Table 1. Incidence of ventricular fibrillation during acute myocardial ischemia, expressed as the number positive of the number tested.

Tylosin pretreated (mg/kg)	Procaine amide for arrhythmia (mg/kg)	Fibrillation incidence
	Group 1	
0	0	13/15
	Group 2	
0	19 ± 1.5*	3/11
	Group 3	
5 per day, for 2 days	22 ± 1.8*	12/12

* Standard errors of mean.

cordial electrocardiogram followed by ventricular ectopic beats (*4*). Within 4 hours of the onset of ischemia, approximately 90 percent of untreated animals had a ventricular tachycardia and fibrillation (group 1, Table 1). Dogs in group 2 received the antiarrhythmic agent procaine amide, intravenously, at the onset of ventricular ectopic beats, and this resulted in a significant reduction of the 4-hour mortality rate to less than 30 percent. Group 3 animals were pretreated with tylosin (5 mg/day per kilogram of body weight) for 2 days prior to ischemia. Since significant enhancement of the incidence of ventricular fibrillation would be difficult under the circumstances of the group 1 study, the animals pretreated with antibiotic were compared by the method of study in group 2. All 12 animals of group 3 succumbed, usually relatively soon after the onset of the initial ventricular ectopic beat, despite receiving an amount of antiarrhythmic equivalent to that in group 2.

To investigate the role of potassium ion in this process, an experimental design was selected in which analysis of ion transfer is more readily performed. Seven intact anesthetized animals, pretreated with tylosin, received a therapeutic nontoxic dose of acetyl strophanthidin, 0.03 mg/kg, and the net movement of K^+ in the myocardium was studied by serial sampling of arterial and coronary sinus blood (*4*). Strophanthidin (*5*) was infused in the femoral vein over a 30-second period. In nine controls not receiving antibiotic, there was a modest egress of potassium ion during the first several minutes, which terminated by 6 minutes (Fig. 1). Normal sinus rhythm was maintained and was associated with a positive inotropic response, represented by an increase in the rate of rise of the ventricular pressure (*6*). In the animals treated with tylosin, no abnormality of rhythm was present before injection of the digitalis compound. The same nontoxic dose of strophanthidin produced a significantly larger loss of potassium from the myocardium. Concurrently, ventricular ectopic beats appeared in six out of seven animals, persisting for an average of 6 minutes. The average peak incidence of ectopics was 32 percent of all beats. In addition, five animals received a toxic dose of strophanthidin, 0.05 mg/kg; they exhibited a ventricular tachycardia that had a greater frequency than occurred in eight animals without antibiotic, which also persisted approximately twice as long.

In vitro data have indicated that macrolide antibiotics may produce selective uptake of potassium ion in isolated mitochondria. Alterations of cell metabolism, such as reduced energy production, may reverse this process, resulting in selective loss of potassium ion (*1, 2*). While the locus from which potassium ion is lost has not been identified, it would appear reasonable to assume that strophanthidin has altered K^+ metabolism in a manner that permits the relatively small loss of cation from heart muscle after a nontoxic dose of strophanthidin to be enhanced in the presence of the macrolide antibiotic. Whether this represents an additive effect of the two lactones remains to be evaluated. The results of the studies during myocardial ischemia indicate that the macrolide compound does not require another pharmacologic intervention to affect cardiac rhythm.

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26 March 1969

Crystalline L-Asparaginase from Escherichia coli B

Abstract. *L-Asparaginase has been crystallized from a partially purified extract of* Escherichia coli *B. The crystalline enzyme is homogeneous, as judged by analytical polyacrylamide-gel electrophoresis and sedimentation behavior. This enzyme preparation is active in preventing lymphoma in mice and also has low glutaminase activity.*

L-Asparaginase has been prepared by column chromatography on DEAE-cellulose and hydroxylapatite or by preparative gel electrophoresis (*1*). We have developed a process for purification of L-asparaginase by simple precipitation steps followed by crystallization. The crystalline enzyme is nearly homogeneous. It has a specific activity of 300 ± 15 international units per milligram (I.U./mg) based on the dry weight of the crystals and the assay procedure of Campbell *et al.* (*2*) modified by the incorporation of 0.1 percent bovine serum albumin (BSA) in the buffer. The crystalline enzyme is active in preventing lymphomas in mice. Our purification process should lead to the availability of crystalline L-asparaginase for further clinical evaluation of its effectiveness in cancer chemotherapy.

The enzyme was extracted from a paste of *Escherichia coli* B cells (*3*). The solution was filtered through Hyflo, and the *p*H of the effluent was adjusted to 5.0 by the addition of 2*M* acetic acid. The suspension was clarified by filtration, and the filtrate was adjusted to *p*H 8.0. The protein fraction precipitating in $(NH_4)_2SO_4$ (between concentrations of 2 and 4*M*) was collected and dissolved in $10^{-3}M$ NH_4HCO_3; it was dialyzed against $10^{-4}M$ NH_4HCO_3 overnight. Ethanol (one volume) was added to the dialyzed solution, and the resulting precipitate was discarded. Another volume of ethanol was then added to the supernatant solution. The resulting precipitate was dissolved in $10^{-3}M$ NH_4HCO_3 and lyophilized. The enzyme powder was then suspended in distilled water and dialyzed overnight against $10^{-4}M$ NH_4HCO_3. Insoluble material was removed, and one volume of ethanol was added to the supernatant. After standing at 4°C for 1 hour, the solution was made 0.02*M* in $(NH_4)_2SO_4$. Upon standing at −20°C for 4 hours, a precipitate formed. The

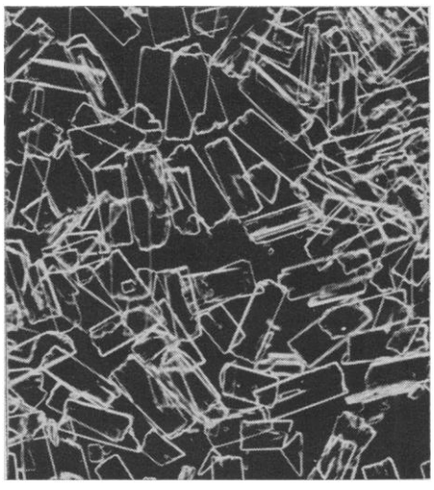

Fig. 1. Crystals of L-asparaginase (× 360).

aqueous extract of the precipitate was made 0.02*M* in $(NH_4)_2SO_4$, and then ethanol was added with constant mixing until incipient cloudiness (one-third to one-half volume of ethanol). The mixture was kept at 4°C overnight; during this time crystals formed, although an amorphous precipitate was also seen. The crystals and amorphous material were collected and washed with 50 percent ethanol. Between 30 to 40 percent of the units in the crude extract were recovered in the crude crystals (Table 1). The above procedures were performed at 4°C unless indicated otherwise.

The crystalline enzyme may be recrystallized in the presence of metal ions, yielding larger crystals of higher specific activity. The crude crystals were dissolved in distilled water made $10^{-2}M$ in magnesium acetate. Ethanol was then added dropwise at room temperature with constant mixing until incipient cloudiness occurred. The solution was immediately clarified by centrifugation. Crystals appeared shortly after centrifugation and, after standing several hours at room temperature, had grown to 100 to 200 μ in length (Fig. 1). The crystals were separated from the solution by centrifugation, washed several times with 50 percent ethanol, and dried in a vacuum. The dried crystals are stable in the cold for at least 4 months. Repeated crystallization (five times) yielded a product with constant specific activity (300 ± 15 I.U./mg) based on the dry weight of the crystals.

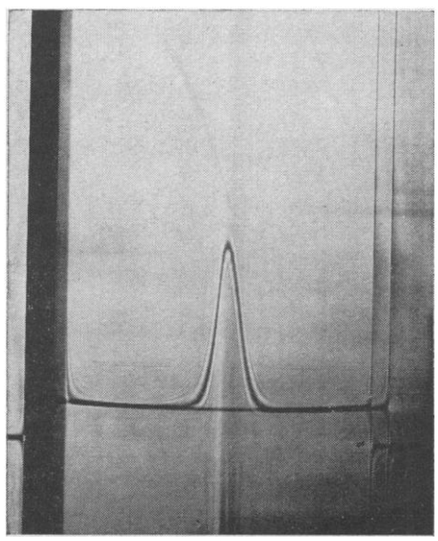

Fig. 2. Ultracentrifugal schlieren pattern of a 1 percent crystalline L-asparaginase solution in 0.015*M* phosphate, 0.10*M* NaCl, *p*H 7.3, bar angle 75°, 60,000 rev/min at 48 minutes after reaching speed; $s^0_{20,w} = 7.6$.

Some tests were performed to determine the purity of the crystalline enzyme. The ultracentrifuge pattern was symmetrical (Fig. 2), although a minor peak which sedimented at a more rapid rate was observed in some preparations. This minor peak never represented more than 5 percent of the total material present. In addition, this faster moving material was not observed until the enzyme had been crystallized. Similar results were also observed in disc-gel electrophoresis (Fig. 3). However, upon assaying the gel in fractions, both the major component and minor components exhibited enzymatic activity. Further evidence that minor components were aggregates of L-asparaginase was demonstrated by the absence of minor components when the electrophoresis was performed in 7.5*M* urea (Fig. 3). On the basis of these results it was concluded that the crystalline enzyme was essentially pure.

The crystalline enzyme was further characterized by thermogravimetric analysis, by atomic absorption spectroscopy, and by ultraviolet absorption. The crystals contained 6 percent water and about 1 g atom of Mg per 30,000 g of enzyme; the absorbancy at 278 nm of a 1 percent solution of the dry crystalline enzyme was 7.1.

Specific activities of 300 to 620 I.U./mg have been reported by other laboratories with the use of an indirect protein standard such as BSA or egg white lysozyme (*1*). Our enzyme preparations, assayed at each stage over five crystallizations, gave a constant value of 300 ± 15 I.U./mg based on dry weight, although a specific activity of 425 ± 20 I.U./mg was found when BSA was used as a standard. Therefore, we suggest that 300 I.U./mg represents the specific activity of essentially homogeneous L-asparaginase.

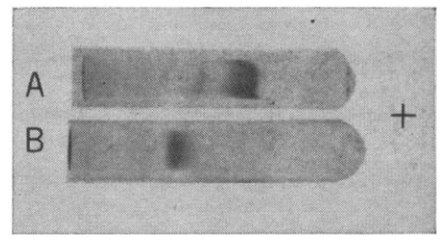

Fig. 3. Polyacrylamide-gel electrophoresis of crystalline L-asparaginase. (A) Electrophoresis was carried out on 5 percent polyacrylamide gel for 75 minutes at room temperature at 2 ma per tube in 0.05*M* tris(hydroxymethyl)aminomethane (tris)-glycine buffer, *p*H 8.9. (B) Electrophoresis was carried out on 3 percent polyacrylamide gel containing 7.5*M* urea prepared according to the method of Reisfeld *et al.* (*7*) for 4 hours at room temperature at 2 ma per tube in 0.05*M* tris-glycine buffer, *p*H 8.9, containing 10*M* urea.

Table 1. Purification summary. L-Asparaginase activity was determined by the method of Campbell *et al.* (*2*), protein by the Biuret method of Gornall *et al.* (*5*) or its absorbancy at 280 nm and 260 nm according to the method of Layne *et al.* (*6*).

Fractions	Total activity (I.U. × 10^6)	Specific activity (I.U./mg protein)	Yield (%)
Crude extract	1.9	1.75	
*p*H 5.0 treatment	1.7	5.0	90
$(NH_4)_2SO_4$ fractionation	1.52	27.0	80
1st ethanol fractionation	0.95	91.0	50
2nd ethanol fractionation	0.75	270	39.5
1st crystallization	0.70	385	36.8
2nd crystallization	0.575	425	30.0

The crystalline L-asparaginase has been demonstrated to be effective in inhibiting the growth of Gardner lymphosarcoma in mice. At *p*H 8.5, the glutaminase activity of the crystalline enzyme was 1 percent of the L-asparaginase activity (*2*). The significance of this relatively low glutaminase activity as compared to the reported value of 2 to 4 percent (*4*) remains to be elucidated.

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Potential Energy Fields about Nitrogen in Choline and Ethanolamine: Biological Function at Cellular Surfaces

Abstract. *Partial charge distribution on first and second neighbor atoms to nitrogen in choline and ethanolamine have been calculated. Coulombic and steric parameters were then utilized to evaluate the interaction of a negative test charge with the two molecules. Both the position and the magnitude of the maximum of interaction energy in the two systems were significantly different. The results suggest that ethanolamine interacts more strongly with anions than choline does. This is due principally to steric repulsion of the negative charge by the methyl groups in choline.*

Compounds containing choline and ethanolamine seem to influence characteristics of cellular surfaces. The distribution and amount of phosphatidyl choline and phosphatidyl ethanolamine are characteristic and specific in a particular membrane or cell (*1*). Furthermore, the charged functional groups of phospholipids appear to be located directly at the cell surface (*2*).

Material from the cell wall of pneumococcus contains a polysaccharide which carries convalently linked choline residues (*3*). Substitution of ethanolamine for choline in this cell wall significantly alters cellular adhesion, bacterial transformation, and cellular autolysis characteristics of this organism (*4*). Since at neutral *p*H the formal charge on ethanolamine and choline is the same, the difference in surface behavior might be due to differences in molecular size or conformation. However, the charge distribution could differ significantly in the two moieties and thereby provide a unique potential energy field about nitrogen in each molecule. We have investigated the latter possibility.

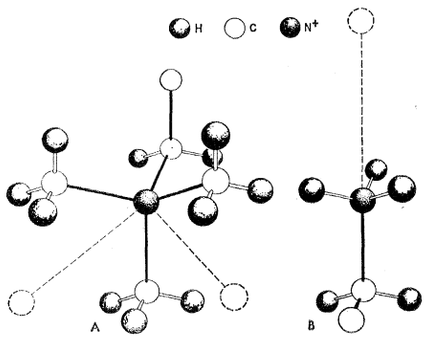

Fig. 1. Spatial location of maximum interaction (energy minimum) of a test anion with choline (A) and ethanolamine (B). Dotted circles represent the position of the negative test charge.

We evaluated the potential energy field about the positive nitrogen atom in the two molecules by using a test particle of negative charge and by calculating the total potential energy of the test particle at any position (x,y,z). This potential energy is approximated by the equation

$$P = \sum_{i=1}^{m} \frac{A_i}{r_i^6} + \frac{B_i}{r_i^{n_i}} + \frac{KQ_iQ}{D\,r_i} \qquad (1)$$

where

$$r_i = [(x - x_i)^2 + (y - y_i)^2 + (z - z_i)^2]^{1/2}$$

and Q_i is the partial charge on ith atom in the molecule; Q is the partial charge on test particle; D is the dielectric constant of the medium; A_i, B_i, n_i are the steric energy parameters of the ith atom interaction with the test particle; and m is the number of atoms in the molecule.

Partial charges on the atoms in the choline and ethanolamine molecules (Q_i) were calculated by the method of Del Re (*5*), which is based upon electron induction due to different electronegativities in different atoms. The partial charges calculated by this technique have been used to predict conformations of many polypeptides (*6–10*) and have also been used to predict nuclear magnetic resonance spectrums of amino acids (*5*).

The calculated partial charges on the first and second neighbor atoms to nitrogen in choline and ethanolamine are shown in Table 1. In choline, although there is some polarization due to the inductive effect, most of the positive charge is localized on the nitrogen atom buried within the methyl groups. In ethanolamine, most of the positive charge is distributed among the three hydrogen atoms attached to nitrogen. Thus, in ethanolamine the positive charge is more diffuse, but it is more accessible to approach by a counterion than that in the choline molecule.

With the values for Q_i shown in Table 1, we evaluated the potential energy field in each system according to Eq. 1. For this analysis, the molecules were positioned in the *trans*, all-staggered configuration (as in Fig. 1). The steric energy parameters were similar to the respective sets described by Scheraga, Flory, Liquori, and Huggins (*7, 8, 11, 12*). To include the effect of a solute-solvent medium on the final potential values, we assigned an effective dielectric of 3.5 to the coulombic potential term (*7, 8*). Although there is some criticism of the steric portion of the potential function (*12*), such "6-12" (or Lennard-Jones) functions have become widely accepted in the calculation of nonbonded interactions in gases (*13*), in the calculation of torsional potentials in various polymers (*14*), and in conformational analysis of polypeptides (*6–8, 11, 15*). However, the choice of an effective dielectric is uncertain and the value of 3.5 should be considered as only a crude approximation. Complete neglect of dielectric effects (vacuum) would only enhance energy differences, and the conclusions would not change.

A systematic digital scan was carried out by movement of the test negative charge in spherical coordinate space around nitrogen with the distance from nitrogen (r) varying from 2.5 to 6.0 Å at 0.1-Å intervals. Both spatial coordinates ϕ and θ were varied at 30° increments from 0° to 330°. The test charge

Table 1. Charge distribution in atoms around nitrogen in choline and ethanolamine.

Atom	Partial charge	
	Choline	Ethanolamine
Methylene carbon	−0.021	+0.003
Methylene hydrogen	+ .048	+ .054
Nitrogen	+ .685	+ .131
Methyl carbon	− .013	
Methyl hydrogen	+ .050	
Amine hydrogen		+ .248