TABLE II

ANALYSES OF CHOLEIC ACIDS BY ULTRAVIOLET OR VISIBLE LIGHT ABSORPTION

	Choleic acid soln. Concn., mg./l.	Choleic acid soln. $\log_{10} I_0/I$	λ, mμ	Pure acholic soln. Concn., mg./l.	Pure acholic soln. $\log_{10} I_0/I$	% Acholic component in choleic acid Calcd.	% Acholic component in choleic acid Found	Co-ordin. No.
	141.0	0.851	275[a]	20.5	0.900		13.7	
Naphthalene	110.4	.669	275	16.3	.712	14.0	13.9	2
	108.5	.658	275	16.3	.712		13.9	
	52.8	1.415	240	3.58	0.744		12.9	
Phenanthrene	24.0	1.159	250[a]	3.92	1.453	13.1	13.0	3
	24.1	1.162	250	3.92	1.453		13.0	
p-Aminoazobenzene	100.5	1.342	385[a]	10.1	1.298		10.4[b]	
	80.4	1.050	385	10.1	1.298	11.1	10.2[b]	4

[a] Approximately, wave length of the absorption maximum. [b] Calcd. for $C_{12}H_{11}N_3 + 5C_{24}H_{40}O_4$: 9.1. Calcd. for $C_{12}H_{11}N_3 + 6C_{24}H_{40}O_4$: 7.7.

puted from the extinction of the main band (around 250 mμ), the expected value for a coördination number of three[4,10] was obtained.

Next, we applied the method to the choleic acids of *p*-aminoazobenzene and its N-dimethyl derivative, which were prepared in connection with a research in azo-carcinogenesis. From the binary "thaw–melting point diagram" of these azo compounds with desoxycholic acid,[11] it was impossible to decide in both cases whether the coördination number in their choleic acids was four or six—five being *a priori* ruled out by symmetry considerations.[12]

It has been found that both choleic acids give the spectrum of the pure azo compound and that *p*-aminoazobenzene combines with four molecules of the biliary acid. However, no consistent results were obtained with *p*-dimethylaminoazobenzenecholeic acid. In this case different samples showed a different content of the azo compound which, however, was consistent within each sample. This choleic acid is still under investigation.

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Experimental

Naphthalene and phenanthrene were several times recrystallized from absolute ethyl alcohol. Naphthalene was dried in Abderhalden drying apparatus at 60°, m. p. 80.2–80.5°. Phenanthrene was dried in a vacuum desiccator over calcium chloride; m. p. 99.4–100.4°. *p*-Aminoazobenzene was a sample of a product prepared by Dr. Lucy L. Nazario; m. p. 123.7–124.8°. Desoxycholic acid was purified according to Sobotka and Goldberg,[13] m. p. 171–173°. Absolute ethyl alcohol was used in both preparative and spectrophotometric work.

Choleic acids were prepared by pouring a hot alcoholic solution of the acholic component (0.3–0.4 g. in 5 ml.) into a filtered hot alcoholic solution of desoxycholic acid (3 g. in 13 ml.). The choleic acid crystallized upon cooling. Naphthalenecholeic acid was dried in an Abderhalden drying apparatus at 60°, m. p. 180–183°, reported m. p. 181–182°,[8] 182°,[6,7] 181.5–182.5°.[9] Phenanthrenecholeic acid was dried in a vacuum desiccator over calcium chloride; m. p. 183.8–185°, reported m. p. 184–185°,[10] 184–186°,[9] 186–187°.[4] *p*-Aminoazobenzenecholeic acid was dried in the Abderhalden apparatus at 94°, yellow crystals melting at 180–183°.

The extinction curves were measured by means of a D. U. Beckman quartz spectrophotometer, using 1.002-cm. silica cells. Absorption data for a solution of desoxycholic acid having a concentration of 14 g./liter are shown in Table I. In Table II are summarized the analyses of choleic acids by light absorption.

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(11) G. Cilento, unpublished data.

(12) H. Rheinboldt, *Ann.*, **451**, 256 (1927).

(13) H. Sobotka and A. Goldberg, *Biochem. J.*, **26**, 555 (1932).

The Separation of Adenosine Polyphosphates by Ion Exchange and Paper Chromatography[1]

BY WALDO E. COHN AND C. E. CARTER

The purity of preparations of ADP[2] and ATP[2] is usually established by the ratio of acid-labile to total phosphate[3] and enzymatic assay,[4] while the preparation of either in pure form, from a mixture of the two, is achieved by differential solubility of their salts.[5] Application of ion exchange and paper chromatography to the resolution and quantitation of a series of adenine compounds encountered in preparations of ATP and ADP is reported in this paper. The principles of these methods have been previously described.[6,7] The chromatographic separation of adenine nucleotides has also been studied by Chargaff, *et al.*,[8] Crammer,[9] and Hummel and Lindberg,[10] although the systems employed by them did not permit the present application. The results reported below indicate that both techniques have certain advantages in the analysis of mixtures of AMP[1], ADP, and ATP and that ion exchange can, in addition, be used as an exact preparative method

(1) Work performed under Contract Number W-7405-Eng-26 for the Atomic Energy Commission.

(2) ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; AMP, Adenosine monophosphate.

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

(4) (a) H. M. Kalckar, *ibid.*, **167**, 445 (1947); (b) G. A. LePage, and V. R. Potter, *ibid.*, **179**, 1229 (1949).

(5) H. M. Kalckar, *ibid.*, **148**, 127 (1943).

(6) W. E. Cohn, THIS JOURNAL, **72**, 1471 (1950) (also *ibid.*, **71**, 2275 (1949)).

(7) C. E. Carter, *ibid.*, **72**, 1466 (1950); Oak Ridge National Laboratory Report ORNL-313 (unclassified).

(8) E. Chargaff, *et al.*, *ibid.*, **71**, 1513 (1949).

(9) J. L. Crammer, *Nature*, **161**, 349 (1948).

(10) J. P. Hummel and O. Lindberg, *J. Biol. Chem.*, **180**, 1 (1949).

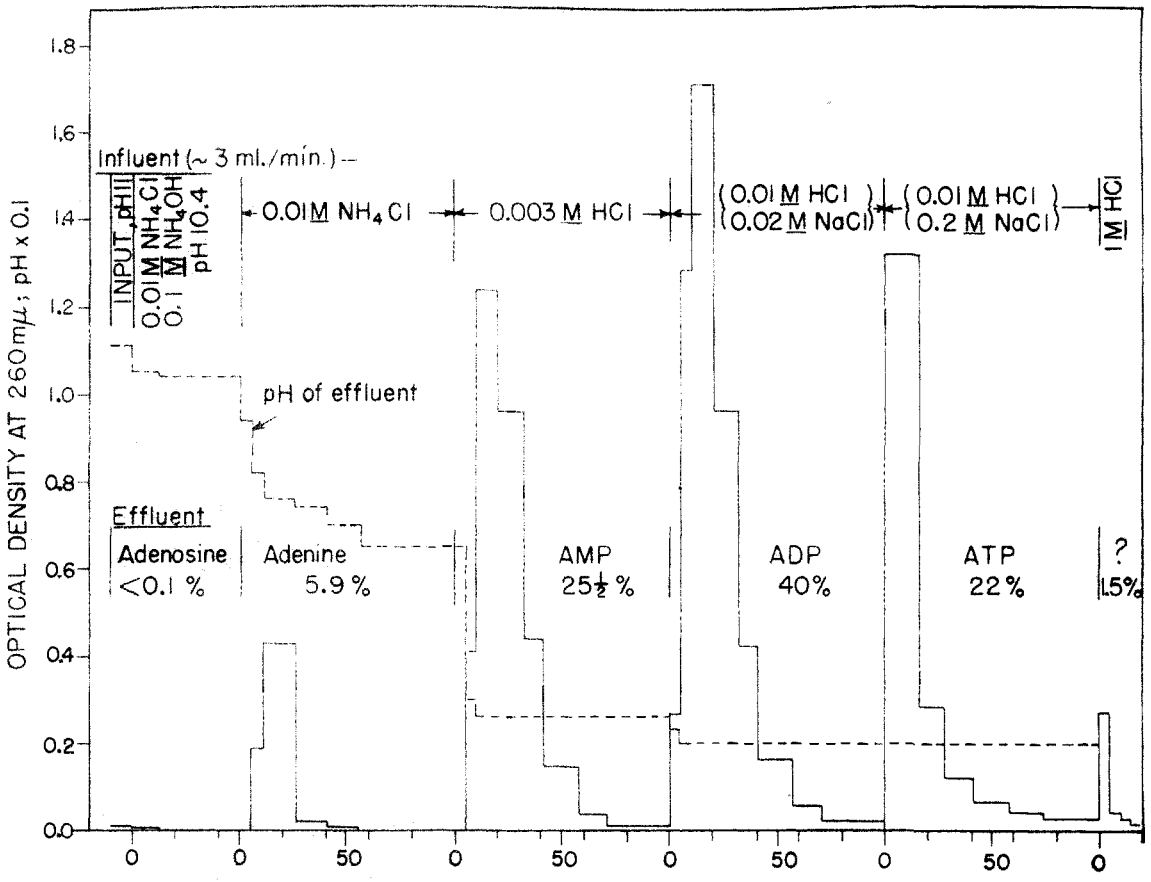

Fig. 1.—Ion-exchange separation of adenosine phosphates: bed, Dowex-1, 200–400 mesh, 1 sq. cm. × 1 cm.; test material, commercial H_4ATP; abscissa, ml. of reagent through column.

for either of the latter two compounds from their mixtures.

Experimental

The methods of ion exchange and paper chromatographic analysis were adaptations of those previously described for the analysis and separation of purine and pyrimidine compounds derived from nucleic acid.[6,7]

Barium salts of ATP and ADP, procured from various commercial sources, were dissolved in water by the addition of an equivalent amount of 0.1 *N* hydrochloric acid, treated with a slight excess of sodium sulfate and immediately neutralized. ATP, similarly obtained as the sodium salt or as the free acid, was dissolved in water and adjusted to *p*H 7.0.

Ion-Exchange Analysis.—For analysis, approximately 1–10 mg. of ATP or ADP were used in a solution made *ca.* 1 *M* with respect to ammonium hydroxide and containing less than 0.01 *M* anion (Cl^- or $SO_4^=$). This solution was passed through a column of 200–400 mesh Dowex-1 anion-exchange resin of bed size 1 cm. × 1 cm.^{2} at flow rates up to 3 ml./min. After a preliminary water wash, which removed no ultraviolet-absorbing material from the column, 100-ml. portions of the following reagents were passed through the column to remove the components and contaminants, if present, indicated (see Fig. 1): (1) 0.01 *M* NH_4Cl in 0.1 *M* NH_4OH (adenosine); (2) 0.01 *M* NH_4Cl in H_2O (adenine); (3) 0.003 *M* HCl (adenosine monophosphate); (4) 0.02 *M* NaCl in 0.01 *M* HCl solution (adenosine diphosphate, plus any inorganic orthophosphate); (5) 0.2 *M* NaCl in 0.01 *M* HCl solution (adenosine triphosphate). The identity of each fraction was established by both chemical (adenine:acid labile P:total P) and chromatographic analysis.[7] Any step in the process can be omitted; the substance not removed will then appear in the following fraction.

Paper Chromatography.—It was previously reported[11] that ATP could be resolved on a paper chromatogram from a mixture of adenosine-5′-phosphate, adenosine and adenine in a two-layer isoamyl alcohol–monopotassium phosphate solvent system. When ADP became available, it was found that this compound migrated to the ATP spot in the acid phosphate system but could be separated from ATP, AMP, adenosine and adenine when an isoamyl–5% disodium phosphate solvent system was employed. Adequate resolution of ATP and ADP in this system is obtained when not more than 50 μg. of each component is delivered onto the starting spot in a volume of 0.010 to 0.015 ml. For determination of the amount of each adenine component, the spots, located by ultraviolet fluorescence, were cut from the paper chromatogram and eluted with 0.01 *N* hydrochloric acid. The solutions were then examined spectrophotometrically at 260 mμ.

Results

The analytical results, given in Table I, are expressed as the per cent. of the total 260 mμ absorption (in 0.01 *N* hydrochloric acid) accounted for by each component in a Beckman ultraviolet spectrophotometer. The concentration of each component may be calculated from extinction coefficients[12] and the per cent. composition of the

(11) C. E. Carter, THIS JOURNAL, **72**, 1835 (1950).

(12) The figure of 14,200, at *p*H 2, has been found in this laboratory for the molecular extinction coefficient of adenylic acid.

TABLE I

PER CENT. COMPOSITION BASED ON TOTAL 260 mμ ABSORPTION[a]

Component	R_f value, paper chromatogram	H_4ATP Paper	H_4ATP Ion exch.	Ba_2ATP Paper	Ba_2ATP Ion exch.	Na_4ATP #1[c] Paper	Na_4ATP #1[c] Ion exch.	Na_4ATP #2[d] Paper	Na_4ATP #2[d] Ion exch.[e]	BaADP Paper	BaADP Ion exch.
ATP	0.83	20.6	22.2	81.5	76.5	(±)[b]	2.0	75	61	(±)	0.1
ADP	.77	42.6	40.0	18.5	14.9	(±)	7.2	23	11	90	84
AMP	.69	35.5	25.4	(±)	2.3	95	80.5	(±)	2.2	(±)	5.9
Adenosine	.52	0	0	0	0	0	0.9		0	0	0
Adenine	.38	(±)	5.9	0	0	0	0		0	0	0

[a] Recovery of components from paper chromatogram and ion-exchange resin was between 90–97% based on 260 mμ. absorption of starting material. [b] ± This sign designates 5% or less, an amount detectable but difficult to quantitate with certainty (2–5 μg.) [c] Na salt which had stood at room temperature for about six months (see text). [d] Na salt which had been kept at −35°. [e] Twenty-five per cent. of the material absorbing at 260 mμ was not recovered in any of the expected fractions.

mixture based on dry weight of the starting material. In most samples examined, the total adenine compounds thus determined accounted for 90–95% of the dry weight. No purine or pyrimidine derivatives other than those reported in Table I were found in the commercial preparations examined.

Discussion

Although good agreement on the assays reported in Table I was achieved with the two analytical schemes, it should be pointed out that the ion-exchange analysis is inherently a more sensitive and rigorous analytical technique than paper chromatography and has the additional advantage of permitting a wide range in the concentrations of components to be separated. Paper chromatography appears to be most useful when a rapid, semiquantitative technique for the analysis of a large number of samples is desired, as in following the composition of a preparation of ATP during isolation procedures.

It is clear that the ion-exchange separation may be used equally well as a preparative method; however, no attention has been paid to the removal of impurities not detected by ultraviolet light absorption. Hence, the method must at present be used in conjunction with enzymatic assay to establish the presence or absence of inhibitors, etc., of this kind.

The instability of the sodium salt of ATP has been noted by others[13] and makes imperative the analytical control of this compound before use in enzyme systems.

(13) F. Lipman, personal communication.

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Transetherification Reactions. Thiophenols with Ethyl β-Ethoxyacrylates[1]

BY W. J. CROXALL, L. R. FREIMILLER AND E. Y. SHROPSHIRE

The transetherification of ethyl β-ethoxyacrylate with alcohols and mercaptans yields ethyl β-alkoxyacrylates and ethyl β-alkylthioacrylates, respectively.[2] Attempts to apply this reaction to phenol were unsuccessful. However, various thiophenols with ethyl β-ethoxyacrylate in the presence of sodium bisulfate catalyst readily undergo the transetherification reaction to give the corresponding ethyl β-phenylthioacrylates in good yields. The various ethyl β-phenylthioacrylates are listed in Table I.

Saponification of these esters gave the β-phenylthioacrylic acids. All the acids as isolated from the saponification experiments melted over a wide range. Fractional crystallization of two of these acids, β-phenylthioacrylic acid and β-(*p*-tolylthio)-acrylic acid, gave in each case two fractions of narrow melting range, the major portion being the higher melting forms. These individual components as well as the original mixtures had the same neutral equivalent values, indicating that the fractions of different melting points are *cis–trans* isomers.

β-Phenylthioacrylic acid was converted to the acid chloride by treatment with phosphorus pentachloride and the resulting acid chloride cyclized with aluminum chloride to 1-thiochromone.

Experimental

The following experiment is typical of all the transetherifications.

Ethyl β-Phenylthioacrylate.—Distillation of 110 g. (1 mole) of thiophenol and 144 g. (1 mole) of ethyl β-ethoxyacrylate from 1 g. of sodium bisulfate gave 42 g. (91%) of ethanol and 180 g. of the thioacrylate.

β-Phenylthioacrylic Acid.—A two-phase mixture consisting of 50 g. (0.24 mole) of ethyl β-phenylthioacrylate and 15.8 g. of potassium hydroxide dissolved in 100 ml. of water was stirred and refluxed for two hours to give a homogeneous solution. Acidification of the solution with dilute hydrochloric acid yielded a white solid which was collected on the filter and washed with water; yield 39 g. (90%). A portion of the acid was recrystallized from petroleum ether (b. p. 90–100°), m. p. 75–103°. *Anal.* Calcd. for $C_9H_8O_2S$: S, 17.8; neut. equiv., 180. Found: S, 17.6; neut. equiv., 181.

Fractional recrystallization of 80 g. of the above acid, m. p. 75–103° (obtained in other experiments), from petroleum ether–acetone mixtures gave 63 g. of white crystalline material, m. p. 127–128.5°; neutral equiv. 181.5. Evaporation of the mother liquors gave a solid which was

(1) For the previous paper of this series, see Croxall and Fegley, THIS JOURNAL, **72**, 2888 (1950).

(2) Croxall, Van Hook and Luckenbaugh, THIS JOURNAL, **71**, 2736 (1949).