Biochemistry VOLUME 2, NUMBER 3

May 10, 1963

The Use of Amines in Liquid-Liquid Extractions of Nucleic Acids and Related Compounds

JOSEPH X. KHYM

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee*

Received November 9, 1962

Liquid anion exchange procedures were developed and tested as a method of investigating nucleic acids and lower-weight components that are associated with them. The liquid exchangers are high-molecular-weight amine salts dissolved in water-immiscible solvents. Upon contact with an aqueous salt solution, the anions in the organic phase can be replaced by those contained in the aqueous phase. Di-, tri-, and tetranucleoside phosphates, RNA, DNA, and lower-weight oligonucleotides are extracted readily from dilute acetate or formate buffer solutions and are recovered from the organic phases by increasing the aqueous buffer concentration or by using dilute alkaline solutions that neutralize the amines and allow extracted compounds to reappear in the aqueous phase. Inherent differences in the extraction coefficients for a given series of compounds indicate that this technique can be used to make practical separations.

Paraffin chain quaternary ammonium salts were first used in nucleic acid chemistry by Jones (1953), who prepared nucleic acids *via* an insoluble cetyltrimethylammonium complex. Aubel-Sadron *et al.* (1960, 1961) and, from the same laboratory, Weil and Ebel (1962) have systematically studied the action of several types of quaternary ammonium salts and have shown a correlation between the molecular weight of a given series of quaternary amines and their ability to precipitate nucleic acids from aqueous solutions. In addition these workers showed that the precipitated quaternary ammonium salts of nucleic acid are soluble in several organic solvents. Their results indicate that a separation of different types of RNA may be possible because of differences in the solubilities of quaternary RNA complexes in a given organic solvent.

Liquid ion exchange was first used by Smith and Page (1948), who used long-chain high-molecular-weight amines as acid-binding extractants. This technique has been used with remarkable success by Coleman *et al.* (1958) for developing methods for recovering uranium and related metals from ore leach liquors. This technique is similar in approach to, but different in principle from, the countercurrent distribution system initiated by Warner and Vaimberg (1958) for the partition of RNA and also polynucleotides between two liquid phases. The partition technique was further developed by Holley *et al.* (1960) and also Kirby (1960) and refined for the fractionation of yeast amino acid acceptor RNA by Doctor *et al.* (1961).

In the liquid anion exchange method, amines dissolved in water-immiscible solvents are converted to a desired salt form either by direct treatment with concentrated acid or by shaking with aqueous solutions of a given acid or buffer solution. Thus converted, the anion of the amine salt can be replaced by another anion by subsequent equilibration with a fresh aqueous phase. As pointed out by Kunin (1961), liquid ion exchange differs from solvent extraction in that liquid ion exchange depends upon a reaction or energy of binding between the solute and the extractant. The over-all reaction is analogous to those of conventional solid anion exchangers.

Liquid anion exchange studies have been initiated to test the feasibility of this technique for (1) the extraction of RNA, DNA, nucleoside mono-, di-, and triphosphates, and intermediate-molecular-weight oligonucleotides from aqueous salt solutions, and (2) the separation of the compounds listed from each other as well as further separations within a given group.

EXPERIMENTAL PROCEDURES

General Considerations.—In this paper, only single-batch distribution studies are reported. The compounds used were dissolved in aqueous salt solutions and shaken with equal volumes of organic phases containing water-immiscible amine salts. Most of the extractions were carried out in small separatory funnels equipped with Teflon drainage plugs. The phases were agitated with a wrist-action shaking machine. For smaller volumes (<10 ml total volume) the phases were agitated either in small beakers by magnetic stirring or in test tubes, where the extractions were made by swirling the phases with a Vortex Jr. mixer. After equilibration, the samples were centrifuged and one or both layers examined for absorbancy at 260 mμ. The aqueous layer was examined directly, and the concentration of material in the organic phase was determined by noting the disappearance of material in the aqueous phase or by contacting the organic phase (usually 0.1 M or less in amine concentration) with 1 M NaCl or with 0.5 M alkaline solutions such as $NaHCO_3$, NH_4HCO_3, or $K_2B_4O_7$ to neutralize the amine and permit the extracted material to reappear in the aqueous phase.

* Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

Many amines have been tested as extractants by using them as described above. Chloride, bromide, and nitrate salts of the amines were unsatisfactory in several of the practical solvents that are available, but sulfate, citrate, formate, and acetate salts are suitable for extractions because emulsions, third-phase formation, and organic salt solubility are reasonably controlled. Thus far it appears that this technique is carried out best when the nucleic acids and related products are dissolved in acetate or formate buffer solutions (*p*H 2.7 to 7.0), and the corresponding amine acetate or formate salts are used as extractants in the organic phase.

Choice of Amine.—Several classes of amines have been tested as extractants. Thus far only the aliphatic branched chain primary amines have been useful reagents. Long straight chain primary amines (*e.g.*, C_{16}, C_{18}) have limited use because of their low solubility in many organic diluents (Coleman *et al.*, 1958), and, of the many secondary or tertiary amines screened, either branched or straight chain, several are too weakly basic to be used in the *p*H range 2.7 to 7.0. Several quaternary ammonium salts, such as those described elsewhere (Scott, 1960), were tried and were found to give emulsions, possibly because of their mutual solubility in both aqueous and organic diluents. However, two high-molecular-weight quaternary salts, "Aliquats 336 and 604,"[1] show good phase-separation qualities and are potentially good extractants, particularly for use at high *p*H. Cursory examinations showed that the borate and carbonate forms of these amines extract RNA, DNA, and ATP.

Choice of Solvent.—The ability of a given amine to extract a particular compound depends greatly on the solvent used as the diluent. The main characteristics for choosing a particular solvent are immiscibility with the aqueous solution, ability to dissolve the amine reagent plus the extracted amine compound complex, and freedom from interfering interactions with the reagent (Brown *et al.*, 1954). Isoamyl acetate and butyl ether have been most useful in these studies thus far, although hydrocarbons such as decane and dodecane and ortho esters such as butyl orthoformate have been suitable solvents for a few of the amine salts.

Nucleic Acid Preparations.—RNA-I (protein ~ 1%) was prepared from calf liver with guanidine hydrochloride used as the protein denaturant and is described by Volkin and Carter (1951). RNA-II (protein ~ 3%) was prepared from calf liver by using a combination of the detergent-Aerosol OT procedure (Astrachan *et al.*, 1957) followed by extractions with chloroform-octanol (Sevag *et al.*, 1938) to remove protein. Calf thymus DNA (protein ~ 1%) was prepared by purifying a well-washed DNA-protein fraction with a detergent (Astrachan *et al.*, 1957). Protein was determined by the method of Lowry *et al.* (1951).

Results

Comparison of Different Amine Types for the Extraction of ATP.—A series of amines at a concentration of 0.1 M was dissolved in isoamyl acetate and converted to their formate and acetate salts by adding the appropriate acid. ATP at approximately 1 μmole/ml was dissolved in either 0.5 M sodium formate or acetate buffer at *p*H 2.7 and 3.7, respectively. Five ml of each phase was placed in a separatory funnel and agitated on a wrist-action shaker. After 10 minutes the phases were separated and one or both layers assayed for ATP content. The results given in Table I show that the primary amines of molecular weight greater than 200 are efficient extractants for ATP.

TABLE I
EXTRACTION OF ATP WITH DIFFERENT AMINES

Amine	Type	m.w.	% Extracted into Organic Phase: 0.5 M Formate	0.5 M Acetate
3,5,5-Trimethylhexyl	Primary	143	<1	<1
81-R[a]	Primary	191	15	55
3-Ethyl-1-isobutyl-octyl	Primary	213	93	98
1-(3-Ethylpentyl)-4-ethyloctyl	Primary	255	96	99
1-Nonyldecyl	Primary	283	98	99
JM-T[a]	Primary	315	94	98
bis(1-Isobutyl-3,5-dimethylhexyl)	Secondary	354	<1	18
N,*N*-Dimethyl-dodecyl	Tertiary	213	7	7
Triisooctyl	Tertiary	353	0	0

[a] 81-R and JM-T obtained from Rohm and Haas Co. are *t*-alkyl primary amines with highly branched alkyl chains of the structure $R,R',R''\text{-}C'NH_2$ where the R's contain 12-14(81-R) and 18-22(JM-T) carbon atoms.

Effect of Amine Concentration on Extractions of UTP, ATP, and RNA.—ATP and UTP at a concentration of about 1.5 μmoles/ml were dissolved in 0.5 M sodium formate buffer, *p*H 3.5, and a series of these samples was shaken for 10 minutes against varying concentrations of 3-ethyl-1-isobutyloctyl formate (used for UTP) and of 1-(3-ethylpentyl)-4-ethyloctyl formate (used for ATP), both dissolved in *n*-butyl ether. RNA-I (about 3 μmoles/ml) in 0.5 M sodium formate buffer (*p*H = 4.7) was mixed with varying concentrations of JM-T formate in isoamyl acetate. An extraction coefficient, E_A^o, defined as the concentration of compound extracted into the organic phase divided by the concentration remaining in the aqueous phase, was calculated for each of the batch extractions. As shown in Figure 1, the E_A^o for each compound increased in direct proportion to the increase of amine concentration.

Effect of Formate Concentration on Extractions of UTP, ATP, and RNA.—In these experiments solutions of UTP, ATP, and RNA at different concentrations of buffer were contacted with solutions of the amine salts as used above, but the concentration of amine in the organic phase was held constant at 0.1 M. As the concentration of buffer was increased in the aqueous phase, the E_A^o for all three compounds, correspondingly, was found to decrease (Figs. 2 and 3).

UTP and ATP Extraction Isotherms.—To obtain the stoichiometry of the resulting triphosphate-amine complex, the saturation concentration of nucleoside triphosphate in the organic phase was determined. A fixed concentration of amine in a single organic phase was shaken with successive volumes of aqueous solutions containing UTP and ATP in known initial concentrations. The cascade extractions were continued until the aqueous phase concentration of UTP and ATP was the same as that of the initial stock solutions. Saturation in the organic phase was confirmed by taking small aliquots (25 to 50 μl), placing them in 1 ml of butyl ether, and then agitating them with

[1] Products of General Mills, Kankakee, Ill., available as chloride salts. Aliquat 336 (tricapyrylyl methyl ammonium chloride), 604 (lauryl dimethyl benzyl ammonium chloride).

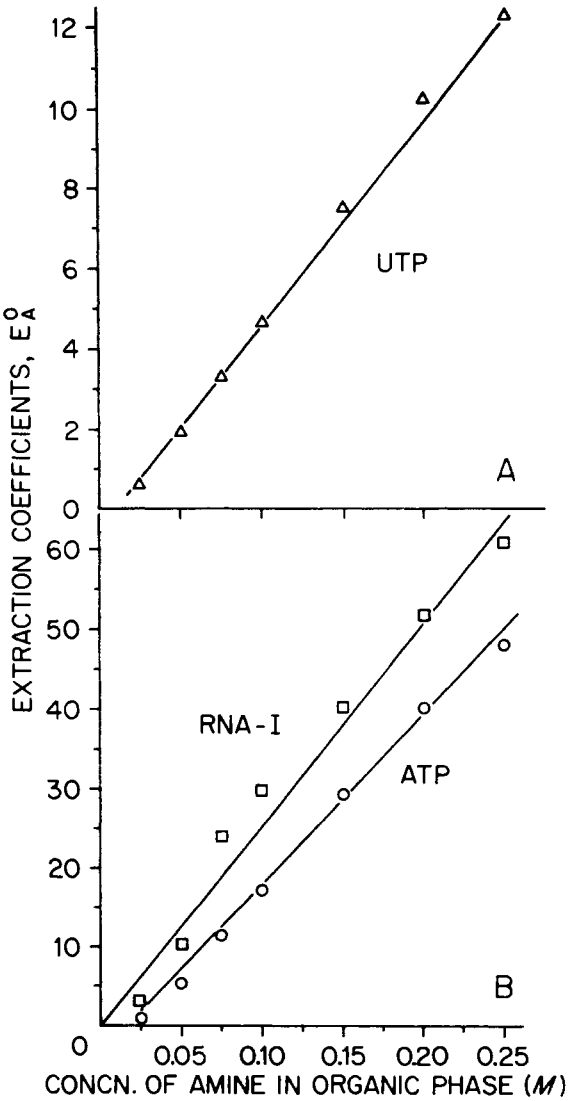

FIG. 1.—Effect of amine concentration on extraction of UTP, ATP, and RNA. *A:* aqueous phase, UTP in 0.5 M formate buffer, *p*H 3.5; organic phase, 3-ethyl-1-isobutyl-octyl amine formate in butyl ether at concentrations shown. *B:* □, RNA in 0.5 M formate buffer, *p*H 4.7, *vs.* JM-T formate in isoamyl acetate at concentrations shown. ○ ATP in 0.5 M formate buffer, *p*H 3.5, *vs.* 1-(3-ethylpentyl)-4-ethyloctyl amine formate in butyl ether at concentrations shown. Phase ratio for both *A* and *B* was 1:1 and agitation was for 10 minutes on wrist-action shaker.

an equal volume of 0.5 M NH_4HCO_3. Dilutions of the aqueous phase were read at 260 mμ. After saturation was reached, an additional extraction was carried out with concentrations of UTP and ATP well in excess of the initial stock solutions; no additional UTP or ATP was extracted, which further confirmed saturation of the organic phase. These results are demonstrated in Figure 4. Both compounds show a limiting association number of about 7 moles of amine to 1 mole of triphosphate.

Extraction Coefficients for Nucleoside Di- and Triphosphates.—As the charge is increased in a given nucleoside phosphate series, extraction coefficients increase rapidly, as seen from the data of Table II. It is evident from the Table II that by continuous extraction techniques the separation of any of these compounds from the others is feasible. Monophosphates and inorganic phosphate do not extract to any appreciable extent in any of the many solvent systems explored.

TABLE II
EXTRACTION COEFFICIENTS FOR NUCLEOSIDE DI- AND TRIPHOSPHATES

Organic phase: 0.1 M JM-T formate in isoamyl acetate. Aqueous phase: phosphate compound in formate buffer. 10 minutes of rapid stirring, phase ratio 1:1.

Compound	0.1 M Formate *p*H 4.7	0.5 M Formate *p*H 2.7
CDP	0.5	0.2
CTP	10.3	1.9
ADP	3.5	0.2
ATP	27.0	15.0
UDP	1.7	0.7
UTP	19.0	15.0
GDP	2.7	0.8
GTP	12.0	7.5

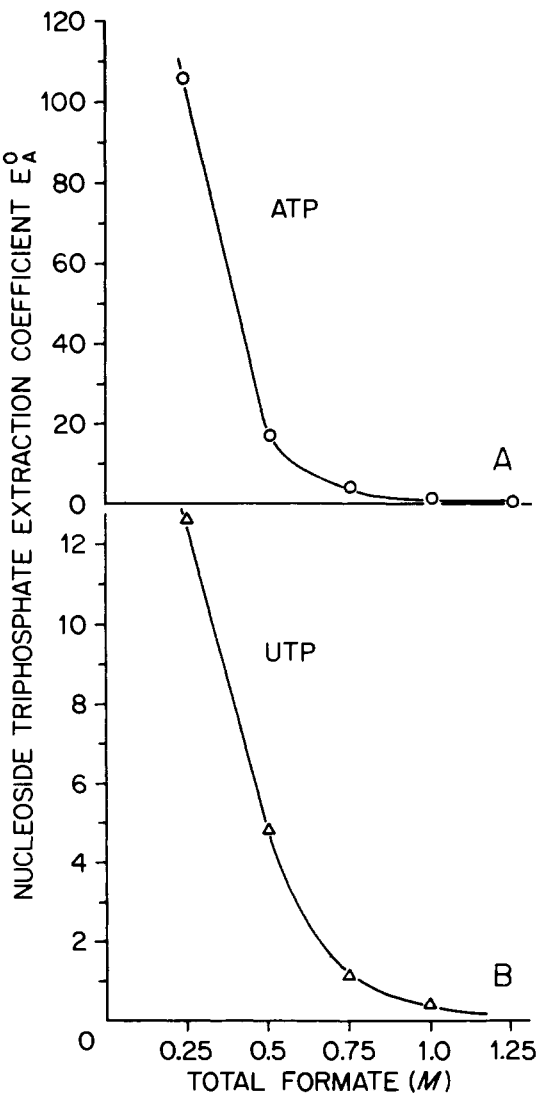

FIG. 2.—Effect of formate concentration on extraction of ATP and UTP. *A:* aqueous phase, ATP in formate buffer, *p*H 3.5; organic phase, 0.1 M 1-(3-ethylpentyl)4-ethyloctyl amine formate in butyl ether. *B:* aqueous phase, UTP in formate buffer *p*H 3.5; organic phase, 0.1 M 3-ethyl-1-isobutyloctyl amine in butyl ether. Phase ratio for both *A* and *B* was 1:1 and agitation was for 10 minutes on wrist-action shaker.

Behavior of RNA-II and DNA with Primary Amines.—High-molecular-weight RNA and DNA also can be extracted into organic solutions containing amines. None of the amines used, however, gives two clear

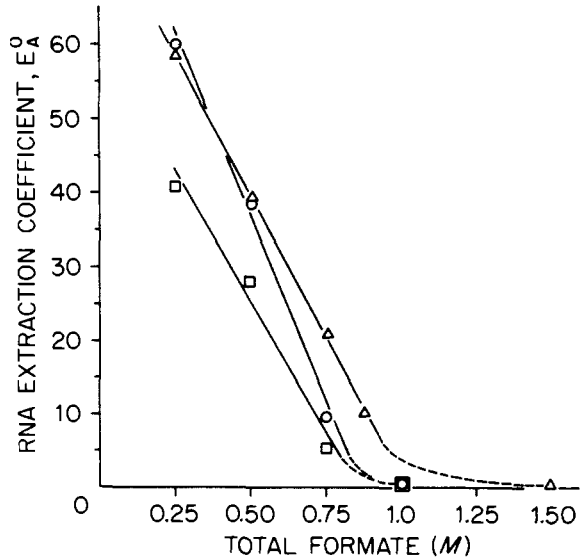

FIG. 3.—Effect of formate concentration on extraction of RNA. Aqueous phase, RNA in formate buffers; organic phase, 0.1 M JM-T formate in isoamyl acetate. Phase ratio 1:1, 10 minutes of agitation by wrist-action shaker. Δ, formate at *p*H 3.6; O, at *p*H 4.7; □, at *p*H 5.6.

phases if the amount of protein in a given nucleic acid preparation is much greater than 3%. In RNA, a protein concentration of >3% in the preparation gives a gel-like aqueous phase with several of the amine-solvent systems tested, but more than 80% of the RNA can be extracted into the organic phase. For DNA preparations high in protein, it is difficult to distinguish any phase separation owing to the formation of a bulky precipitate that extends into both phases. Furthermore, when nucleic acid of low protein content is extracted, it is difficult to maintain two clear phases when the aqueous salt concentration is increased to obtain a series of decreasing extraction coefficients. At a buffer concentration in the range of 0.8 to 1.0 M, precipitates are formed which layer at the interface and may contain as much as 15% of the nucleic acid. In extractions of nucleoside triphosphates several primary amines can be used interchangeably without apparent changes in their extraction behavior; however, the same amines differ markedly in their ability to extract the nucleic acids. Table III demonstrates the extraction behavior of RNA-II (protein ~ 3%) and DNA (protein ~ 1%) with several primary amines.

Extraction of Polyadenylic Nucleotides.—Preliminary experiments were carried out on some nucleotides given to us by Dr. Leon Heppel. Only small quantities were available, and the extraction of these compounds was carried out by mixing 1 ml of 0.1 M 3-ethyl-1-isobutyloctyl formate in butyl ether with the compounds dissolved in 1 ml of 0.5 M formate buffer, *p*H 3.5. For comparison, adenosine tetraphosphate was also included in this series. These results are given in Table IV.

DISCUSSION

Structure and type of amine are two variables that are being tested for any selectivity that amines may have for the extraction of a particular class of compounds. At present the only indication that structure may be related to selectivity is the different behavior of a series of primary amines in their reactions with RNA and DNA (Table III). However, with a given amine-solvent system, inherent differences in extraction coefficients are readily obtained in the lower-

TABLE III

EXTRACTION OF RNA AND DNA WITH PRIMARY AMINES

Organic phase: 0.1 M amines in solvent listed. Aqueous phase: 0.5 M acetate buffer, *p*H 4.7. Mixing of phases by wrist-action shaker for 10 minutes. Phase ratio 1:1.

Nucleic Acid	Amine[a]	Solvent	Phase Observations[b]	% Extracted
DNA	JM-T	Butyl ether	T.F.	99
RNA	JM-T	Butyl ether	T.F.	97
DNA	1	Butyl ether	T.F.	92
RNA	1	Butyl ether	T.F.	97
DNA	1	Isoamyl acetate	Ppt. I	54
RNA	1	Isoamyl acetate	Ppt. I	90
DNA	2	Isoamyl acetate	Ppt. O	—
RNA	2	Isoamyl acetate	Ppt. I	0
DNA	3	Isoamyl acetate	Ppt. I	25
RNA	3	Isoamyl acetate	Ppt. I	0
RNA	4	Isoamyl acetate	Ppt. A	79
DNA	4	Isoamyl acetate	Ppt. I	50

[a] 1 = 3-ethyl-1-isobutyloctyl; 2 = 3,5,5-trimethylhexyl; 3 = 1-(3-ethylpentyl)-4-ethyloctyl; 4 = 1-nonyldecyl. [b] T.F. = thin film at interface; ppt. I = precipitate at interface; ppt. O = precipitate in organic phase; ppt. A = precipitate in aqueous phase.

TABLE IV

EXTRACTION OF POLYADENYLIC NUCLEOTIDES AND ADENOSINE TETRAPHOSPHATE

Organic phase: 0.1 M 3-ethyl-1-isobutyloctyl formate in butyl ether. Aqueous phase: 0.5 M formate buffer, *p*H 3.5. Mixing of phases by a Vortex Jr. mixer for 2 minutes, phase ratio 1:1.

Compound	% Extracted	E_A^0
$(pA)_2$	7	0.08
$(pA)_3$	75	3
$(pA)_4$	81	4
$(pA)_5$	88	7
Adenosine tetraPO_4	98.5	68

molecular-weight phosphate compounds, so that a given pair of compounds, *e.g.*, ADP-ATP or $(pA)_2$-$(pA)_5$ can be separated easily with a few batch extractions. For other pairs or groups of compounds with separation factors (ratio of E_A^0 of one compound to another) of much lower magnitude, countercurrent distribution would be necessary to effect practical separations.

It is also obvious from the data that the molecular weight is an important factor to consider in using an amine as an extractant. Amines of molecular weight as low as 185 can remove RNA and other compounds from dilute buffer solution, but this is about the lowest limit that is practical. Losses of amine salt to the aqueous phase and complete loss of extractant power occur when lower-weight amines are used.

The mechanism of extraction by this technique is not completely understood (Coleman *et al.*, 1958). However the analogy of conventional ion exchange involving sorptions by alkylammonium groups in solid anion exchanges is very striking (Kunin, 1961). For instance, different salt forms of an amine show particular affinities in their extractability for a given compound, and, as the charge in a particular class of compounds is increased, correspondingly higher extraction coefficients are obtained. In an extraction sequence, a series of decreasing extraction coefficients can be obtained when the aqueous salt concentration is increased. Also conversion of an amine salt to free amine im-

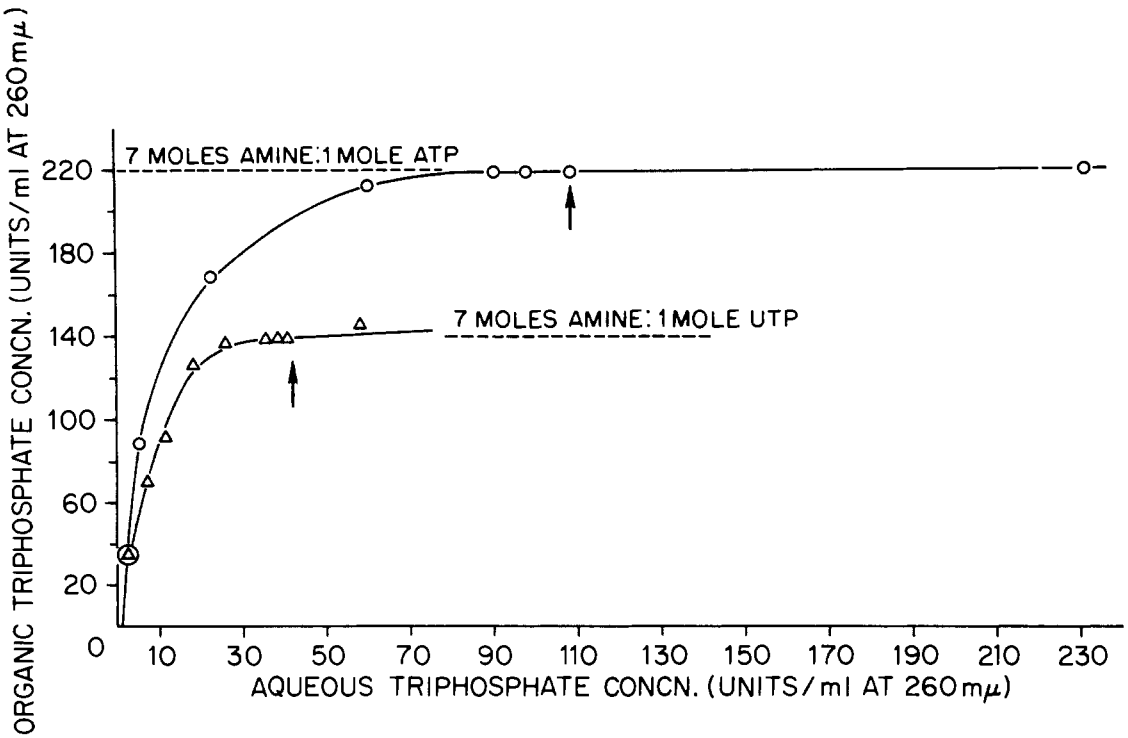

FIG. 4.—Nucleoside triphosphate isotherms. Cascade extractions (organic phase contacting successive volumes of aqueous phase) at phase ratio 1:1 and contact time of 10 minutes per stage by wrist-action shaker O, ATP in 0.5 M formate buffer, *p*H 3.5, *vs.* 0.1 M 1-(3-ethylpentyl)-4-ethyloctyl amine formate in butyl ether. △, UTP in 0.25 M formate buffer, *p*H 3.5, *vs.* 0.1 M 3-ethyl-1-isobutyloctyl amine formate in butyl ether. Arrows indicate stage at which aqueous phase concentration of nucleoside triphosphates equals initial concentration. Additional points after arrows are the concentrations of a different solution of triphosphates in which the aqueous phase far exceeded initial stock concentrations.

mediately releases extracted compounds and prevents the amine from extracting any additional material.

All the foregoing observations fit qualitatively an

$$a\text{R-NH}_3\text{F}_{org} + X_{aq}^{-a} \rightleftarrows (\text{R-NH}_3)_aX_{org} + a\text{F}_{aq}^-$$

$$K = \frac{[(\text{R-NH}_3)_aX][\text{F}^-]^a}{[X^{-a}][\text{RNH}_3\text{F}]^a}, \quad E_A^O = \frac{C_x \text{ in org}}{C_x \text{ in aq}} = \frac{[(\text{R-NH}_3)_aX]_{org}}{[X^{-a}]_{aq}}$$

$$E_A^O = \frac{K(\text{RNH}_3\text{F})^a}{(\text{F}^-)^a}$$

equation which can be derived for the liquid ion exchange reaction. Thus if F is equal to formate and X is equal to a substance such as UTP (where C_x = concentration of UTP in a given phase), then at a given formate concentration the E_A^O for UTP would be expected to be proportional to some power of the concentration of free amine salt used as the extractant, and at a given amine concentration the E_A^O should vary inversely with the formate concentration. At *p*H lower than 3.5, UTP would be expected to have a negative charge of at least 3, and therefore the extraction coefficient should vary as the cube of the free amine salt concentration. However, as seen in Figure 1, the extraction coefficient of UTP and of both RNA and ATP varies directly to the first power of the amine salt concentration. Also, as shown in Figure 4, a limiting association number of about seven moles of amine to one mole of triphosphate was determined from the extraction isotherms. This same limiting number was found for calf liver RNA saturated in the organic phase with the amine JM-T. Thus a simplified interpretation of liquid ion exchange does not adequately explain the empirical relationship of the data as found, but the reactions considered as liquid ion exchange have been useful in designing and correlating the various extraction experiments.

Liquid-liquid extractions involving the use of amines as extractants are ideally suited for countercurrent distribution or partition chromatography techniques. However some means of overcoming the occurrence of gel, or third phase, formation must be developed for the adoption of this technique in the separation of high-molecular-weight phosphate polymers especially when they are contaminated with protein.

ACKNOWLEDGMENTS

The author wishes to thank Dr. E. Volkin for his interest and cooperation during the course of these investigations and in particular acknowledge the suggestions and direction given me during the isolation of various nucleic acid preparations. Gratitude is expressed to Dr. C. F. Coleman, W. D. Arnold, F. D. Seeley, and D. J. Crouse of the Chemical Technology Division of Oak Ridge National Laboratory for their generous supply of several amines used in these studies. Also for the same reason to the following commercial establishments: General Mills, Rohm and Haas, Universal Oil, Archer-Daniels-Midland, Foremost Chemical, and the Armour Chemical Companies.

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Preparation of Isoionic Protein by Electrodialysis with Permselective Membranes*

SAM KATZ AND F. ELLINGER†

From the Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland

Received November 26, 1962

A water-jacketed apparatus employing permselective membranes for the electrodialysis of water-soluble polymers is described. This apparatus, characterized by its simplicity of construction and operation, is singularly free from leaks because of the incorporation of O-rings in its design. Heat production, type and concentration of salts, electrolysis products, geometry, membrane type and resistance, stirring, etc., are evaluated with respect to the rate and efficiency of the electrodialysis process. Routinely, a 2% solution of albumin in 0.10 M NaCl can be deionized in 4 hours. Two voltage effects, namely, coulombic heat and membrane polarization, are discussed in relation to the denaturation of protein. The values for the isoionic points of some proteins were determined and subjected to a critical analysis.

In spite of the obvious advantage of preparing salt-free protein by electrodialysis this technique has failed to gain wide acceptance for several reasons: electrodialysis with cellophane membranes generally required from 10 to 30 hours for adequate desalting, and the high voltages employed often resulted in denatured product. Frequently the conventional apparatus was prone to leak, resulting in a material loss and the hazard associated with electrical malfunction.

With the advent of commercially available permselective membranes the initial objections are no longer applicable. The instrument described is simple in design and operation, free from leaks, and can readily accommodate volumes ranging from 5 to 35 ml. An analysis is made of the electrodialysis process, with particular reference to the factors which serve to set an upper limit to the current and or voltage which can be applied to this system.

APPARATUS

A water-jacketed electrodialysis unit for use with permselective membranes consisting of two identical glass electrode vessels and a Plexiglass center or sample cell was designed (Fig. 1). Each electrode cell, with a capacity of 15 ml, has shiny platinum electrodes, 28 mm diameter, mounted 6 mm from the membrane surface. In operation, influent distilled water is directed laterally against the membrane and electrode surfaces to prevent the accumulation of electrolysis products and to minimize temperature gradients.

The capacity of the water-jacketed center cell is 35 ml. Its contents can be stirred by either magnetic or mechanical means. For processing smaller volumes, cylindrical glass inserts are placed in this chamber. Burets or electrodes can be introduced through the top ports for *p*H monitoring, while the bottom port serves as the outlet. The membranes used were Nepton-AR-111-A (anion exchange) and Nepton

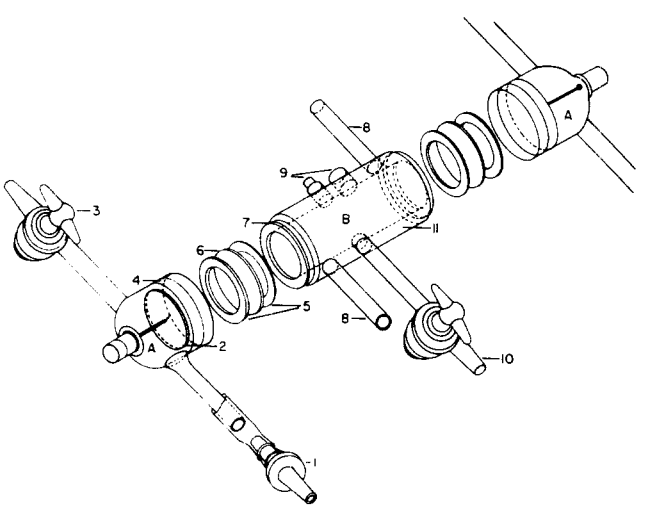

FIG. 1.—An expanded view of the electrodialysis unit. *A*, electrode cell; *B*, center or sample cell; *1*, polyethylene connector for water inlet; *2*, platinum electrode; *3*, needle valve for electrode water outlet; *4*, polyethylene support for membrane; *5*, rubber gaskets; *6*, permselective membrane; *7*, O-ring; *8*, cooling water inlet and outlet; *9*, electrode ports; *10*, sample outlet; *11*, water jacket. The remainder of the unit is identical to the labeled portion.

* This paper was presented in part at the 140th Meeting of the American Chemical Society at Chicago, Illinois, September, 1961. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

† Deceased.