spun off again. Paper chromatography showed the barium salt to be of mainly ATP, with ADP and a higher phosphate (R_f 0.06, in 1% ammonium sulfate–isopropyl alcohol, 1:2, v./v.) as the impurities. It was converted to the mercury salt (precipitation with Lohmann reagent) and the mercury salt decomposed as usual. Final precipitation of the barium salt at *p*H 3.8 and repeated washing with 0.2% acetic acid gave 350 mg. of a sample which was slightly contaminated by ADP and a small amount of the slower travelling higher phosphate.

The supernatant from the precipitation at *p*H 4 was brought to *p*H 8 with alkali and the precipitated barium salt, mostly of ADP, was collected by centrifugation after keeping the mixture at 0° for some hours, and washed with water; yield 120 mg. This sample of ADP was slightly contaminated by AMP and ATP.

Acknowledgments.—The work was carried out under a consolidated grant from National Research Council of Canada, Ottawa. The author is indebted to Drs. Takagi and Potter for an enzymatic test on a synthetic sample of ATP, and Dr. R. H. Wright for the determination of the infrared spectra. Gratitude is expressed to Dr. G. M. Shrum for his generous encouragement of this work.

VANCOUVER 8, CANADA

[CONTRIBUTION FROM THE BRITISH COLUMBIA RESEARCH COUNCIL (CHEMISTRY DIVISION) AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Carbodiimides. VI. The Reaction of Dicyclohexylcarbodiimide with Yeast Adenylic Acid. A New Method for the Preparation of Monoesters of Ribonucleoside 2′- and 3′-Phosphates

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The reaction of adenylic acids *a* and *b* and their mixture (yeast adenylic acid) with dicyclohexylcarbodiimide in aqueous pyridine at room temperature has been studied. The corresponding 2′,3′-cyclic phosphate which in every case is the initial product reacts further to give two products, presumably the 2′- and the 3′-isomers, having the novel structures III and IV. Although stable to aqueous ammonia, III and IV yield adenylic acids *a* and *b* and dicyclohexylurea as the ultimate products when treated with either dilute sodium hydroxide or dilute hydrochloric acid. The action of sodium benzoxide on either III or IV gives in good yield a mixture of the monobenzyl esters of the isomeric adenylic acids *a* and *b*. These results demonstrate that the cyclic phosphate is an intermediate in all of these reactions. The significance of these findings in the problem of synthesis of dinucleoside phosphates containing 2′,5′- and 3′,5′-phosphodiester linkages has been pointed out.

It has been reported quite recently[3] that the prolonged treatment of adenosine-5′-phosphate (muscle adenylic acid) in aqueous pyridine with excess of dicyclohexylcarbodiimide (DCC) affords in very good yield the symmetrical P^1,P^2-diadenosine-5′-pyrophosphate. The reaction conditions[4] employed are particularly suitable for work in the general nucleotide field and the one-step synthesis of adenosine di- and triphosphate (ADP and ATP[3]) demonstrates the practical application of this new and attractive approach to the synthesis of nucleotide-derived coenzymes. In seeking to extend the study of the reactions of carbodiimides with nucleotides we have investigated the reaction of DCC with the isomeric adenylic acids *a* and *b*[5] (I) which, from the known reactions of carbodiimides,[6] could lead either to pyrophosphate formation or by virtue of the adjacent free hydroxyl group to internal diester (cyclic phosphate) formation. A preliminary study[7] of the reaction of DCC with yeast uridylic acid in dimethylformamide had indeed suggested the formation of the corresponding 2′,3′-cyclic phosphate. Compounds of the latter type, since their discovery as intermediates in the alkaline and ribonuclease catalyzed hydrolysis of ribonucleic acid,[8–10] have been the subject of extensive chemical and enzymatic investigation.[11–16]

By the treatment of yeast adenylic acid[17] (I) (a mixture of adenosine-2′- and 3′-phosphate), adenylic acid *a*[17] or adenylic *b*[17] with an excess of DCC in aqueous pyridine, it was found that the course of reaction was identical irrespective of the particular isomer or mixture of isomers employed. After a reaction period of about 30 minutes, adenosine 2′,3′-cyclic phosphate (II) was the major product (70–75%) contaminated by starting material. The identification of this expected reaction product was readily accomplished by synthesis by an independent route[11] and comparison of physical properties. However, with a slightly prolonged

(1) Biochemistry and Virus Laboratory, University of California, Berkeley.

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(3) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954).

(4) No protecting groups are required. Anhydrous conditions are not mandatory. The reagents are stable and easy to handle.

(5) The initial separation of two isomeric adenylic acids from alkaline hydrolysates of yeast ribonucleic acid was accomplished jointly by C. E. Carter, THIS JOURNAL, **72**, 1466 (1950), and W. E. Cohn, *ibid.*, **72**, 1471 (1950). These compounds, termed adenylic acids *a* and *b*, were later shown to be the 2′-, and 3′-phosphates but not necessarily respectively (D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952)). Recent evidence (J. X. Khym, D. G. Doherty, E. Volkin and W. E. Cohn, THIS JOURNAL, **75**, 1262 (1953), and the references cited therein; L. F. Cavalieri, *ibid.*, **75**, 5268 (1953); and D. M. Brown, G. D. Fasman, D. I. Magrath, A. R. Todd, W. Cochran and M. M. Woolfson, *Nature*, **172**, 1184 (1953)) has firmly established the *a*-isomer as the 2′-phosphate and the *b*-isomer as the 3′-phosphate. For the sake of convenience we have used throughout this paper the original terms: adenylic acids *a* and *b*.

(6) H. G. Khorana, *Chem. Revs.*, **53**, 145 (1953).

(7) Unpublished work of D. M. Brown, referred to in ref. 6.

(8) R. Markham and J. D. Smith, *Nature*, **168**, 406 (1951).

(9) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(10) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

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

(12) D. M. Brown, C. A. Dekker and A. R. Todd, *ibid.*, 2715 (1952).

(13) R. Markham and L. A. Heppel, *Nature*, **171**, 1152 (1953).

(14) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

(15) L. A. Heppel, P. R. Whitfield and R. Markham, Abstract of a paper presented at a meeting of the Faraday Society, October, 1953.

(16) C. A. Dekker, *Federation Proc.*, **13**, 197 (1954).

(17) Like muscle adenylic acid, these substances are practically insoluble in anhydrous organic solvents including dimethylformamide and pyridine. Although difficultly soluble even in water, they dissolve readily in aqueous pyridine.

reaction period a new product appeared which could be detected with paper chromatography as a spot travelling near the solvent front in isopropyl alcohol–ammonia–water (70:5:25). Further increase in time of reaction caused the formation of this new product at the expense of the cyclic phosphate so that after 8–10 hours the fast travelling material formed the major ultimate product of the reaction. That this product was composed of two substances was shown by elution of the single spot and rechromatographing the material in the two layer solvent isoamyl alcohol–5% aqueous disodium hydrogen phosphate. Subsequent elution of either of these components followed by chromatography in the same solvent system gave a spot of identical R_f eliminating the possibility of "double-spotting" or similar solvent effects. These unknown substances were tentatively called "x" and "y," "x" having an R_f value in isoamyl alcohol–disodium hydrogen phosphate similar to adenylic acid *b*, and "y" an R_f slightly lower than that of the cyclic compound II.

The secondary reaction of 2′,3′-cyclic phosphate with DCC leading to the formation of "x" and "y" proved to be unique. Evidence showing that "x" and "y" had not arisen from any interaction involving the adenine portion of the nucleotide was obtained from a comparison of the ultraviolet absorption spectra of these substances, as eluted from paper chromatograms run in isoamyl alcohol–disodium hydrogen phosphate, with that of adenosine-2′,3′-cyclic phosphate[18] (Fig. 1). Although the only known reaction of diesters of orthophosphoric acid with carbodiimides is the formation of tetraesterified pyrophosphoric acid,[6,19] the chemical and physical properties of "x" and "y" were unlike those to be expected for a compound of this type. Such a tetraesterified pyrophosphate structure, moreover, would be inconsistent with the finding of two products unless a subsequent partial hydrolysis to a tri- or diesterified pyrophosphate had occurred. All compounds of this type were excluded from consideration when it was observed that "x" and "y" were stable to 14% aqueous ammonia at room temperature for 24 hours. Under these conditions a tetraesterified pyrophosphate should act as a phosphorylating agent[20] and give diesterified orthophosphate (in the present case, adenosine 2′,3′-cyclic phosphate) and diesterified phosphoramidate,[21] while a diesterified pyrophosphate of the type P¹,P²-diadenosine-3′(or 2′)-pyrophosphate would also be expected to yield adenosine-2′,3′-cyclic phosphate by analogy with results obtained on similar treatment of flavin–adenine dinucleotide[22a] and uridine diphosphate glucose.[22b] Finally, the high identical R_f values of "x" and "y" in isopropyl alcohol–ammonia–water (70:5:25) were in conflict with a diesterified pyrophosphate structure since the R_f value observed for P¹,P²-diadenosine-5′-pyrophosphate[3] is very low in this solvent.

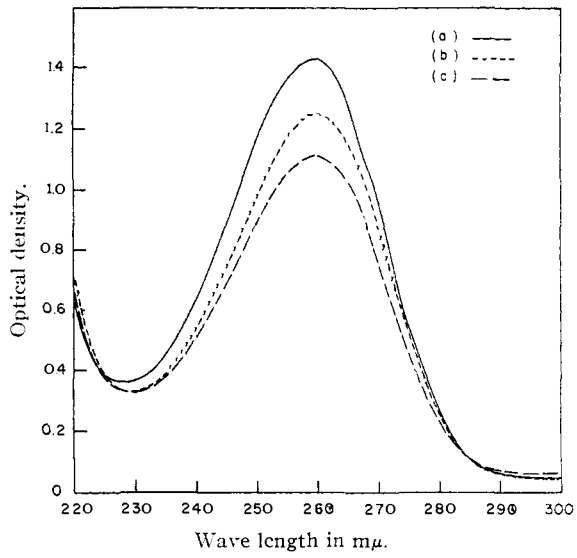


Fig. 1.—Ultraviolet absorption spectra: (a) ——, adenosine-2′,3′-cyclic phosphate; (b) ------ and (c) — —, N-adenylyl-N,N′-dicyclohexylureas, "x" and "y," respectively.

Light was shed on the nature of "x" and "y" by a study of the action of both dilute hydrochloric acid and dilute sodium hydroxide on a mixture of the two compounds purified by cellulose column chromatography. In 0.01 *N* hydrochloric acid, slow decomposition occurred at room temperature to give adenosine 2′,3′-cyclic phosphate and ultimately adenylic acids *a* and *b*. The latter were shown to arise more rapidly from the action of stronger hydrochloric acid or 0.5 *N* sodium hydroxide. Simultaneously in these experiments a water-insoluble material shown to be dicyclohexylurea was obtained. Quantitative determination of the acid degradation products of "x" and "y" showed these substances to be 1:1 adducts of yeast adenylic acid and DCC. The novel structures III and IV were thus assigned to these products.

The similarity of their ultraviolet absorption spectra, their identical reaction with acid and alkali, and (as will be seen later) their identical behavior in transesterification reactions, afford strong presumptive evidence in favor of the isomeric structures III and IV related to the isomeric adenylic acids *a* and *b* themselves. In analogy with the N-acylureas derived from carbodiimides and carboxylic acids, the names N-adenylylureas are given to these compounds. The assignment of a definite structure III or IV to either "x" or "y" is as yet impossible, the ready conversion of these substances into the cyclic compound making this an even more difficult task than the identification of the yeast adenylic acid isomers. One observation is, however, pertinent in this connection. Of the two isomers, "x" was formed in greater amount. It is also known that as a result of hydrolysis of the 2′,3′-cyclic phosphate, adenylic acid *b* is formed in greater amount (see below). A consideration of

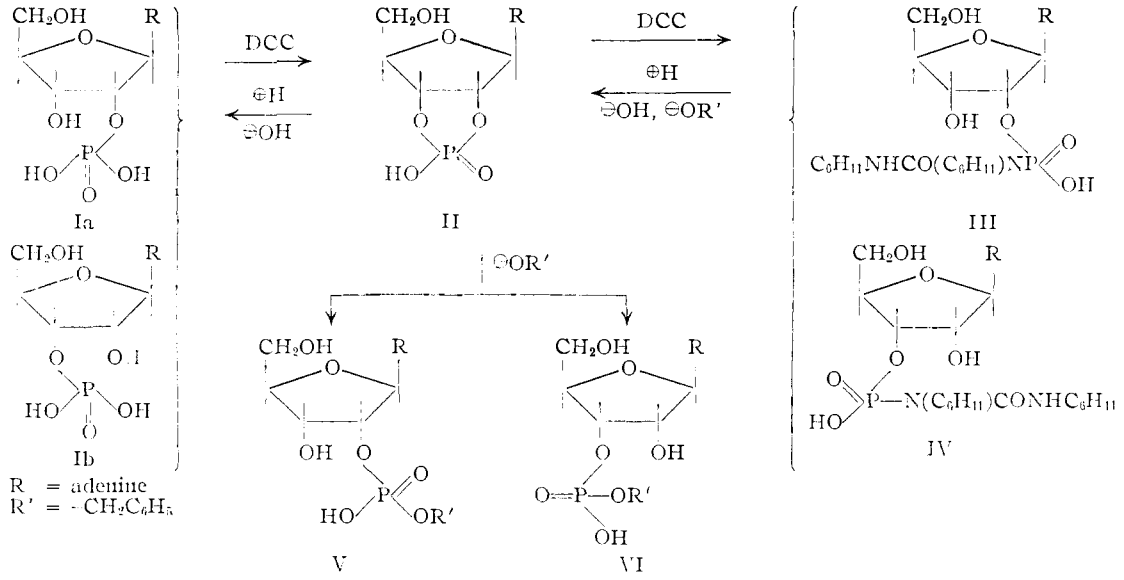
(18) The ultraviolet absorption spectrum of yeast adenylic acid as determined at *p*H 8.5 is also almost identical with those recorded in Fig. 1.

(19) H. G. Khorana and A. R. Todd, *J. Chem. Soc.*, 2259 (1953).

(20) F. R. Atherton and A. R. Todd, *ibid.*, 674 (1947).

(21) New nomenclature of phosphorus compounds adopted by the International Union of Pure and Applied Chemistry; *e.g.*, *J. Chem. Soc.*, 5122 (1952).

(22) (a) H. S. Forrest and A. R. Todd, *ibid.*, 3295 (1950); (b) R. Caputto, L. F. Leloir, C. E. Cardini and A. C. Paladini, *J. Biol. Chem.*, **184**, 333 (1950).



the possible mechanism of formation of "x" and "y" (see below) and its relation to the hydrolysis of the 2′,3′-cyclic phosphate offers some justification for relating "x" to the *b* series.

The structures III and IV are supported by elemental analysis of a crystalline preparation of the free acids (>90% "x") and by a molecular weight estimation, calculated both from the neutral equivalent and from the optical density of a mild acid hydrolysate. An electrometric titration revealed no secondary phosphoryl dissociation and no basic group other than the 6-amino group of the adenine. The absence of a more strongly basic group would seem to eliminate the alternate O-phosphoryliso-urea structures (P—O—C(NHR)=NR formulas) in which the double bonded nitrogen would be expected to be a fairly strong proton attractor. Related compounds of the type A—O—C(R′)=N—R are known to be unstable when AOH is a strong acid and most attempts to prepare them result in spontaneous ketonization, giving R′–CO–N(R)–A.[23] The infrared spectra of a mixture of III and IV, *sym*-dicyclohexylurea and yeast adenylic acid[24] are recorded in Fig. 2. The bands at 1628 cm.$^{-1}$ (6.14 μ) and 1577 cm.$^{-1}$ (6.35 μ), presumably due to the NHCONH grouping,[25] have shifted in the spectrum of III and IV to 1650 cm.$^{-1}$ (6.06 μ) and 1605 cm.$^{-1}$ (6.24 μ). Further, from the work of Bellamy and Beecher,[26] it seems certain that the band at 1225 cm.$^{-1}$ (8.17 μ) in the spectrum of yeast adenylic acid is due to P=O stretching; this band seems to have shifted to lower frequency (1205 cm.$^{-1}$ or 8.3 μ) in the spectrum of III and IV. Owing to the complexity of the molecules and lack of definitive data on compounds of this general type it is not possible to interpret the absorption frequencies in the fingerprint region.

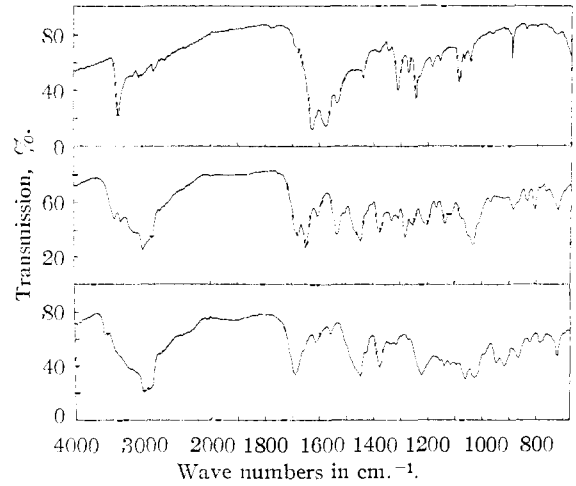


Fig. 2.—Infrared spectra of: *sym*-dicyclohexylurea (top curve); mixture of N-adenylyl-N,N′-dicyclohexylureas "x" and "y" (middle curve); yeast adenylic acid (bottom curve). The spectra were taken in Perkin–Elmer infrared spectrophotometer model 21 B using nujol mulls.

Treatment with sodium benzoxide of a solution of the mixed pyridinium salts of III and IV (or of the pyridinium salt of chromatographically purified III or IV) in anhydrous benzyl alcohol gave a mixture of the monobenzyl esters V and VI of adenylic acids *a* and *b*. The R_f values of these esters in several solvents were similar to those recorded by Brown and Todd.[24] These results are formally similar to those previously obtained on the alkaline hydrolysis of the above mentioned benzyl esters (*cf.* Brown and Todd, ref. 24, p. 48) and of ribonucleic acid[8–10] in that the cyclic phosphate is the intermediate in all these reactions. This conclusion was further confirmed by a study of the reaction of adenosine 2′,3′-cyclic phosphate with sodium benzoxide; results identical with those reported above were obtained.

(23) E. Alexander, "Ionic Organic Reactions," John Wiley and Sons, Inc., New York, N. Y., 1950, p. 75.

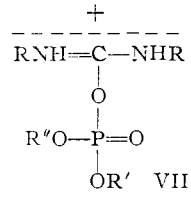
(24) The infrared spectra of adenylic acids *a* and *b* have previously been recorded by D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952), and by E. R. Blout and M. Fields, *J. Biol. Chem.*, **178**, 335 (1949).

(25) H. M. Randall, R. G. Fowler, N. Fuson and J. R. Dangl, "Infrared Determination of Organic Structures," D. Van Nostrand Co., Inc., New York, N. Y., 1949. For spectra of disubstituted ureas see also H. G. Khorana, *Can. J. Chem.*, **32**, 261 (1954).

(26) L. J. Bellamy and L. Beecher, *J. Chem. Soc.*, 475, 1701 (1952) 728 (1953).

It is clear that these "transesterification" experiments establish a new method for the synthesis of monoesters of ribonucleoside phosphates, which have so far been prepared through the reaction of nucleoside phosphates with diazo compounds (*e.g.*, phenyldiazomethane for the preparation of benzyl esters[14]). Although the cyclic phosphates can be employed directly in such reactions, the use of the derivatives of the type III and IV appears to offer advantage on the grounds of solubility. Further it is interesting to compare the relative proportions of the two benzyl esters (*b*, 80–85%; *a*, 15–20%) formed consistently in the above mentioned experiments with the proportions of the isomeric adenylic acids (*b*, 60–65%; *a* 35–40%) obtained after alkaline hydrolysis of ribonucleic acid[27] or adenosine 2′,3′-cyclic phosphate.[28] After the initial nucleophilic attack of hydroxyl or alkoxyl ion on the phosphorus atom of the cyclic phosphate, the opening of the ring (the two processes may be simultaneous) occurs so as to form the *b*-nucleotide or its ester preferentially. The high proportion of adenylic acid-*b* benzyl ester in the latter case (4:1) as compared with the proportion of adenylic-*b* in the former case (3:2) relates the steric effect, which seems to be operative, directly to the size of the initially attacking ion. These observations appear to be of particular significance for the synthesis by this method of dinucleoside phosphates containing *a*-5- and *b*-5-phosphodiester linkages. Work along these lines will be reported later, but it is relevant to point out that the bulky ions derived from suitably protected nucleosides (*e.g.*, 2′,3′-isopropylideneadenosine) should yield predominantly the *b* to 5 linked dinucleoside phosphates. The latter type of linkage has been shown to occur in ribonucleic acids.[13,14]

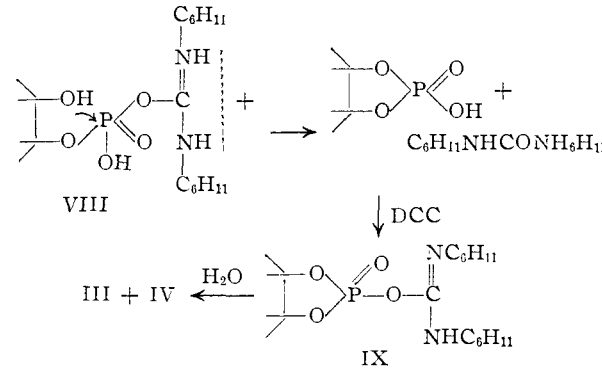
An obligatory intermediate in the described reactions of carbodiimides with mono- and diesters of phosphoric acid[6,19] was postulated to be a cation of the general structure VII, which on the subsequent attack of another acid anion on the phosphorus atom gave urea and a pyrophosphate.



VII

The reaction of adenosine-5′-phosphate which was studied[3] under the conditions employed in the present work is in accord with this mechanism. The initial formation of the cyclic phosphate in the reaction of yeast adenylic acid with DCC is clearly due to the attack of the vicinal hydroxyl group on the phosphorus atom of the intermediate VIII, analogous to VII above. This intramolecular reaction occurs much faster than the bimolecular reaction yielding a pyrophosphate (*cf.* the much slower formation of P^1,P^2-diadenosine-5′-pyrophosphate[3]).

The subsequent reaction of the cyclic phosphate



VIII IX

is probably a 1,2-addition to DCC, forming the intermediate O-phosphorylisourea IX, followed by a rapid sequence of events leading to III and IV. The over-all reaction is reminiscent of the formation of N-acylureas from carbodiimides and carboxylic acids[6] and appears to be general for the cyclic diesters of phosphoric acid. Thus all the 2′,3′-cyclic phosphates derived from yeast ribonucleotides and the 1,2-cyclic phosphates of glycerol and propanediol give "phosphorylureas" under similar conditions.[28] In the reaction of non-cyclic diesters of phosphoric acid with carbodiimides it has so far not been possible to isolate 1:1 adducts containing P to N linkages as in III and IV.

Experimental

Reaction of Yeast Adenylic Acid with DCC.[29] General Method.—Ten mg. of yeast adenylic acid[30] was dissolved in a mixture of 0.1 cc. of water and 0.2 cc. of pyridine. A solution of 200 mg. of DCC in 0.5 cc. of pyridine was added and the clear solution kept at room temperature. Dicyclohexylurea began to crystallize after approximately 30 minutes. Aliquots of 0.1 cc. were removed at intervals, diluted with 0.2 cc. of water and extracted repeatedly with ether. The residual aqueous layer (*p*H 3–4) was examined on paper chromatograms in the solvent system isopropyl alcohol–ammonia–water (70:5:25)[9] (solvent 1). Three spots located by their absorption of ultraviolet light had R_f values corresponding to unchanged yeast adenylic acid (0.12), adenosine 2′,3′-cyclic phosphate (0.41) and the "adenylylureas" (0.95). Their relative concentrations were determined by elution with 3 cc. of 0.1 *N* hydrochloric acid and measurement of the optical density of the resulting solutions at 260 mμ. Table I records the results thus obtained as well as those obtained in experiments employing the individual isomers, adenylic acid *a* and adenylic acid *b*, as starting material.

Preparation of N-Adenylylureas (Method A).—To a solution of 200 mg. of yeast adenylic acid in aqueous pyridine (1.5 cc. of water and 10 cc. of pyridine) was added 2 g. of DCC. The clear solution was kept at room temperature for 24 hr. and then filtered to remove dicyclohexylurea, the latter being washed thrice with small portions of water (total volume, 10 cc.). The combined filtrate was extracted repeatedly with ether and the residual aqueous solution dried over calcium chloride in a vacuum desiccator. The glassy residue was dissolved in 5 cc. of a mixture of isopropyl alcohol–pyridine–water (70:5:30) and the solution was transferred to the top of a cellulose column (2.5 cm. in diameter by 21 cm. in length) previously washed with the same solvent. The materials were eluted with this solvent and collected in fractions of 8 cc. Examination of these fractions by paper chromatography (solvent 1) revealed that the adenylylureas III and IV were obtained in the first 16 cc. of the eluate (exclusive of hold-up volume), with the cyclic phosphate and the yeast adenylic acid following in

(27) W. E. Cohn, *J. Cell. Comp. Physiol.*, **38** (Suppl. 1), 21 (1951).
(28) Unpublished work of C. A. Dekker.
(29) DCC was prepared as previously described (H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954)).
(30) Commercially available material was used after it was shown by paper chromatography to contain only adenylic acids *a* and *b*.

TABLE I

Time, hr.	Yeast adenylic acid 1.5	4	6	47[a]	Adenylic acid *a* 0.25	0.5	1	2.15	3.75	9.5	20.5	Adenylic acid *b* 0.25	0.5	1	2
Adenylic acids *a* and/or *b*, %[b]	6.9	4.0	3.1	8.4	22.7	16.4	13.0	10.0	5.8	7.5	4.0	26.6	12.3	11.4	8.4
Cyclic phosphate, %	49.2	33.1	23.9	29.3	73.1	74.0	69.6	54.6	48.2	37.7	25.8	70.4	75.0	62.8	42.6
Phosphorylureas, %[c]	43.9	62.9	73.0	62.3	4.2	9.6	17.1	35.4	46.0	54.8	70.2	3.1	12.6	25.9	49.0

[a] Additional DCC (100 mg.) was added after 24 hours. [b] In the solvent system isoamyl alcohol–5% disodium hydrogen phosphate, it could be demonstrated that this spot was primarily unreacted starting material. After 24 hours, there was slight isomerization probably due to slow hydrolysis of the phosphorylureas. [c] In the solvent system isoamyl alcohol–5% disodium hydrogen phosphate, this spot was resolved into two components. In all cases the faster travelling material ("x") was present in greater amount.

that order. The fractions containing III and IV were concentrated under reduced pressure below 30°, and finally evaporated to dryness over calcium chloride *in vacuo*. The residue (110 mg.) was triturated with 2 cc. of water, and a small amount of insoluble urea was removed by filtration. The filtrate was cooled to 0° and acidified with 1 cc. of cold 0.115 *N* hydrochloric acid. After standing for approximately 30 minutes at 0°, the clear solution deposited a microcrystalline precipitate which was collected, washed twice with ice-cold water and dried *in vacuo* at room temperature over phosphorus pentoxide; yield 35 mg. Paper chromatography in solvent 1 revealed a single spot travelling close to the solvent front. In isoamyl alcohol–5% disodium hydrogen phosphate[31] (solvent 2) the material resolved into two components with R_f values[31] of 0.66 ("x") and 0.39 ("y"). The relative amounts of "x" and "y" were 75 and 25%, respectively. On heating the substance in an open capillary tubing it began to darken at 195° and charred above 210°.

Method B.—Yeast adenylic acid and DCC reacted on a fivefold larger scale than described in method A. After 29 hr. standing at room temperature, the urea was removed by filtration and washed with 50 cc. of water. The aqueous pyridine solution was extracted with three 200-cc. portions of toluene and the remaining aqueous solution was concentrated *in vacuo* below 30° to 33 cc. The presence of large amounts of III and IV and of smaller amounts of the cyclic phosphate and yeast adenylic acid was revealed by paper chromatography. After adjusting the *p*H to 3 with 0.1 *N* hydrochloric acid and filtering to remove a trace of dicyclohexylurea, the mixture was allowed to stand at 5°. A copious precipitate had accumulated after 3 days and the *p*H of the supernatant had risen to 3.4. The precipitate was collected in a centrifuge[32] and the centrifugate was washed in the tube with 3 cc. of ice-water (twice) and 2 cc. of cold methanol (twice). The insoluble residue was immediately placed in a vacuum desiccator and dried over phosphorus pentoxide; wt. 60 mg. Paper chromatographic examination in solvent 1 of this material and the washings showed the former to be only phosphorylurea and the latter to contain, in addition, small amounts of the cyclic phosphate and yeast adenylic acid. Paper chromatography of the residue in solvent 2 followed by spectrophotometric estimation showed it to be a mixture of "x" (73%) and "y" (27%).

23.8 mg. of this material was suspended in 10 cc. of water and dissolved by the controlled addition of 0.3 *N* sodium hydroxide to *p*H 11.[33] The solution was then titrated with 0.31 *N* hydrochloric acid using an automatic electrometric titration apparatus.[34] This revealed no titratable group in the *p*H range 5–10, the primary amino group of the ring titrating normally. Assuming the pK_a of the amino group to be the same as that in yeast adenylic acid (3.8), the neutral equivalent was calculated to be 595,[33] 20 μmoles of the acid being required to titrate the amino group.

The solution was quantitatively recovered and diluted to 25 cc. A 1-cc. aliquot was further diluted to 25 cc. with 0.1 *N* hydrochloric acid.[35] The optical density of this solution at 257 mμ (Beckman instrument, model DU) was 0.950 thus giving a figure of 577[33] for the mol. wt. of the N-adenylylureas. The calculated mol. wt. for $C_{23}H_{36}O_7N_7P$ is 553.6. A second 1-cc. aliquot from the original solution was diluted to 10 cc. with water, and 1-cc. aliquots of this solution were used for the estimation of total phosphorus by the method of Bonar.[36] *Anal.* Found: P, 5.3. Calcd. for $C_{23}H_{36}O_7N_7P$: P, 5.6.[33]

A sample of material prepared by Method B (from 500 mg. of yeast adenylic acid) was taken up in 3 cc. of 0.25 *N* ammonium hydroxide and filtered carefully to remove traces of urea. The clear ammoniacal solution (concentration, 150–200 mg. of adenylylurea in 2 cc.) was lyophilized and the residue redissolved in 2 cc. of water. After filtration and cooling, ice-cold 0.15 *N* hydrochloric acid was added dropwise with shaking to *p*H 3. After 2 hr. at 5°, a fine white powder separated. This was collected, washed with water and immediately dried over phosphorus pentoxide at room temperature. This preparation was completely soluble in dilute ammonium hydroxide, and in chromatographic solvent 1 travelled as a single component. In solvent 2, it proved to be almost pure "x" (95%) with only a slight contamination by "y" (5%). *Anal.* Calcd. for $C_{23}H_{36}O_7N_7P$: C, 49.9; H, 6.4; N, 17.7; P, 5.6. Found: C, 49.1; H, 6.3; N, 17.3; P, 5.9. In McIlvaine buffer (*p*H 7), spectrophotometric examination revealed $\lambda_{max.}$ to be 259–260 mμ and $\epsilon_{max.}$ to be 13,300.[37]

Action of Acid and Alkali on N-Adenylylureas.—An aqueous solution of the ammonium salts of the adenylylureas (concentration, 20–30 mg. per cc.) purified by chromatography on large paper sheets and elution of the fast-running components ("x" and "y"), was used in the following experiments. (a) To 0.1 cc. of this solution was added 0.1 cc. of concentrated ammonium hydroxide (sp. gr. 0.900) and the resulting solution was allowed to stand in a stoppered tube for 28 hr. Paper chromatography in solvent 1 revealed only unchanged starting material.[38]

(b) A mixture of 0.1 cc. of the stock solution and 0.1 cc. of 0.5 *N* sodium hydroxide on standing slowly deposited a precipitate. Paper chromatography of aliquots of the reaction mixture removed after 1.2 hr. and 24 hr. showed that the first aliquot contained primarily unchanged starting

(31) This is the solvent originally used by Carter to separate the yeast adenylic acid isomers (see ref. 5). The R_f values of "x" and "y" were found to be influenced considerably by the thickness of the overlying layer of isoamyl alcohol. The R_f's recorded above were those obtained when the top layer was 0.5 cm. and the aqueous layer 1 cm. thick. Using a top layer of 1 cm. thickness, the R_f's of "x" and "y" were 0.51 (same as that of adenosine 2,3-cyclic phosphate, run simultaneously) and 0.18, respectively; in 5% disodium hydrogen phosphate alone, the R_f's were: "x," 0.67 (same as that of adenylic acid *a*, run simultaneously) and "y," 0.51 (same as that of the 2',3'-cyclic phosphate). In contrast, these modifications of the solvent system caused little change in the R_f's of adenylic acids *a* and *b* or the cyclic phosphate.

(32) Further crops of material, predominantly III and IV, were obtained from the mother liquor by readjusting the *p*H to 2.8–3.0 and cooling to 5°.

(33) Faint cloudiness was observed at this stage. This and the analytical data recorded below for this sample indicate the presence of a small amount of dicyclohexylurea as impurity.

(34) The unit employs a Model R Beckman *p*H meter, a Minneapolis–Honeywell Brown Electronik Recorder and a Gilmont Ultramicroburet.

(35) Under these conditions the conversion to yeast adenylic acid should by very rapid and the use of the figure 14,400 for the ϵ_{max} of yeast adenylic acid at 257 mμ will then be valid.

(36) Described in the dissertation submitted by R. Bonar to the Graduate School, University of California, Berkeley, in partial fulfillment of the requirements for the Ph.D. Degree. This is a modification of the micro-method of B. L. Griswold, F. L. Humoller and A. R. McIntyre, *Anal. Chem.*, **23**, 192 (1951), in which *p*-methylaminophenol sulfate (G. Gomori, *J. Lab. Clin. Med.*, **27**, 955 (1942)) has been used as reducing agent in place of aminonaphtholsulfonic acid.

(37) Based on the molecular weight of the anhydrous compound.

(38) This treatment partially hydrolyzes adenosine 2',3'-cyclic phosphate.

material and some yeast adenylic acid whereas the latter contained mostly yeast adenylic acid. In neither case was any 2′,3′-cyclic phosphate detected. The precipitate was collected, washed with water and dried; m.p. 226° undepressed on admixture with authentic N,N′-dicyclohexylurea.

(c) 0.1 cc. of the stock solution acidified with 0.1 cc. of 0.01 *N* hydrochloric acid was allowed to stand at room temperature. After 9 hr., spherical crystals were observed which were shown by paper chromatography to be pure starting material (as the free acid). The supernatant also contained the adenylylureas as well as a large amount of adenosine 2′,3′-cyclic phosphate and a small amount of yeast adenylic acid. In a similar experiment employing 0.1 *N* hydrochloric acid, complete degradation to N,N′-dicyclohexylurea (m.p. and mixed m.p. with authentic sample 226–227°) occurred within one hour at room temperature.

One hundred mg. of the pyridinium salts of III and IV, obtained by cellulose column chromatography (Method A above) and dried *in vacuo* over phosphorus pentoxide, was triturated thoroughly with 1 cc. of *N* hydrochloric acid and the insoluble dicyclohexylurea was collected after one hour, washed with water and dried over phosphorus pentoxide; wt. 37 mg.; theoretical yield for the pyridinium salts, 35.2 mg.[39]

Action of Sodium Benzoxide on "x," "y" and Adenosine-2′,3′-cyclic Phosphate.—Twenty-five mg. of the pyridinium salts of a mixture of III and IV obtained by cellulose column chromatography were dried *in vacuo* over phosphorus pentoxide and dissolved in 0.3 cc. of anhydrous benzyl alcohol; 0.1 cc. of a solution of sodium benzoxide in benzyl alcohol (from 100 mg. of sodium in 5 cc. of anhydrous benzyl alcohol) was added and the reaction vessel was sealed and allowed to stand at room temperature. Aliquots of 0.1 cc. were removed at intervals, diluted with 0.2 cc. of 2.5% acetic acid (to *p*H 3–4) and then extracted twice with ether. The residual aqueous solutions were examined by paper chromatography in solvent 1 and spectrophotometric estimation was made of the amounts of the reaction products and the unreacted adenylylureas. The results are summarized in Table II.

TABLE II

	1 hr.	7 hr.	17 hr.[40]
"x" and "y," %	70.0	25.0	7.4
Benzyl esters, %	26.4	49.8	31.7
Yeast adenylic acid, %	3.6	25.2	60.9

Table II. The benzyl esters were separated by large scale paper chromatography in solvent 1 and after elution and concentration were rechromatographed in solvent 2 and in butanol–acetic acid–water (4:1:5). In solvent 2 the reported[24] R_f values for the benzyl esters of *a* and *b* are, respectively, 0.67 and 0.56; found: 0.64 and 0.52. In butanol–acetic acid–water the reported[24] values are: 0.44 and 0.50; found: 0.49 and 0.57. Elution of the benzyl esters followed by spectrophotometric estimation revealed that the ester of adenylic acid *b* was present in much larger amount than that of adenylic acid *a* (4:1). Adenosine 2′,3′-cyclic phosphate and the individual isomeric adenylylureas were treated also with sodium benzoxide under similar conditions to those described above. The benzyl esters were formed in good yield (40–55%) in each instance. The determination of the relative amounts of the two benzyl esters revealed that in every case the *b* ester predominated (80–85%). Further identification of the mixed benzyl esters was made by acid (80% acetic acid at 100°) and alkaline (0.5 *N* sodium hydroxide at 30°) hydrolysis and chromatographic examination of the products of the reaction in solvent 2 and in butanol–acetic acid–water.[24] In both cases a mixture of adenylic acids *a* and *b* resulted.

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(39) The slightly higher yield indicates some loss of pyridine from the pyridinium compounds.

(40) The absolute exclusion of moisture is difficult and will profoundly influence such a small scale reaction. This probably accounts for the increased formation of yeast adenylic acid with time.

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The Electrical Effect of the N-Oxide Group in Pyridine 1-Oxide[1]

BY H. H. JAFFÉ

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Pyridine 1-oxide is shown to violate Brown's "chemical non-crossing rule."[3] The two major semi-empirical molecular orbital methods of calculating the reactivity of this compound are considered. The *static* method cannot correctly predict the reactivity in both electrophilic and nucleophilic substitution in this case. Hammett substituent constants for the replacement of a CH group in benzene by an $N^+ \rightarrow O^-$ group are derived from the pK's of the N-oxides of nicotinic acid, isonicotinic acid, 3- and 4-hydroxy- and 4-aminopyridine, and are used in calculation, by the *localization* method, of the relative reactivity of the various positions in pyridine 1-oxide. The electronic structure and the dipole moment of this compound are also discussed.

Two main semi-empirical wave mechanical methods are available for the prediction of reactivity of conjugated organic compounds. These methods are referred to as the *localization* and the *static* methods[2]; both have been used extensively, and are generally found to lead to the same conclusions.[3] Unfortunately, neither method achieves a calculation of the true activation energy. In the *static* method the π-electron contribution to the total potential energy of the reacting system is calculated at some point along the reaction path before the transition state is reached. The *localization* method, on the other hand, is concerned with the same quantity at some point on the reaction path beyond the transition state.[3] The fact that predictions made by both methods usually agree with each other and with the experimental findings has led Brown to propose the "chemical non-crossing rule." This rule expresses the notion that curves representing the variation of the potential energy of the reacting system along the reaction path for similar compounds, or for different positions in a single compound, usually do not cross.[3] This rule is not postulated to be universally valid; it has received some theoretical support from the

(1) Paper V in the Series: Theoretical Considerations Concerning the Hammett Equation. For Paper IV see H. H. Jaffé, *J. Chem. Phys.*, **21**, 415 (1953).

(2) H. H. Greenwood, *Trans. Faraday Soc.*, **48**, 585 (1952).

(3) R. D. Brown, *Quart. Rev.*, **6**, 63 (1952).