with ϕX174 in vitro. This culture system preserves the morphological integrity of the tissues and supports cellular differentiation (*2*). Our results, therefore, may be explained by a maintenance in vitro of the various cell types participating in the antibody response as well as the excellent immunogenicity of ϕX174. From other experiments an apparent primary antibody response to flagellar protein of *Salmonella adelaide* can also be induced and maintained in vitro in the same manner (*13*).

Note added in proof: Globerson and Auerbach (*14*) have recently reported that spleen explants from mice treated with either phytohemagglutinin or adjuvant could be stimulated in vitro to form antibody to sheep red blood cells. No antibody formation occurred, however, following similar antigenic stimulation of explants of spleens from normal untreated mice.

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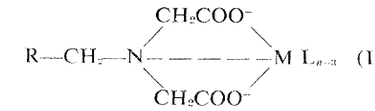
Concentration of Dissolved Amino Acids from Saline Waters by Ligand-Exchange Chromatography

Abstract. *Amino acids dissolved in salt solutions may be concentrated and removed from the solution by ligand exchange on copper–Chelex 100 resin. Competing inorganic ligands do not interfere, and ion exchange with cations does not occur; thus loss of metal ion from this column is avoided. To test the potentiality of ligand exchange for chromatography, the type and nature of the dissolved amino compounds in sea water were investigated. The data revealed that the bulk of the dissolved amino compounds is present in a combined rather than a free state.*

This study focuses attention on "ligand-exchange chromatography" for the removal of dissolved organic constituents from electrolyte solutions (*1*). The method is highly selective in concentrating a major portion of the organic substances from strong electrolyte solutions such as sea water. In this procedure a cation-exchange resin saturated with a complex-forming heavy metal (Cu^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, or Zn^{2+}) acts as a solid sorbent. Ligands which may be anions or neutral molecules such as ammonia, amines, amino acids, or olefins are removed from the liquid phase by formation of complexes with the metal attached to the resin and subsequent displacement of water or other liquids coordinated to the metal ion. The advantages of this type of ligand exchange lie in the high selectivity of the metal-resin ion exchanger for ligands that form complexes and chelates. This permits quantitative concentration of the organic ligand from very dilute solutions even in the presence of the competing inorganic ligands that are normally found in sea water.

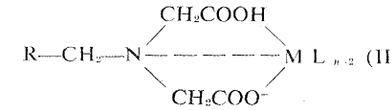
Previous uses of ligand exchange have involved resins of low specificity for the transition metals (*2*). As a result, only dilute electrolyte solutions could be used in extracting organic materials because competing cations would displace the complexed metal ion from the resin by ion exchange. This in turn would cause the loss of metal ion from the column (metal-ion bleed), lower the ligand-exchange capacity, and prevent a complete sorption of the organic ligands onto the resin.

We have used Chelex 100 resin (Bio-Rad Laboratories, Richmond, California) because of its extraordinary selectivities for the transition metals. The active sites of the resin are iminodiacetic acid groups similar to those found on ethylenediaminetetraacetic acid (EDTA). The structure of the complex formed in the resin is either:



(I)

or



(II)

where M is the metal ion, n is the coordination number of the metal, and L denotes the additional ligand bound to the metal ion (*3*). The chelating groups on the resin bind the transition metal so strongly that metal-ion bleed does not occur when saline waters are passed through the resin. Consequently, this resin extends the use of ligand-exchange techniques to strong electrolyte solutions such as sea water, brines, urine, or blood serum.

In the preparation of the metal resins, the following steps were taken. First, the resin (sodium form) was stirred with the appropriate metal chloride solution; we prepared Cu^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, and Zn^{2+} resins. Second, the excess metal chloride was removed by exhaustive washing with distilled water until the wash water was free of metal ions (*4*). Third, the resins were stirred with 3.0*M* NH_4OH and subsequently placed in columns (10 by 1 cm). Finally, the excess ammonia was removed by flushing the columns with distilled water until the *p*H of the effluent was between 8 and 9. After this, the columns were ready for chromatographic use.

The first group of organic compounds tested for their ligand-exchange properties was the amino acids; copper-Chelex resin served as the solid sorbent. To get some idea regarding the quantity of resin needed for an individual run, 1.25 μmole of each of the common amino acids (Beckman amino acid standard type 1) was added to 1 liter of artificial sea water. Five medicine droppers (2 ml each) filled with the copper resin were connected one after the other, and the solution of amino acid and salt water was subse-

quently passed through this set of columns. A flow rate of about 5 ml/min was maintained. After the sea water had passed through, the droppers were disconnected, and each was individually washed with distilled water until the wash water was free of chloride ions. The droppers were then eluted with 50 ml of 3.0*M* ammonia to free the sorbed amino acids. Some copper was eluted by the ammonia (*5*) and, after the effluent volume was reduced under vacuum, the amino acids were taken up in 3 ml of 6*N* HCl. The HCl was removed on a Buchler Rotary Evapo-Mix, and the volume was adjusted with a citrate buffer (*p*H 2.2) to 1 ml. Quantitative estimations of the amino acids were made by automatic high-pressure ion-exchange chromatography (*6*).

In Fig. 1 the upper curve is a chromatogram of standards, and it is presented for the identification of the peaks in the chromatograms below. Those standards not included in our "medicine-dropper" run are identified by a dark spot above the standard peak. The following information was obtained:

1) All amino acids, with the exception of cystine, were recovered completely (100 percent). In the case of cystine the amino acid was converted to cysteic acid and recovered in that form. The peak occurring farthest to the left is cysteic acid and is particularly noticeable in the chromatogram of the amino acids retained on the fourth medicine dropper. We did not include cysteic acid in the standards

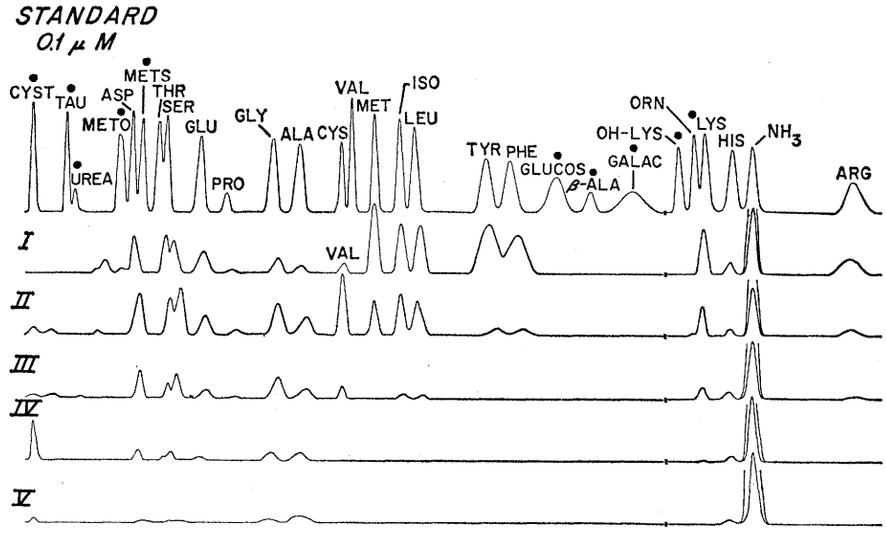


Fig. 1. Ion-exchange chromatograms of amino acids; the individual traces show the amino acids retained on each of the five micro columns in the copper-Chelex train. Those standards indicated by a spot were not introduced in the run. Abbreviations as for Table 1; in addition: *glucos*, glucosamine; *galac*, galactosamine; *tau*, taurine.

used in this run, and the appearance of this peak is matched by the disappearance of the cystine peak.

2) Partition effects between amino acids occur; for instance, glycine and alanine are retained the least, whereas hydrophobic amino acids are retained to the greatest extent.

3) About 10 ml of resin is sufficient to hold back all the amino acids added in this test.

It is noteworthy that contamination from distilled water or from the Chelex resin is insignificant. When the amino acid recorder is set on expanded scale (ten times scale expansion), two larger peaks occur at the beginning of the run (Fig. 2, *blank*) and can be attributed to imino groups released from the resin. These, however, can be eliminated by repeated use of the resins; the low-level amino acid contaminations (Fig. 2) can also be avoided by passing the distilled water through copper-Chelex columns prior to use. Both of these precautions are now routinely applied, but were not used during the test runs.

As a first application of ligand-exchange chromatography a problem

Table 1. Amino acid analysis of Buzzards Bay sea water; data are percentages of total. Arg, arginine; lys, lysine; his, histidine; orn, ornithine; glu, glutamic acid; asp, aspartic acid; thr, threonine; ser, serine; pro, proline; gly, glycine; ala, alanine; iso, isoleucine; leu, leucine; cyst, cysteic acid; cys, cysteine; mets, methionine sulfone; met, methionine; tyr, tyrosine; phe, phenylalanine. S, sample; tr, trace; n.d., not done.

S (No.)	Basic				Acidic		OH + Imino			Neutral					Sulfur				Aromatic		Total
	Arg	Lys	His	Orn	Glu	Asp	Thr	Ser	Pro	Gly	Ala	Val	Iso	Leu	Cyst	Cys	Mets	Met	Tyr	Phe	μg/liter
										Particulate matter											
1																					
2	8.4	8.4	2.1	0.2	16.0	8.4	5.1	6.7	3.8	7.3	6.7	5.6	3.9	7.3	1.2	tr		1.8	3.7	3.9	179
3	12.0	14.0	3.7	.4	11.0	3.6	2.3	2.0	8.4	6.5	6.4	1.5	4.5	7.7	1.0	tr		2.5	5.8	6.8	144
4	13.0	9.6	3.9	3.5	14.0	6.6	4.6	6.1	3.8	6.6	6.1	2.1	3.7	7.1	0.4	tr		0.3	4.83	4.8	198
5	13.0	10.0	3.7	0.7	14.0	7.0	4.3	5.6	4.6	6.6	6.1	4.6	2.9	6.6	.5	tr		1.5	3.9	4.4	213
6	8.7	7.6	2.9	.5	19.0	10.0	6.4	9.3	1.4	7.6	6.9	5.7	2.9	6.4	1.2	tr		0.2	1.1	1.9	172
7	13.0	13.0	4.9	.2	13.0	7.7	3.9	6.8	1.6	7.2	4.9	4.5	3.1	5.9	0.6	tr		1.8	3.7	4.0	221
8	6.2	8.9	1.6	.4	16.0	8.9	5.2	7.9	1.6	8.4	6.2	5.2	4.0	7.9	1.0	tr		1.8	3.8	5.1	178
										Total dissolved											
1	7.9	11.0	17.9	12.0	2.7	2.7	3.5	5.7	2.9	5.9	8.5	4.9	4.9	7.4	1.1				3.9	4.5	401
2	2.2	5.9	7.0	3.6	1.7	2.4	2.4	12.0	7.0	17.0	10.0	2.8	5.9	10.0	2.0	2.0	3.6		3.2	4.6	185
3	7.5	10.0	1.6	3.5	1.9	2.4	3.4	8.0	5.1	16.0	9.6	2.8	5.9	10.0	2.4	1.9	1.7		3.2	4.6	187
4	6.9	11.0	4.6	3.9	1.8	1.7	5.7	9.3	7.7	14.0	7.7	2.3	5.7	8.5	3.2		tr		1.2	4.9	129
5	13.0	12.0	6.3	11.0	1.9	1.6	3.8	5.8	6.7	6.3	6.7	1.5	5.4	7.1	0.7		0.4		3.8	5.8	240
6	12.0	11.0	4.3	6.7	2.1	1.8	4.4	8.1	5.3	13.0	7.2	2.3	7.2	9.6	1.5		1.1		1.2	1.6	209
7	8.1	7.1	7.1	7.6	1.6	1.9	3.8	9.2	10.0	14.0	6.6	2.9	5.6	6.6	0.8		4.7		tr	1.9	197
8	11.0	12.0	8.1	6.5	2.3	1.8	4.1	6.0	6.5	11.0	4.7	1.8	6.3	9.1	1.9		1.2		tr	6.9	199
										Free											
1		6.1	1.5	1.6	7.7	20.0	3.8	16.0	tr	29.0	6.1	2.5	2.7	2.3	tr				0.5	0.7	56
2		0.9	tr	4.0	21.0	8.5	3.2	15.0	2.2	27.0	9.6	5.2	1.5	1.4	0.6				tr	tr	72
3		5.9	6.9	13.0	12.0	9.2	4.7	9.6	tr	18.0	6.6	6.7	1.4	2.3	tr				2.1	1.6	77
4		17.0	n.d.	24.0	6.9	6.4	1.8	8.6	tr	22.0	5.4	6.2	tr	tr	1.9				tr	tr	50
5		5.0	3.9	6.6	8.9	6.2	4.8	15.0	3.6	19.0	4.7	5.2	4.5	2.2	1.7				3.8	4.3	58
6		8.4	n.d.	1.9	3.9	8.1	2.9	13.0	tr	26.0	16.0	5.8	2.0	2.2	4.4				2.6	2.4	38
7		3.3	2.5	2.7	9.6	12.0	4.1	12.0	tr	29.0	7.8	10.0	1.2	1.0	4.5				tr	tr	69
8		11.0	5.6	9.6	6.5	11.0	3.2	12.0	1.9	18.0	5.6	5.5	1.8	1.5	4.9				0.7	1.3	66

was selected which is of significance to the general field of oceanography. The extraction and concentration of organic materials from sea water is formidable owing to the presence of vast amounts of inorganic materials; the ratio of inorganic to organic matter in sea water is of the order of 3×10^4. Methods applied to this problem were reviewed by Jeffrey and Hood (*7*), who concluded that coprecipitation of organic matter from sea water with iron hydroxide showed the most promise. However, others (*8*) found that the amount of organic carbon removed from sea water by this method was only 40 to 60 percent. In addition, this method introduces the problem of removing the iron without altering the state of the organic compound *in situ*.

Most of these difficulties have now been overcome by ligand-exchange chromatography. Runs were performed with standards added to sea water (Buzzards Bay), with recoveries equal to that obtained from artificial sea water. The method was then applied to determinations of the amino acid content of sea water.

In order to provide information on the amino acid composition of both the dissolved and particulate matter, eight surface water samples (4 liters each) were collected in Buzzards Bay at 1.6-km intervals about 1.6 km off the shore of Elizabeth Islands, Massachusetts. The waters were passed through Gelman Type A glass-fiber filters which had been cleaned in a furnace. In following the terminology generally adopted by biologists, the materials retained on the filter are referred to as particulate matter, whereas the dissolved organic constituents are those present in the filtrate. To obtain the actual free amino acids, 1 liter of the filtered sea water was passed through a 10-ml copper-Chelex column. The standard procedure, as outlined above, was then followed. For the total dissolved amino acids, another liter of the filtered sea water was subjected for 22 hours to hydrolysis in 6*N* HCl after the volume had been reduced to about 50 ml. Subsequent to hydrolysis, the acid was neutralized with NaOH, and the sample was adjusted again to 1 liter. The amino acids were removed and analyzed as described for the free sample. In the case of the particulate matter no initial treatment was necessary; thus the hydrolyzed sample could be added directly to the ion-exchange column of the amino acid analyzer (Table 1 and Fig. 2).

Because of the comparatively small volumes of sea water analyzed the concentrations of some of the amino acids measured and reported are at the analytical limits of the capabilities of the chromatographic procedure, and no particular importance is ascribed to any one sample. However, certain conclusions can be drawn. Pronounced differences in both concentration and type of amino acids are established between free, total dissolved, and particulate organic matter. Of significance is the observation that the bulk of the amino acids dissolved in the sea are in a combined rather than a free form. In following the concept proposed (*9*) it is suggested that, aside from peptides, sizeable portions of the dissolved amino acids in the sea are tied up in complexes of the phenol-quinone type and require acid hydrolysis for release. The presence of a variety of phenols, in particular *p*- and *m*-hydroxybenzoic acids, in the hydrolyzed but not in the

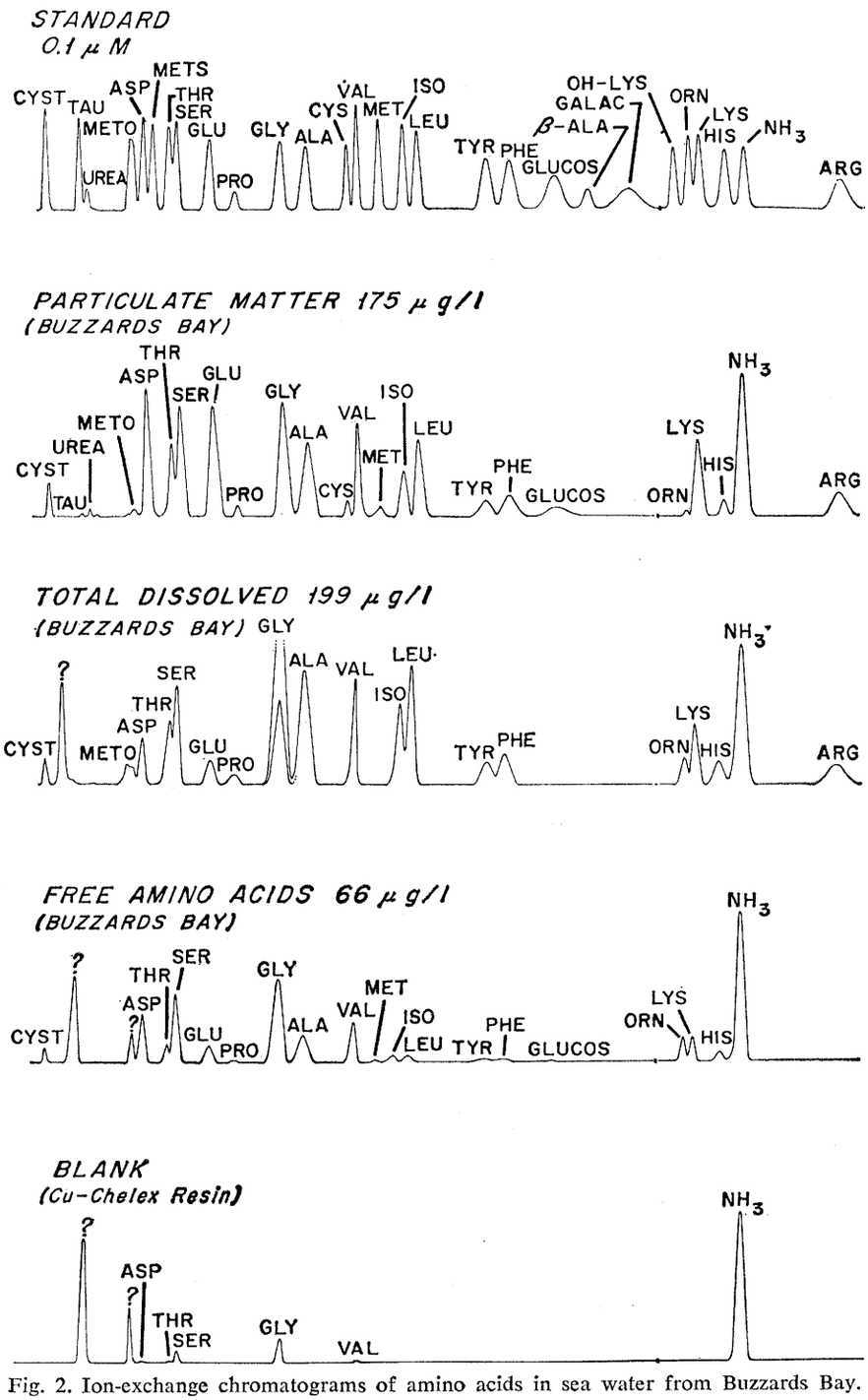


Fig. 2. Ion-exchange chromatograms of amino acids in sea water from Buzzards Bay.

unhydrolyzed sea water samples supports this inference.

We are checking the ligand-exchange properties of a wide number of organic constituents. These include amines, phenols, the bases of the purines and pyrimidines, hydrocarbons, sugars, peptides, and humic acids. In addition to copper-Chelex resins, we have prepared other metal resins as mentioned. All exhibit different ligand sorption characteristics; iron resins, for example, only retain the acidic and hydrophobic amino acids, but leave the others unaffected.

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4. Metal ions in the wash water were determined with KCNS for Fe^{3+}, eriochrome black T for Zn^{2+}, and pyrocatechol violet for Cu^{2+} and Ni^{2+}. In some cases the wash required up to 60 liters of distilled water.
5. The slight elution of copper from the resin by 3.0*M* NH_4OH occurs only after the passage of a strong electrolyte solution through the column. It does not occur after passage of distilled water through the column, for example. This copper interference is now eliminated by the introduction of a Chelex column (NH_4^+ form) immediately after the copper column during the ammonia elution. The copper is caught on the NH_4–Chelex resin, and the amino acids continue on, eluted by more ammonia. This use of a Chelex column to retain the copper eliminates the necessity for the use of the HCl. The ammonia eluent is evaporated in a vacuum, and the amino acids are taken up in citrate buffer.
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Thiosemicarbazide Injection Followed by Electric Shock Increases Resistance to Stress in Rats

Abstract. *Adult rats that had been raised with a minimum of stimulation were injected with thiosemicarbazide, a drug that lowers γ-aminobutyric acid concentrations. Fifteen minutes later the animals were given 30 mild electric shocks over a half-hour period. Two weeks later they were tested for their resistance to gastric ulceration induced by immobilization. The experimental animals showed a much greater resistance to stress than did the appropriate control groups. A replication confirmed the results of the first study.*

The fact that stimulation in infancy can reduce emotionality and physiological response to stress in the adult animal is well documented (*1*). Although many such studies have examined the stimulus conditions that are optimal for producing the effect, the underlying physiological mechanism has received little direct attention beyond Bovard's theoretical discussion (*2*). A clue to this mechanism was suggested during discussion of a Macy Symposium paper on biochemical maturation by Roberts (*3*). He had noted that the concentrations of γ-aminobutyric acid (GABA) and the enzyme involved in its synthesis, glutamic acid decarboxylase (GAD), were very low immediately after birth but rose to approximately adult values after several days; the exact time varied with the species. These time periods proved quite similar to those observed as critical periods for the establishment of imprinting; the same periods seem to define the developmental state during which the early stimulation manipulations have maximum effect.

Although there seems to be some question regarding identification of GABA as the inhibitory synaptic transmitter, substance I, it is generally agreed that GABA does inhibit activity of cells within the central nervous system. A period during which GABA is at relatively low concentrations, therefore, should be a period of reduced threshold for activation of the neural systems that are normally inhibited by GABA. If those systems in turn are normally implicated in the control of emotional responsiveness and of resistance to stress, they may be permanently and positively conditioned by stimulation during the critical period when threshold is low.

Table 1. Number of gastric ulcers in each rat, injected or uninjected (with or without subsequent shocking), after subjection to immobilization stress. TSC, thiosemicarbazide.

Injected with TSC		Not injected	
Shock	No shock	Shock	No shock
Experiment			
1	4	9	7
1	4	3	4
0	3	3	3
0	2	2	2
0	1	0	1
0	0	0	1
0	0	0	1
0	0	0	0
0			
0			
Replication			
0	3	6	6
0	2	4	4
0	1	1	3
0	1	1	0
0	1	1	0
0	1	1	0
	0	0	0
	0	0	0
		0	

Direct substantiation of the hypothesis relating the GABA system to the phenomenon of early stimulation would require demonstration that the phenomenon does not result from stimulation during the critical period if GABA and GAD concentrations are raised to adult levels immediately at birth; and, conversely, that the critical stimulation period can be prolonged by concomitant prolongation of the period during which GABA and GAD are at low concentrations. Technical problems associated with biochemical manipulations in the newborn that do not induce experimentally confounding stimulation so far preclude this direct approach. As a first approximation, however, an alternative test of the hypothesis was made by examining the resistance to stress of adult animals that had been raised with a minimum of stimulation and then stimulated during a period when GABA concentrations were reduced by injection of thiosemicarbazide, which reduces concentrations in the central nervous system by blocking synthesis of pyridoxal phosphate, the cofactor necessary for the normal function of GAD in the synthesis of GABA (*3*).

The first experiment used, in groups balanced as to sex, 34 albino rats derived from the Berkeley-Pacific strain but born and raised in our laboratory; 31 similar rats were used for subsequent replication. The rats were raised