Ultraviolet Absorption Spectra.—The spectra were measured with a Beckman spectrophotometer, model DU, using solutions containing 10 mg. per liter. For solutions of *p*H 1, 0.1 *N* hydrochloric acid was used, for *p*H 11, a glycine–sodium hydroxide buffer.

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

The Catalytic Hydrogenation of Pyrimidine Nucleosides and Nucleotides and the Isolation of their Ribose and Respective Ribose Phosphates[1,2]

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The complete catalytic hydrogenation of the 4,5-double bond of pyrimidine nucleotides has been carried out under mild conditions with a rhodium catalyst. Dihydrouridylic acid, formed from either uridylic or cytidylic acid, is cleaved by dilute alkali at room temperature to give the N-ribosyl phosphate of β-ureidopropionic acid. Dilute acid at room temperature hydrolyzes this substance to ribose phosphate and β-ureidopropionic acid without appreciable isomerization of the phosphate group, thus making available the sugar phosphates of pyrimidine nucleotides. Similar reductions and degradations have been carried out on cytidine, thymidine and deoxycytidylic and thymidylic acids. From uridylic acids a and b, ribose 2- and 3-phosphates, respectively, were obtained, thus confirming the identity of the pyrimidine nucleotide isomers.

Introduction

The accessibility of the sugars of pyrimidine nucleosides, or of the sugar phosphates of pyrimidine nucleotides, is severely limited by the resistance to acid hydrolysis of the N-glycosidic linkage. It has long been known that this stability is dependent on the ethylenic unsaturation between the adjacent carbon atoms in the ring; reduction or bromination of the 4,5-double bond destroys the resonating structure and renders the N-glycosidic linkage susceptible to acid hydrolysis. In this manner Levene and LaForge[3] identified ribonic acid from cytidine, following bromination and oxidation, establishing ribose as the sugar in these substances as well as the purine nucleotides. Bromination has also been used to render the ribose susceptible in colorimetric procedures used to identify pentose reducing groups.[3–5] Hydrogenation of pyrimidine nucleoside, although as old as bromination, was originally used to identify only the dihydrouracil component[3,6] and subsequently to show that the dihydropyrimidine nucleotide had the same rate of acid hydrolysis as purine nucleotides as judged from the appearance of reducing groups.[7] A variety of procedures have been utilized to effect the reduction of pyrimidines, *e.g.*, hydrogenation under pressure with colloidal platinum[7–9] or palladium,[3,6] the dropping mercury cathode[10] and sodium and ethanol in liquid ammonia.[11–13] In only a few cases has reduction of nucleosides or of nucleotides been successful.[3,6,11,12]

Now the sugar moiety has been isolated as such, for the first time without oxidation or other significant change, from both natural and synthetic pentosides and from deoxypentosides reduced by sodium and ethanol in liquid ammonia[11] and cleaved with a sulfonic acid cation-exchange resin.[12] In the deoxyribosides, this marked the first chemical identification of deoxyribose in the pyrimidine compounds. In all other cases recorded, the sugar has been identified indirectly, and in no case has a sugar phosphate been isolated. The purpose of this investigation was to recover quantitatively the sugar phosphate component of pyrimidine nucleotides, under conditions mild enough to avoid phosphomigration, in order to identify it both as to sugar type and phosphate location. The availability of a new rhodium catalyst,[14] which is particularly suited for the reduction of heterocyclic compounds, made a reinvestigation of this route seem feasible. With it, it was possible to achieve complete reduction of the 4,5-double bond at 1–1.2 atmospheres of hydrogen at *p*H 2–5 and at room temperature. Disappearance of the ultraviolet absorption at 260 mμ was used as a criterion of reduction, for it has been shown that the same double bond that is critical for the integrity of the N-glycosidic linkage is also critical for the characteristic ultraviolet spectrum of the pyrimidine nucleotides.[15] It was found that the increase in pentose concentration as detected by conventional orcinol reaction could be directly correlated with the disappearance of ultraviolet spectrum, as expected. It

(1) This work was performed under USAEC Contract No. W-7405-eng-26.

(2) Presented in part at the Federation of American Societies for Experimental Biology, San Francisco, April 11–15, 1955.

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(4) L. Massart and J. Hoste, *Biochim. Biophys. Acta*, **1**, 83 (1947).

(5) T. G. Brady and F. McEvoy-Bowe, *Nature*, **168**, 299 (1951).

(6) P. A. Levene and F. B. LaForge, *J. Biol. Chem.*, **13**, 508 (1912).

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(9) R. D. Batt, J. K. Martin, J. M. Ploeser and J. Murray, *ibid.*, **76**, 3663 (1954).

(10) L. F. Cavalieri and B. A. Lowy, *Arch. Biochem. Biophys.*, **35**, 83 (1952).

(11) D. C. Burke, *J. Org. Chem.*, **20**, 643 (1955).

(12) W. Bergmann and D. F. Burke, *Angew. Chem.*, **67**, 127 (1955).

(13) A. Bendich in "The Nucleic Acids," Vol. I, E. Chargaff and J. N. Davidson, Editors, Academic Press, New York, N. Y., 1955, p. 81.

(14) A 5% rhodium-on-alumina, procured from Baker & Co., Inc., Newark, N. J.

(15) L. F. Cavalieri and A. Bendich, THIS JOURNAL, **72**, 2587 (1950); L. F. Cavalieri, A. Bendich, J. F. Tinker and G. B. Brown, *ibid.*, **70**, 3875 (1948).

was also found that the cleavage of the 1,6-bond[16] of dihydrouridylic acid by mild alkali markedly predisposes the N-ribosidic linkage to acid hydrolysis, making it possible to recover the sugar phosphates under sufficiently mild conditions (*p*H extremes of 1–13 for short duration at room temperature) to avoid isomerization as well as decomposition. The uracil (or cytosine) moiety of the original was recovered as ureidopropionic acid (plus ammonia, if cytosine was used).

Experimental

Reduction.—The catalytic hydrogenation of the 4,5-double bond of the pyrimidine nucleosides and nucleotides was carried out in dilute aqueous solution in a low pressure (1.0–1.5 atm.) hydrogenation apparatus[19] with a 5% Rh on alumina catalyst.[14] The theoretical amount of hydrogen (for 1–3 mmoles of compound) was taken up in 1–5 hr. at *p*H's of 2.0 to 5.0. The conditions used for uridylic acid were 0.1–3.1 mmoles of nucleotide in 5–25 ml. of water and 5–250 mg. of Rh catalyst. Cytidylic acid, 0.3 to 1.5 mmoles in 10 to 60 ml. of water, was reduced with 20 to 200 mg. of catalyst at a noticeably slower rate than uridylic acid. At *p*H 6 to 7 the reduction was slow; only 60% of the theoretical amount of hydrogen was absorbed in 24 hr. Barium ion, in one experiment, inhibited the hydrogen uptake as did adenylic acid in three of the cytidylic acid experiments. The catalyst was removed by centrifugation and the dihydrouridylic acid (H_2URP) isolated by ion-exchange chromatography[20] on a Dowex-1-chloride column (10 cm. × 0.9 sq. cm., 200–400 mesh, 2–3 ml. 0.01 *N* HCl/min.) (Fig. 2A).

Hydrolysis.—(a) The 1,6-linkage of the dihydrouridylic acid (obtained from the Dowex-1-chloride column) was opened[16] at room temperature by the addition of sufficient alkali to bring the free OH^- concentration to 0.016–0.1 *N* (Fig. 2B-E); the conversion to ureidopropionyl ribose phosphate (UPRP) being essentially complete in 1 hr. at the higher alkalinity. The UPRP was separated on a Dowex-1-chloride column in a manner similar to the H_2URP. (b) The hydrolysis of the N-ribose linkage of UPRP was accomplished with dilute (0.02–0.1 *N*) HCl, Dowex-50-H^+ or Amberlite IR-120-H^+ at room temperature (Fig. 2 F-H) and the ureidopropionic acid (N) separated by ion-exchange chromatography.

Isolation of Ureidopropionic Acid.—Uridylic acid, 1.01 g. (3.1 mmoles) was dissolved in 25 ml. of water (*p*H *ca.* 3) and hydrogenated in the presence of 50 ml. of catalyst. The reduction was complete (disappearance of ultraviolet spectrum, theoretical hydrogen absorption) in 3 hr. The catalyst was removed by centrifugation and the solution made 0.1 *N* in OH^- by the addition of alkali. After 1 hr. at room temperature Dowex-50-H^+ was added to *p*H 2.3, filtered off, the filtrate made 0.1 *N* in HCl and heated on the steam-bath 15 min. The β-ureidopropionic acid was absorbed on a Dowex-1-OH^- column and eluted with 0.01 *N* HCl. The eluate contained 2.6 mmoles (84% based on uridylic acid) ureidopropionic acid. After concentration *in vacuo* to a small volume and crystallization at 5° for 2 days, 210 mg. (1.6 mmoles) of crude crystals, m.p. 155–160°, were obtained. Extraction with and recrystallization from hot absolute methanol yielded 50 mg. melting at 169–170°. The melting point of β-ureidopropionic acid, prepared by the method of Lengfeld and Stieglitz,[21] was 170–171°. The mixed melting point was 169–170°.

Uridylic acids a and b, thymidylic acid, deoxycytidylic acid, cytidine and thymidine were reduced and chromatographed in a similar manner. The ribose phosphates obtained were chromatographed in a sulfate–borate system on a Dowex-1-sulfate column.[20,22,23]

Nitrogen was determined spectrophotometrically according to a Nesslerization procedure[24] and phosphorus, ribose and deoxyribose spectrophotometrically assayed by a modification of the method of Griswold, *et al.*,[25] and the conventional orcinol and diphenylamine methods,[26] respectively. The nucleotides used were either commercial products or were prepared by chromatography[27,28] from hydrolyzates of ribonucleic acids.

Results

The course of a typical hydrogenation of uridylic acid is shown in Fig. 1. After an initial rapid uptake by the catalyst, the rate is essentially linear until 80% reduction is achieved.

The dihydrouridylic acid produced in the experiment described in Fig. 1 was freed of catalyst by centrifugation, made 0.016*N* in free hydroxide ion, and allowed to stand at room temperature. Samples of the solution were absorbed on ion-exchange columns at various times and chromatographed with the results indicated in charts A through E in Fig. 2. A continuous and complete transformation of the dihydrouridylic acid (H_2URP) to a substance of higher cationic affinity, containing all the nitrogen, phosphorus and ribose reactivity of the original, was thus observed. The rate of this transformation was such as to reach 25% in 5 min. and 75% in 60 min. (charts B and C, Fig. 2), based on the orcinol assay of the effluents. The product, called ureidopropionylribose phosphate (UPRP) was stable in the alkaline solution up to at least 50 hr. (charts D and E). Dihydrouridylic acid allowed to stand in 0.1 *N* HCl, on the other hand, did not change in 9 days.

The remaining solution was then made 0.1 *N* in free H^+ with HCl, and fractions of this were similarly chromatographed. As shown in charts F, G and H of Fig. 2, a conversion of the ureidopropionylribose phosphate to substances identified as ribose phosphate (cross-hatched peak) and to ureidopropionic acid (arrow N) and a small fraction of other components, including some dihydrouridylic acid, was observed. The amount of ribose phosphate formed was 47% at 40 min. and 82% at 24 hr. (charts F, G and H, respectively. Fig. 2). In each case, 2 moles of organic nitrogen (1 mole of ureidopropionic acid) per mole of ribose phosphate formed appeared in the effluent at the *p*H break in the elution sequence. This lability in HCl is in

(16) A strict interpretation of the nomenclature rules set forth in Chemical Abstracts[17] and The Ring Index[18] leads to the designation of this linkage as 3,4 and that of the ethylenic bond as 5,6. However, the weight of the argument for this[12] is only slightly greater than that for the classical system, in which these would be 1,6 and 4,5, respectively. The classical system has the advantage, as has been pointed out,[18] of indicating the chemical and biochemical relations of pyrimidines and purines more clearly and is therefore used here.

(17) *C. A.*, **39**, 5867 (1945).

(18) A. M. Patterson and T. L. Capell, "The Ring Index," The Reinhold Publishing Co., New York, N. Y., 1940, p. 52.

(19) L. M. Joshel, *Ind. Eng. Chem., Anal. Ed.*, **15**, 590 (1943).

(20) W. E. Cohn, in "The Nucleic Acids," Vol. I, E. Chargaff and J. N. Davidson, Editors, Academic Press, New York, N. Y., 1955, p. 211; also THIS JOURNAL, **72**, 1471 (1950).

(21) F. Lengfeld and J. Stieglitz, *Am. Chem. J.*, **15**, 504 (1893).

(22) J. X. Khym, D. G. Doherty and W. E. Cohn, THIS JOURNAL, **76**, 5523 (1954).

(23) J. X. Khym and W. E. Cohn, *ibid.*, **76**, 1818 (1954).

(24) O. Folin and H. Wu, *J. Biol. Chem.*, **38**, 81 (1919); J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," Vol. II, Williams and Wilkins Co., Baltimore, Md., 1932.

(25) B. L. Griswold, F. L. Humoller and A. R. McIntyre, *Anal. Chem.*, **23**, 192 (1951).

(26) A. H. Brown, *Arch. Biochem.*, **11**, 269 (1946); E. Volkin and W. E. Cohn, in "Methods of Biochemical Analysis," Vol. I, D. Glick, Editor, Interscience Publishers, New York, N. Y., 1954, p. 287.

(27) W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 2606 (1950); E. Volkin, J. X. Khym and W. E. Cohn, *ibid.*, **73**, 1533 (1951).

(28) W. E. Cohn and J. X. Khym, in "Biochemical Preparations," Vol. V, E. Shemin, Editor, John Wiley and Sons, Inc., New York, N. Y., in press.

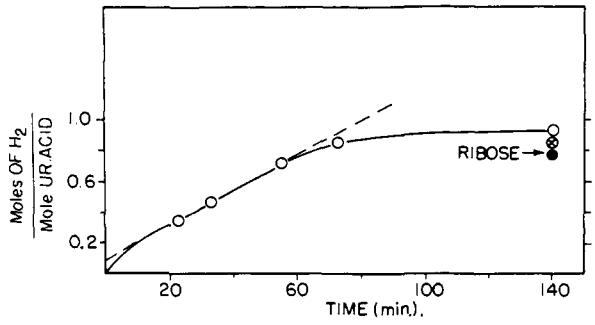

Fig. 1.—Catalytic hydrogenation of uridylic acid: 3.0 mmoles in 20 ml.; *p*H 5.4; 50 mg. of Rh catalyst; 25°; 1.15 atm. of H_2; ⊗, final point corrected for catalyst H_2 consumption; ●, ribose assay.

contrast to the stability observed without prior alkali treatment.

In experiments in which the separate a and b isomers of uridylic acid[20,28] were used, the ribose phosphates derived from them, as described, were analyzed in the sulfate–borate system of Khym, Doherty and Cohn[22] in order to determine which was ribose 2-phosphate and which the isomeric 3-phosphate. Only partial reductions and degradations were achieved but the results (Fig. 3) indicate a predominance of ribose 2-phosphate from the a uridylic acid and of the 3-phosphate from the b (Fig. 4). Other experiments in which the degradations were carried out more completely (overall ribose phosphate yields of 80 and 60%, respectively) gave similar results (70% ribose 2-phosphate from the a, 95% ribose 3-phosphate from the b). In these experiments, any reconstituted dihydrouridylic acid was again treated with alkali and acid to obtain its ribose phosphate. Thus the designation of uridylic acids a and b as the 2′- and 3′-isomers, respectively, which has hitherto rested on the physicochemical properties of the cytidylic acids[29] and the deamination linking of a cytidylic to a uridylic and of b to b[30,31] is now confirmed by direct isolation and identification of the ribose phosphate moiety as with the purine nucleotides.[22,23]

Thymidylic acid was reduced under the same conditions, but at a slower rate, as were the ribotides. Consumption of hydrogen, disappearance of spectrum and appearance of reactivity in the diphenylamine reaction were used as the criteria of reduction. Deoxyribose phosphate was demonstrated but not isolated. Cytidine, thymidine and deoxycytidylic acid were also reduced, yielding compounds which underwent the same alkali and acid degradations although at varying rates (*e.g.*, the methyl group of thymidylic acid retards the alkali degradation; the deoxy products are more labile).

Evidence for the formation of dihydrocytidylic acid in experiments involving cytidylic acid was the elution, just ahead of the position of cytidylic acid, of a small amount of a substance assaying for nitrogen, phosphorus and ribose in the ratio 3:1:1. Most of the material was usually recovered as dihydrouridylic acid, and free NH_3 in equimolar amount could be demonstrated. Presumably, the amino group of dihydrocytidylic acid is hydrolyzable under the mild conditions of the reduction and absorption steps.

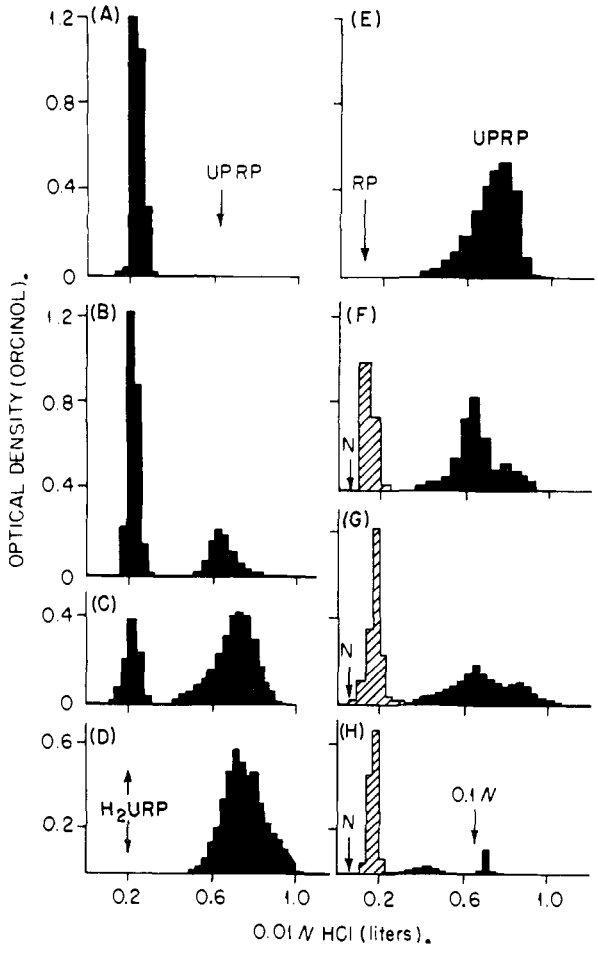

Fig. 2.—Ion-exchange chromatography of dihydrouridylic acid and derivatives: dihydrouridylic acid (H_2URP) (A) treated with 0.016 *N* NaOH at 25° for 5 min., (B); 1 hr. (C); 24 hr., (D), and 50 hr. (E) to yield ureidopropionylribose phosphate (UPRP). UPRP treated with 0.1 *N* HCl for 20 min. (F), 40 min. (G), 24 hr. (H) to yield ureidopropionic acid (N) and ribose phosphate (RP), cross hatching is orcinol assay.

Discussion

No previous isolation of a sugar phosphate from a pyrimidine nucleotide has been reported, and even the reduction of such substances, presumably necessary to achieve labilization of the N-glycosidic linkage, has been achieved only rarely.[7] The sodium–ethanol–liquid ammonia procedure, so effective with nucleosides, is seemingly ineffective with nucleotides.[12]

The conversion of β-ureidopropionic acid to dihydrouracil[21,32] and of the unsaturated analog to uracil[33] are the reverse of the alkali lability shown

(29) L. F. Cavalieri, THIS JOURNAL, **74**, 5804 (1952); **75**, 5268 (1953); J. J. Fox, L. F. Cavalieri and N. Chang, *ibid.*, **75**, 4315 (1953).

(30) H. S. Loring and N. G. Luthy, *ibid.*, **73**, 4215 (1951).

(31) D. M. Brown, C. A. Dekker and A. R. Todd, *J. Chem. Soc.*, 715 (1952).

(32) S. Gabriel, *Ber.*, **38**, 631 (1905).

(33) R. Behrend, *Ann.*, **229**, 5 (1885), quoted by J. J. Donleavy and M. A. Kise in "Organic Synthesis," Col. Vol. II, A. H. Blatt, Editor John Wiley and Sons, Inc., New York, N. Y., 1947, p. 422.

by the reduced pyrimidine bases.[9,34] That this may occur also with dihydrouridylic acid, to give the nucleotide analog (β-ureidopropionylribose phosphate) is now demonstrated and the rate is similar to that for the alkaline decomposition of dihydrouracil.[9] The additional carboxyl group formed is presumably responsible for the greater ion-exchanger affinity, just as the further breakdown product, β-ureidopropionic acid, shows more acidic properties than urea or ammonia.

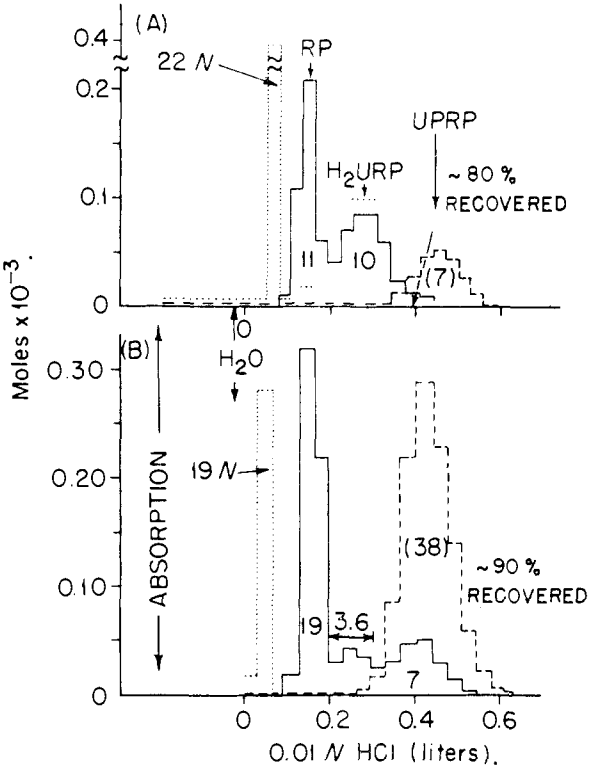

Fig. 3.—Ion-exchange chromatography of reduced uridylic acids: A, 38 μmoles of uridylic acid a (75% reduced) treated with 0.1 N OH^- for 1 hr. and then by 0.1 N H^+ for 2 hr., B, 76 μmoles of uridylic acid b (45% reduced) treated with 0.1 N OH^- for 16 hr. and then by 0.1 N H^+ for 5 hr. Numbers under peaks refer to amounts (μmoles) recovered: ———, ribose phosphate; – – –, uridylic acid; ------, N_2.

(34) P. A. Levene, L. W. Bass and H. S. Simms, *J. Biol. Chem.*, **70**, 229 (1926).

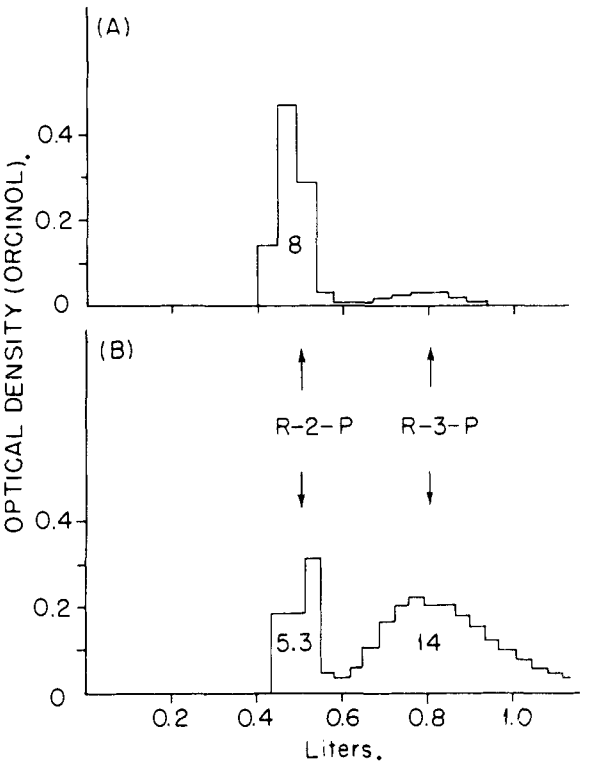

Fig. 4.—Ion-exchange chromatography of ribose phosphate derived from uridylic acid a (A) and uridylic acid b (B). R-2-P, ribose 2-phosphate; R-3-P, ribose 3-phosphate. Eluted with 0.005 M sulfate plus 0.005 M borate. Numbers under peaks refer to amounts (μmoles) recovered.

The increased labilization of the N-glycosidic linkage caused by this alkali-catalyzed splitting of the reduced pyrimidine ring, to the point where it hydrolyzes rapidly in 0.1 N HCl at 25°, is a novel and unexpected finding. This makes possible the cleavage of the glycosidic linkage under conditions mild enough to preclude phosphomigration.[35] Direct cleavage of dihydrouridylic acid would apparently, as judged by its relative stability in acid, require conditions prejudicial to the identification of isomers.

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(35) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952); W. E. Cohn, THIS JOURNAL, **72**, 2811 (1950).