on normal medium. Only 20 to 30 percent of the original potassium content could be found when the cultures were grown in 1.0*N* LiCl medium or were exposed to a 1.0*N* LiCl solution for 24 to 48 hr. The sodium content of the cells did not show a significant change in any case. In cultures grown on LiCl medium, lithium could be demonstrated only in the cells if the cultures were not washed more than twice. Nonresistant strains, grown on normal medium, were also exposed to LiCl solutions for 24 to 48 hr. They showed the same decrease in the potassium content as the resistant strains grown on the salt medium. The sodium content of the nonresistant strains exposed to LiCl solutions was also unchanged in comparison with control cultures.

The decrease of the potassium content after treatment with LiCl is of special interest since it is known that the K^+ ion plays an important role during fermentation in the yeast cell (*6–8*). It has also been pointed out that Li^+ interferes with the carbohydrate metabolism in yeast, sea urchin larvae (*9, 10*), and bacteria (*11*). An antagonistic effect of Li^+ and K^+ in yeast with regard to glucose fermentation was reported by Lindahl (*10*). Whether there is a direct exchange in the yeast cell of K^+ by Li^+ or whether Li^+ acts as a glycolytic inhibitor like iodoacetic acid or sodium fluoride which causes the loss of K^+ (*7*) remains to be determined. The fact that no Li^+ could be found in the yeast cells after several washes does not support the first possibility.

On the basis of these facts, it may be stated that the effect of LiCl on the decrease of the potassium content is the same in nonresistant and resistant strains. The ability of the latter to grow on LiCl medium is under gene control. If potassium is normally essential to the cell (*6–8*), the mutation enables the cell to grow in the presence of LiCl with a considerably decreased amount of potassium. Therefore, it can be assumed that a general difference exists between mutant and normal cells in regard to their requirement for a minimal amount of potassium.

This assumption could be supported by the results of fermentation tests in a glucose medium lacking LiCl and deficient in potassium (*12*). Several LiCl-resistant and nonresistant strains were inoculated into test tubes containing 8 ml of the medium, part of which filled a small inverted test tube (Durham tube). After 1 wk of incubation it appeared that only the LiCl-resistant strains had filled the inverted tubes with gas, whereas the nonresistant strains had produced no gas or only a small amount of it. In addition, it should be mentioned that strains containing the genes L_2, L_3, or L_4 filled the inverted tubes with gas in a shorter period of time than strains with the gene L_1. Further experiments to determine the significance of potassium for normal and mutant strains are in progress (*13*).

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4. The yeast strains used in these experiments are genetic strains that are morphologically like *S. cerevisiae* and were largely derived from this species.
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12. The medium used was a modification of DIFCO yeast nitrogen base medium (with glucose as carbon source) in which the potassium salts were replaced by sodium salts. The possibility remains that traces of K^+ were still present as impurities of other compounds. For the present purpose, however, it was considered as sufficient if the K^+ content was largely decreased in comparison to the standard medium.
13. This investigation was supported in part by funds from grant E-328, National Institutes of Health, Public Health Service, and the Biological and Medical Fund of the State of Washington. I am grateful to R. B. Walker for an introduction to the methods of operating the flame spectrophotometer.

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Hemocyanin and Radioactive Copper

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In view of the recent revival of interest in the binding of copper to the hemocyanins (*1–4*), we wish to report some observations made several years ago in these laboratories (*5*), that emphasize the extremely low degree of dissociability of the copper-protein bond. During the preliminary phase of a series of investigations in which radioactive copper has been used to study the exchangeability of copper in the copper enzymes, ascorbic acid oxidase (*5–7*), and tyrosinase (*8*), we had occasion to carry out an exploratory study of copper exchange in the hemocyanin of *Busycon canaliculatum*.

Filtered whole *Busycon* serum containing 0.22 to 0.26 percent copper based on the dry weight of the serum was employed. The procedures including the methods of radioactivity assay for the hemocyanin-radiocopper exchange experiments were similar to those previously published describing the exchange studies on ascorbic acid oxidase (*6, 9*). Unbuffered columns of Amberlite IR-100 (Na^+), 25 by 0.8 cm, were used for separating from the protein solution the unbound radiocopper remaining in solution after the hemocyanin had been exposed to the radiocopper for the desired length of time. All experiments were carried out at *p*H 6.5 to 7.0, approximating the natural *p*H of *Busycon* serum.

Table 1. Removal of extraneous ionic copper but not native copper from hemocyanin by Amberlite IR-100 (Na^+).

Expt.	Prepn.* (1 ml)	Cu content (μg)	Amt. of Cu^{++} added (μg)	Time of contact with added Cu^{++} (hr)	Cu content of effluent (μg)
1	1H*	1.0	0	0	1.0
2	1H	10.0	10.0	20	9.7
3	1H	10.0	10.0	20	9.8

* For description of this hemocyanin preparation see first footnote of Table 2. In experiment 1 the hemocyanin was diluted (1 : 10). Experiments 2 and 3 were duplicates.

It was found that hemocyanin can be passed slowly through an Amberlite column with no loss in its native copper. This result itself affirms the high stability of the copper-protein linkage, a property long recognized by virtue of the nondialyzability of the copper. It was also shown that when an amount of ionic copper equal to the copper content of the hemocyanin was added to the serum and the mixture passed through the column, the extraneous ionic copper was removed and the effluent solution had a copper content equal to that of the original hemocyanin (Table 1). All copper determinations were performed by the method of Warburg and Krebs (*10*).

The nearly quantitative recovery of nonionic copper has been taken as evidence for the quantitative recovery of hemocyanin protein. Since no measurements of physiological properties, such as oxygen capacity, were performed on the hemocyanin solutions before or after their passage through the resin columns, only qualitative evidence that the protein was not damaged is available. Thus it was observed that all of the effluents still possessed the characteristic blue color of oxyhemocyanin, and had the ability to be decolorized reversibly by sodium hydrosulfite, a property shown only by native hemocyanin (*11*).

When radioactive cupric ions were added to a solution of oxyhemocyanin for periods of time up to 16 hr, it was found that no significant radioactivity was incorporated into the hemocyanin (Experiments *A*, Table 2). It may be concluded, therefore, that under the conditions of the experiment no exchange occurred between radiocupric ions and the oxyhemocyanin copper.

The possibility of an exchange reaction between hemocyanin copper and radiocopper ions while the hemocyanin was undergoing reversible oxygenation–deoxygenation was investigated. It is well known that fresh blue solutions of oxyhemocyanin, when placed under a vacuum, readily lose oxygen and decolorize rapidly. When the decolorized solutions are shaken in air, they become blue again. An amount of radioactive cupric ions equal to the copper content of the hemocyanin was added to freshly prepared hemocyanin solutions. Evacuation of the mixture with a water pump vacuum for 5 min resulted in decolorization; shaking with air for 1 min restored the blue color. This process was repeated 5 times, after which the mixtures were passed through an Amberlite column. The effluents were analyzed for copper content and radioactivity. The results, shown in Table 2 (Experiments *B*), indicate that no significant exchange occurred while the hemocyanin was undergoing reversible oxygenation–deoxygenation.

From the work of Kubowitz (12) it appears that the copper in hemocyanin is in the cuprous form. It has also been suggested that dissociation of the cuprous ion from unoxygenated hemocyanin may proceed more easily than from the oxyhemocyanin (*4*). It is clear, however, that such dissociation did not occur to any significant extent under the conditions of our experiments. If it had occurred, the following equilibrium system would have been established, and radioactivity would have been incorporated into the hemocyanin via the cuprous ion, that is

$$\text{Hemocyanin-Cu}^+ \rightleftarrows \text{Cu}^+ + \text{apohemocyanin}$$
$$\text{Cu}^+ + {}^*\text{Cu}^{++} \rightleftarrows {}^*\text{Cu}^+ + \text{Cu}^{++}$$
$$\text{Apohemocyanin} + {}^*\text{Cu}^+ \rightleftarrows \text{hemocyanin-}{}^*\text{Cu}^+$$

Table 2. Hemocyanin and Cu^{64}. Experiments type *A* involved oxyhemocyanin exposed to radioactive cupric ions for two different periods of contact before passage through an Amberlite IR-100 (Na^+) column to remove the extraneous cupric ion. The *B* experiments are duplicates and involved hemocyanin undergoing reversible oxygenation-deoxygenation in the presence of radioactive cupric ions prior to passage through the column.

Experiment		Prepn.* (1 ml)	Cu content (μg)	Amt. Cu^{64} added (μg)	Time of contact with Cu^{64} (hr)	Cu in effluent (μg)	Radioactivity (counts/min)		Exchange (percent)
Type	No.						Effluent	Comp. stand.†	
A	1	1H	10.0	10.0	1	not detd.	4 ± 3	818 ± 12	0
A	2	1H	10.0	10.0	16	9.8	2 ± 2	324 ± 6	0
B	1	2H	12.1	12.1	0.5	11.7	0	3850 ± 27	0
B	2	2H	12.1	12.1	.5	not detd.	0	3850 ± 27	0

* Preparation 1*H* was obtained from a stock solution of hemocyanin 2 yr old. After dialyzing for 2 wk against copper-free water, the copper content was found to be 0.25 percent. Preparation 2*H* was a fresh solution of hemocyanin (3 days old) ; it contained 0.22 percent copper.

† The method of preparing the comparison standard has been previously described (*6*).

It is known that if both cuprous and cupric ions are present in solution, the radioactivity cannot be localized at either ion, regardless of which ion is originally tagged (*13*).

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Thermodynamic Analysis of the Intracellular Osmotic Gradient Hypothesis of Active Water Transport

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The problem of transporting water from a solution of high osmolarity to one of low osmolarity has engaged the attention of biologic investigators for many years. The intracellular gradient hypothesis, and the mechanism for the maintenance of the gradient, formulated in detail by Franck and Mayer (*1*), seemed to us, on superficial examination, to be a reasonable working hypothesis. An alternative mechanism for the maintenance of osmotic gradients involving the flow of electric current through ion-selective circuits was considered (*2*). Detailed examination of the implications of such a system brought to light certain fundamental objections. It became apparent that such objections applied with equal validity to any intracellular osmotic gradient scheme.

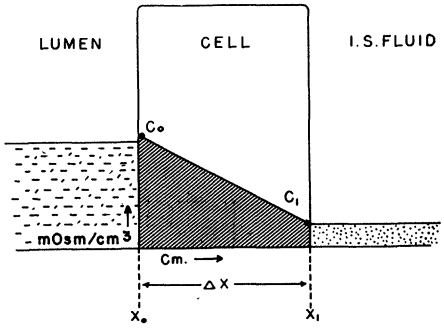

Fig. 1. Scheme of an intracellular osmotic gradient. The osmotic activity C is plotted as a function of cell thickness ΔX.

To illustrate the analysis, consider the application of the osmotic gradient hypothesis to the process of formation of a hypertonic urine. When (Fig. 1) the intracellular osmotic activity at the lumen side of the cell, C_0, is slightly higher than that of the lumen fluid, and the intracellular osmotic activity at the interstitial fluid side of the cell, C_1, is equal to that of the interstitial fluid, water could be transported from the lumen to the interstitial fluid. Consider the analysis applied to those cells concerned with active water transport, under conditions where the gradient is maintained but in which no water is being transported, that is, C_0 is equal to the osmotic activity of the lumen fluid and C_1 is equal to that of the interstitial fluid. The results of analyses applied to both a flat-sheet and a tubular arrangement of water-transporting cells were of a similar order of magnitude. For simplicity the flat-sheet arrangement of cells was chosen for presentation. The number of solute particles diffusing from the lumen side of the cell to the interstitial side must be equal to the number of solutes transported in the opposite direction by the mechanism maintaining the gradient. Under such steady-state conditions, the number of solute particles transported can be estimated from the integrated form of Fick's equation,

$$Q_0 = -D(C_1 - C_0)/\Delta X, \qquad (1)$$

where Q_0 is equal to the number of osmols per square centimeter per second diffusing from X_0 to X_1, D is the diffusion constant, and ΔX is equal to the cell thickness. During the production of a hypertonic urine, reasonable values for the parameters in Eq. 1 are $C_0 = 1.5$ mOsm/cm^3, $C_1 = 0.3$ mOsm/cm^3, $\Delta X = 2 \times 10^{-3}$ cm, and $D = 2.0 \times 10^{-5}$ cm^2/sec. Upon substitution of these values in Eq. 1 and conversion of the units, it is found that Q_0 is equal to 4.3×10^{-2} osmol/cm^2 hr.

The rate of change of free energy for the diffusion process may be evaluated by using the well-known formula

$$\delta(\Delta F)/\delta t = -Q_0 RT \ln(C_0/C_1), \qquad (2)$$

where ΔF is the change in free energy, t the time, R the gas constant, and T the absolute temperature. Since diffusion is an irreversible process, the free-energy decrease of the diffusion process cannot be funneled back into the transporting mechanism. Substitution in Eq. 2 for a temperature of 37°C gives a value of 4.3×10^{-2} kcal/cm^2 hr, which represents the minimum rate of expenditure of free energy for the uphill transport of the solutes.

To calculate the rate of change of free energy per unit volume, a specific gravity of 1.0 for cells was assumed. The minimum rate of expenditure of free energy that is required to maintain the gradient is then found to be 21,000 kcal/kg hr, which is approximately 1000 times the maximal rate for living cells.