of ribose-2- and -3-phosphates was achieved with 0.04 *M* ammonium chloride containing 0.004 *M* potassium tetraborate, about 1 liter being required. Under these conditions, the adenylic acids are retained; these were then separated with dilute hydrochloric acid as previously described, usually on a separate column.

In the chloride system, ribose-4-phosphate (if present) appeared with ribose-2-phosphate. Its separation was achieved in a sulfate system, eluting first with 2 liters of 0.001 *M* sodium sulfate plus 0.001 *M* sodium tetraborate to remove the ribose-4- and -2-phosphates in that order, then with 300 ml. of 0.005 *M* sodium sulfate to remove ribose-3-phosphate. Alternatively, 1.1 liters of 0.0018 *M* sodium sulfate plus 0.0018 *M* sodium tetraborate were used to separate the 4- and 2-isomers, although less completely.

Flow rates were about 3 ml. per minute. The samples, collected on an automatic time-operated sample changer, were assayed by the procedures just described.

## Results[38]

The separation of the products of a typical partial hydrolysis of adenylic acid *a* with Dowex-50-$H^+$ is shown in Fig. 1. The substances eluted from the resin (adenine and absorbed but unreacted adenylic acid *a* and the adenylic acid *b* produced from it during the hydrolysis period) are separated as shown in Fig. 1A. The supernatant from the hydrolysis procedure was absorbed and the ribose phosphates separated as shown in Fig. 1C. Identification was afforded by comparison with known material.[31] The absence of ribose, which would not absorb and would therefore appear in the first effluent, should be noted (inorganic phosphate was also absent; this serves as a check on the ribose). The adenylic acids on this column were eluted with 0.01 *N* hydrochloric acid, absorbed on a separate column and separated as shown in Fig. 1B.[5b,10] The relative preponderance of the starting substance (*a*) in this unabsorbed and unhydrolyzed material should be compared to the absorbed material in Fig. 1A, where the amount *b* already exceeds the amount of *a* (61:39) and is approaching its maximum of 71%.[39]

The hydrolysis of adenylic acid to adenine and ribose phosphates is essentially complete in 150 seconds and the isomerization of the two nucleotides is essentially complete in 20 seconds under these conditions. Periods of the latter magnitude allow very little secondary decomposition or isomerization of the initially produced ribose-2- and -3-phosphates. Longer periods of heating, however, result in the formation of ribose-4-phosphate from ribose-3-phosphate, on the one hand, and the appearance of ribose and inorganic phosphate, on the other, possibly through isomerization to the acid-labile ribose-1-phosphate. The analysis (for ribose and ribose phosphates) of such mixtures in the sulfate system—which permits the isolation of ribose-4-phosphate—is shown in Fig. 2. The starting material, commercial adenylic acid, is essentially an equilibrium mixture of *a* and *b* isomers; even if it were not, the heating period is sufficient to convert it to such a mixture. All

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(39) The ratio of *a* to *b* (*ca.* 29:71) in acid isomerization is not necessarily related to the ratio of *a* to *b* (*ca.* 40:60) produced in the alkaline hydrolysis of diesters.

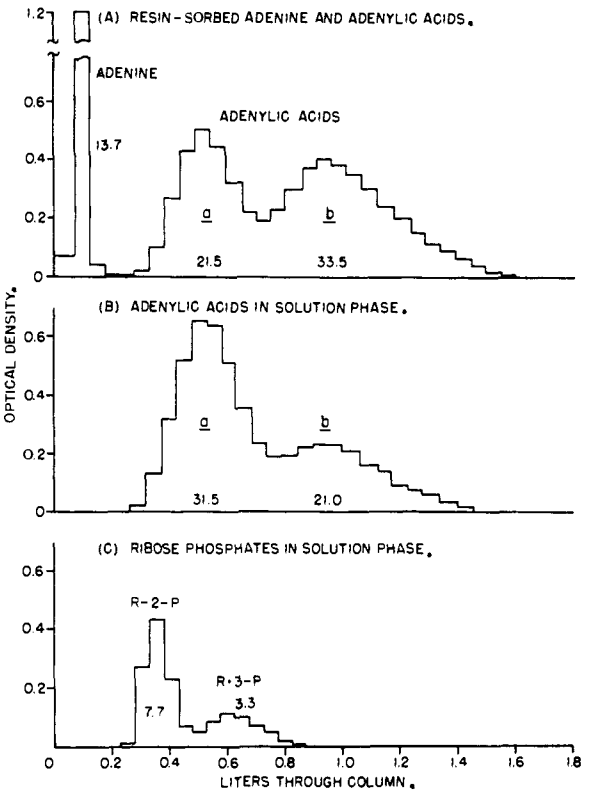


Fig. 1.—Ion-exchange analysis of products of 20-sec. hydrolysis of 60 mg. of adenylic acid *a* in 6 ml. of $H_2O$ with 6 ml. of Dowex-50-$H^+$ at 100°; columns 0.86 cm.$^2$ × 12 cm. Dowex-1-Cl, 300 mesh; A, resin-absorbed adenine and adenylic acids, eluted with 0.002 *N* HCl; B, adenylic acids in solution phase, eluted with 0.002 *N* HCl; C, ribose phosphates (and ribose, if present) in solution phase, eluted with 0.04 *M* $NH_4Cl$ plus 0.004 *M* $K_2B_4O_7$; numbers refer to micromoles recovered.

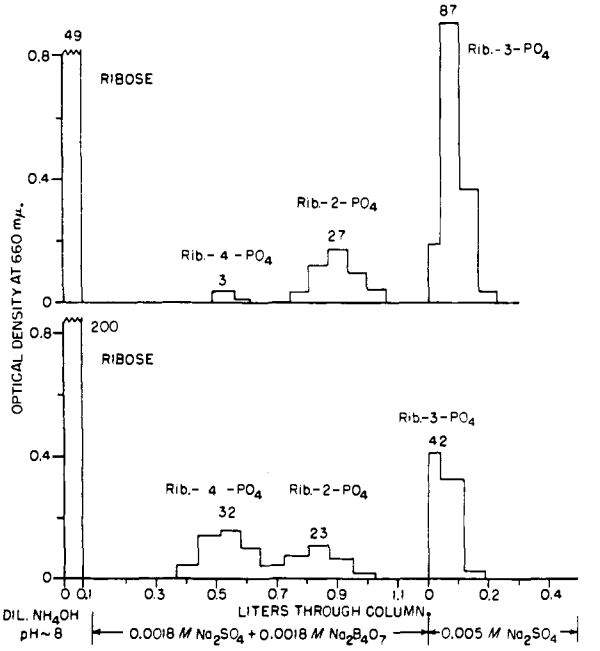


Fig. 2.—Separation of ribose phosphates produced by hydrolysis of commercial adenylic acids with Dowex-50-$H^+$ followed by heating ribose phosphates at their own *p*H at 100°: a, 10 mg. acid, 1 ml. $H_2O$, 1 g. Dowex 50, 20 min.; b, 20 mg. acid, 2 ml. $H_2O$, 2 g. Dowex 50, 120 min.; column 0.86 cm.$^2$ × 12 cm. Dowex-1-$SO_4$, 300 mesh.

adenylic acid has been hydrolyzed and a significant amount of the produced ribose-2- and -3-phosphates has been degraded to ribose-4-phosphate and to ribose plus inorganic phosphate. The increase in these secondary products during the period between 20 and 120 minutes is clearly shown, but the magnitude of the increase indicates their relative insignificance in the 10- to 30-second heating periods employed in the experiments that will be described.

The relative amounts of absorbed adenylic acid *b* and of the ribose-3-phosphate formed from it when adenylic acid *a* is heated with Dowex-50-$H^+$ for varying periods of time are shown by the points in Fig. 3. These points are superimposed upon curves which correspond to the formulas (shown in Fig. 3)[40] derived from the consideration of the process as composed of two parts: (1) a reversible equilibration between adenylic acids *a* and *b*; (2) an irreversible degradation of each adenylic acid to a specific ribose phosphate, these undergoing no further change or decomposition. The adenylic acid points used are those corresponding to the resin-absorbed acids (*e.g.*, Fig. 1A, not 1B). The theoretical amount of ribose-3-phosphate produced is considered to be proportional to the *average* amount of adenylic acid *b* existing throughout the heating period, which is proportional to the integral, with respect to time, of the equation for the instantaneous amount of adenylic acid *b*. The theoretical adenylic acid *b*, curve (B) is normalized to the equilibrium values found ($b/a$ = 71/29) and to one other experimental point. The correspondence of all experimental points with these curves, the predominance of ribose-2-phosphate (A) at early periods and the predominance of ribose-

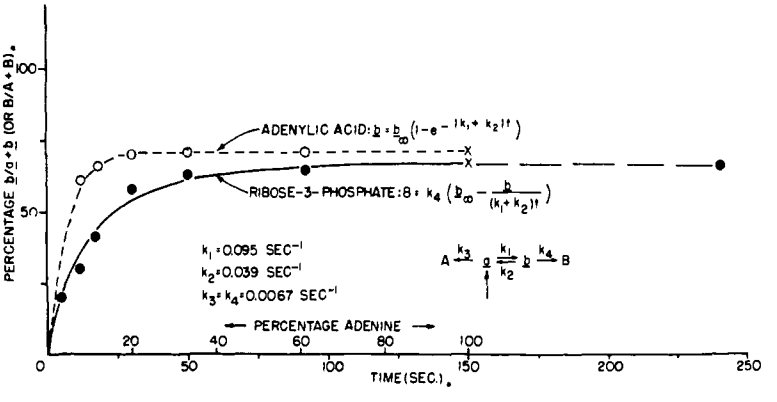


Fig. 3.—Appearance of adenylic acid *b* and of ribose-3-phosphate (B) when adenylic acid *a* is heated with Dowex-50-$H^+$ at 100°.

(40) The assistance of Dr. C. W. Sheppard is gratefully acknowledged.

3-phosphate (B) at late periods of heating conform to those predicted from the process shown, and lead to the conclusion that ribose-3-phosphate is directly derived from adenylic acid *b* and the -2-phosphate from *a*.

The curves for the disappearance of adenylic acid *a* and the corresponding fall in the amount of ribose-2-phosphate produced from it would be shown by the mirror images of the curves in Fig. 3 (*i.e.*, $a = 100 - b$; $A = 100 - B$). When adenylic acid *b* is the starting substance, the corresponding curve for the production of adenylic acid *a* rises rapidly to a maximum of 26% while that for ribose-2-phosphate appears more slowly and approaches a maximum of about 20%. These observations, while somewhat short of the equilibrium values (29 and 28%) predicted from the experiments on adenylic acid *a*, nevertheless serve to confirm the conclusion that ribose-2-phosphate is derived from adenylic acid *a*.

That the degrees of interconversion and of degradation of the ribose phosphates in the hydrolytic system employed are too small to interfere with these conclusions is shown by direct tests upon these substances themselves. The heating of either ribose-2- or -3-phosphate for 30 seconds at 100° with Dowex-50-$H^+$ gives rise to no detectable amounts of the other isomer or of ribose or inorganic phosphate. A heating period of 5 minutes of either isomer produces no significant isomerization, and only traces of ribose, whereas a 15-minute period converts 26% of ribose-2-phosphate to ribose -3-phosphate and ribose. The same period converts 8% of ribose-3-phosphate to ribose-2-phosphate (plus ribose-4-phosphate) and an equal amount to ribose; 84% is recovered unchanged. These degrees of conversion are roughly equivalent to those produced in 10 minutes at 100° at *p*H 1.0 (see Table I).

TABLE I

RIBOSE COMPOUNDS FORMED ON ACID HYDROLYSIS OF RIBOSE-2- AND RIBOSE-3-PHOSPHATES AT 100° IN PERCENTAGE OF TOTAL RIBOSE

| Starting isomer | Time (min.) | Acid | Rib-2-P | Rib-3-P | Ribose |
|---|---|---|---|---|---|
| Rib-2-P | 5 | Dowex-50-$H^+$ | 100(?) | 0 | Trace |
| Rib-3-P | 5 | Dowex-50-$H^+$ | 0 | 100(?) | Trace |
| Rib-2-P | 15 | Dowex-50-$H^+$ | 74 | 10 | 16 |
| Rib-3-P | 15 | Dowex-50-$H^+$ | 8 | 84 | 8 |
| Rib-2-P | 10 | *p*H 1.0 | 75 | 10 | 15 |
| Rib-3-P | 10 | *p*H 1.0 | 14 | 68 | 18 |
| Ad-2′-p[a] | 5 | *p*H 1.0 | 74[a] | 13[a] | 13[b] |
| Ad-3′-p[a] | 5 | *p*H 1.0 | 13[a] | 74[a] | 13[b] |
| Ad-2′-p[a] | 5 | *p*H 2.0 | 2[a] | 94[a] | 4[b] |
| Ad-3′-p[a] | 5 | *p*H 2.0 | 92[a] | 4[a] | 4[b] |
| Rib-2- and -3-P(?)[c] | 175 | *p*H 2.0 | 50 | | 50 |

[a] Adenylic acid. [b] Adenine (= ribose phosphate). [c] From Levene and Harris.[2b]

Four entries in Table I give data for the isomerization and glycosidolysis of adenylic acids in dilute acid. While the periods reported are only 5 minutes, in contradistinction to the one hour used by Levene and Harris,[2b] it should be noted that there is measurable isomerization even at *p*H 2 in this short period. It may be concluded that one hour would bring the mixture close to equilibrium.

The relative amounts of ribose phosphates produced in the resin-catalyzed hydrolysis of guanylic acid isomers are shown in Table II and indicate that guanylic *a* is the parent of ribose-2-phosphate and *b* of the -3-phosphate.

TABLE II

RELATIVE AMOUNTS OF RIBOSE PHOSPHATES PRODUCED BY HYDROLYSIS OF GUANYLIC ACIDS AT 100° WITH DOWEX-50-H

| Starting isomer | Time, min. | Rib-2-P | Rib-3-P |
|---|---|---|---|
| Guanylic acid *a* | 1/2 | 45 | 12 |
| Guanylic acid *a* | 1 | 62 | 88 |
| Guanylic acid *b* | 1/2 | 4 | 68 |
| Guanylic acid *b* | 1 | 25 | 106 |

## Discussion

The original identifications of the purine nucleotides as nucleoside-3′-phosphates were made by Levene and Harris,[2a,b] using adenylic and guanylic acids derived from the alkaline hydrolysis of yeast RNA. In the absence of evidence that these nucleotides are liberated by alkali as mixtures of two isomers, no attempts were made by them to separate single components and, in the absence of information on the physical properties of the guanylic acid isomers, there can be no assurance that the guanylic (or xanthylic) acid isomers used by them might, fortuitously, have been separated in the course of the preparation. Such may actually have been the case in the preparation of their adenylic acid[2b] for, although no properties of their preparation are given, the melting point and solubility data of "yeast adenylic acid" accepted at that time[41] are those which we now know to be associated with the *b* isomer only.

A guanylic acid preparation[2a] (and, subsequently, an adenylic acid preparation)[2b] was deaminated with nitrous acid to obtain xanthylic acid (inosinic acid). The xanthylic acid, which was not isolated was allowed to stand at 50° at its own *p*H (1.9) for 3 to 4 days, whereupon it decomposed to ribose phosphate to the extent of about 50%. This ribose phosphate had an optical rotation practically identical with that subsequently derived from the inosinic acid preparation, which was heated to 95–100° for one hour at its own *p*H (1.6-1.7) in order to sever the hypoxanthine group. In this case, specific mention is made some 20% of the total phosphorus was lost as inorganic phosphate during the degradation. No such mention is made in the two papers on the decomposition of xanthylic acid to ribose phosphate and the degradation of this to ribitol phosphate, but from the data of Levene and Dmochowski[3] (indicating 6% phosphate loss during a similar hydrolysis period, giving a 47% release of xanthine) and from the frequent reference to inorganic phosphate as a contaminant of ribose phosphate,[2a,b] it is probable that significant phosphate hydrolysis did occur. Certainly at least 12% (6%/47%) of the ribose phosphate product had been hydrolyzed, indicating (see Table I) that an equal degree of isomerization took place concurrently. If, as is possible, hydrolysis takes place

(41) See P. A. Levene and L. W. Bass, "The Nucleic Acids," Reinhold Publ. Corp., New York, N. Y., 1931, p. 222.

through ribose-1-phosphate, hydrolysis should be an exact index of isomerization.

Although the conditions used to obtain ribose phosphate from guanylic acid *via* xanthylic acid were relatively mild, the absence of any manipulations to effect a separation of the nucleotide isomers plus the fact that hydrolysis is always accompanied by isomerization (see Table I and Fig. 2), makes it probable that at least two ribose phosphates were produced. Further isomerization of these is possible during the prolonged incubation, and there is no evidence of any attempt to recrystallize or otherwise separate the product isomers. Thus it is likely that both were present. In the case of adenylic acid, it is probable that the *b* isomer was indeed used as the starting material. However, the more drastic methods used to degrade it (attested to by the inorganic phosphate found) make it probable that isomerization took place to yield more than one ribose phosphate isomer. If these preparations (or that from guanylic acid) isomerized at the ribose phosphate stage, ribose-2-, -3- and -4-phosphates would all be present and the reduction of these to ribitol phosphate, even if no further isomerization took place, would yield a combination of optical inactivity (the -3-) and a racemate (the -2- and -4-) as pointed out by Brown and Todd.[11] In this connection, it should be noted (see, for example, Fig. 2) that it requires only about 100 minutes at 100° for ribose-3-phosphate, at its own *p*H (*i.e.*, with Dowex-50 present), to produce equal amounts of 2- and 4-isomers, each about half of the ribose-3-phosphate remaining. This fact and the preponderance of the 3-compound which we observe regardless of starting product (if the conditions are such as to degrade the bulk of the starting nucleotide) indicate that the optical inactivity of the Levene and Harris ribitol phosphate was probably due either to the production of a racemate[11] or to the unwitting selection of ribose-3-phosphate from the mixture and not to the unintentional preservation of isomeric integrity, even if initially present, throughout the degradation to yield ribitol-3-phosphate.[2a]

The existence of inorganic phosphate in preparations of ribose phosphate by nucleotide hydrolysis can be ascribed to acid attack on the phosphate linkage only if alkaline degradation of the product during workup has been avoided. Experiments reported elsewhere[31] indicate a greater degree of alkali lability of ribose-3-phosphate (and the 5-isomer) than of free ribose itself, with the -2-phosphate being more stable than ribose. Alkali degradation releases inorganic phosphate and may thus have been the source of this degradation product in some of the older precipitations of ribose phosphate(s), using barium at high *p*H.[2a,b]

Since similar results have been obtained with guanylic acid (that is, more ribose-2-phosphate from the hydrolysis of guanylic acid *a*, more of the -3- from the *b*), it would appear that both *a* purine nucleotides are the -2′-phosphates of the respective nucleosides while the *b*'s are the -3′-phosphates. The same order of ion-exchange elution (5′, 2′, 3′)[10] is observed in the pyrimidine nucleotides,[19,42] which would indicate the dependence of this order upon the *pK*'s of the ionizable groups.[10,16b,25] It is already known that the 5′-phosphoinosine precedes the 2′- and 3′-isomers[10a]; hence, it may be inferred that the inosinic acids *a* and *b* derived from commercial yeast adenylic acid are also 2′- and 3′-phosphoinosines, respectively. The similarity in physical properties of the corresponding adenylic and cytidylic isomers[25] already indicated and the derivation of the uridylic acid isomers from the cytidylic acid isomers,[18] together with the similarities in enzymic susceptibility of all *a*'s and all *b*'s,[19a] serve to add to the certainty that all *a*'s are the 2′-derivatives and all *b*'s the 3′.

(42) W. E. Cohn, in E. Chargaff and J. N. Davidson (eds.), "The Nucleic Acids," Academic Press, Inc., New York, N. Y., in press.

OAK RIDGE, TENNESSEE

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[CONTRIBUTION FROM THE LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

# The Isolation of Protogen

BY E. L. PATTERSON, J. V. PIERCE, E. L. R. STOKSTAD, C. E. HOFFMANN, JOHN A. BROCKMAN, JR., F. P. DAY, M. E. MACCHI AND T. H. JUKES

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Procedures are described for the isolation of protogen, a growth factor for *Tetrahymena geleii* and *Streptococcus faecalis*. The bound form of protogen occurring in liver was liberated by hydrolysis and extracted into chloroform. The extract contained several forms of protogen. The predominant form, Protogen-A, was purified by solvent countercurrent distribution and chromatographic adsorption. Upon mild oxidation Protogen-A was converted into another form, Protogen-B, which was obtained pure by solvent countercurrent distribution and chromatographic adsorption.

In determining the nutritional requirements of *Tetrahymena geleii* Kidder and Dewey[1-3] found that a supplement such as liver extract was required for growth of the organism. The unknown factor in the supplement designated as Factor II[4] was later shown to contain two components, designated IIA and IIB, the latter of which was found to be replaceable by a mixture of pyridoxine and copper.[3] Stokstad, *et al.*,[5] studied the stability and chemical properties of Factor IIA and showed it existed in more than one form and suggested the name protogen in view of its essentiality for the growth of protozoa.

Snell and Broquist[6] compared the stimulatory effect of concentrates of protogen, the "acetate-

(1) G. W. Kidder and V. C. Dewey, *Biol. Bull.*, **87**, 121 (1944).
(2) G. W. Kidder and V. C. Dewey, *Arch. Biochem.*, **8**, 293 (1945).
(3) G. W. Kidder and V. C. Dewey, *ibid.*, **20**, 433 (1949).
(4) V. C. Dewey, *Biol. Bull.*, **87**, 107 (1944).
(5) E. L. R. Stokstad, C. E. Hoffmann, M. A. Regan, D. Fordham and T. H. Jukes, *Arch. Biochem.*, **20**, 75 (1949).
(6) E. E. Snell and H. P. Broquist, *ibid.*, **23**, 326 (1949).