The inactivity of arginine and histidine strongly suggests that a deficiency in lysine is at least one basic cause of the caries potentiality of diet 636. The specificity of L-lysine as a compound having caries-inhibitory effects in diet 636 is also supported by the evidence that D-lysine in particular, as well as ornithine and cadaverine, failed to reduce the caries produced by this diet.

Continuing studies will attempt to resolve some of the questions raised by these latest results and particularly to clarify the possible role played by L-lysine. The properties of these cariogenic experimental diets permit somewhat new approaches to the resolution of the possible relationship of dietary factors to the etiology of dental caries.

F. J. McCLURE
J. E. FOLK*
National Institute of Dental Resarch, National Institutes of Health, Bethesda, Maryland

References and Notes

1. F. J. McClure, *Science* 116, 229 (1952).
2. F. J. McClure and J. E. Folk, *Proc. Soc. Exptl. Biol. Med.* 83, 21 (1953).
3. F. J. McClure, in *Advances in Experimental Caries Research*, R. F. Sognnaes, Ed. (AAAS, Washington, D.C., 1955).
4. F. J. McClure and J. E. Folk, *J. Nutrition* 55, 589 (1955).
5. R. M. Griswold, *J. Am. Dietetic Assoc.* 27, 85 (1951).
6. R. A. Kraft and A. Morgan, *J. Nutrition* 45, 567 (1951).
7. We are indebted to J. D. Rust for valuable technical assistance.
8. F. L. Losee and J. L. Nemes, *Proc. Soc. Exptl. Biol. Med.* 87, 429 (1954).
9. J. E. Folk, unpublished data.
10. L. A. Bavetta and S. Bernick, *J. Am. Dental Assoc.* 50, 427 (1955).

* Research Associate, American Dental Association.

7 June 1955

Interaction between Oxygen and Oxygen-Carryng Proteins

In a recent article I. M. and T. A. Klotz (*1*) state that they have elucidated the nature of the electronic changes responsible for color and the oxygen-carrying ability of certain metal-proteins. Inspection of the observations and deductions of these authors leads to a somewhat different impression.

The article (*1*) starts with a summary of earlier studies of hemocyanin, a copper-containing protein that carries oxygen. Although the general inference is drawn that the protein contains cuprous copper, it is correctly stated that the evidence from magnetic and spectrophotometric data, as well as from the experiments of the authors on the oxidation-reduction potentials of the protein, is inconclusive. Some chemical evidence is then discussed. The experiments were carried out by the authors in the following fashion. Hemocyanin and oxyhemocyanin were separately prepared, and the copper content of the proteins was estimated by means of diquinolyl, a reagent for cuprous copper. A separate experiment was made so that the total copper content of the protein was known. Then, the authors continue, the difference between total copper content and copper content determined by diquinolyl gives the content of cupric copper.

Two points deserve consideration here. First, the reaction with diquinolyl is not instantaneous, on the authors' evidence, and no proof that the reaction goes to completion is given. It is less likely to go to completion with oxyhemocyanin than with hemocyanin, for the copper is bound with much greater affinity in the oxyhemocyanin. Diquinolyl can act only as a competing ligand for cuprous copper against the protein and oxygen, assuming, as the authors do, that there is a direct oxygen-copper bond, and it seems more reasonable to assume that the reagent fails to extract all the coppen than to assume that all it fails to extract is cupric ions. Klotz and Klotz do not test for cupric ions. If their assumptions are allowed, however, the conclusion is reached that half of the copper is as cupric and half as cuprous in the oxyhemocyanin. (In passing it should be observed that the figure of a half is derived from a comparison of the cuprous content of hemocyanin and of oxyhemocyanin, but the latter is only 39 percent of the *total* copper in the protein.) Surely, on removal of the cuprous copper from the oxygenated protein, the oxygen must be released, especially if the stability of the oxygen complex depends on the structures given. But the uptake of oxygen is reversible in the protein, and release of oxygen should lead to the "cupric" ions of oxyhemocyanin reverting to cuprous. Klotz and Klotz do not observe this. Does their procedure irreversibly oxidize the cuprous ion?

Turning next to hemerythrin, the authors present an identical argument. This protein is iron-containing, and the estimation of the ferrous and ferric content is made by use of phenanthroline, which gives a color with ferrous ions. No color developed in the reaction between the reagent and oxyhemerythrin; therefore, state Klotz and Klotz, all the iron is in the ferric state. The more likely conclusion is that the phenanthroline extracted no ferrous iron from the protein. The authors do not test for ferric ions. In hemerythrin itself about two-thirds of the iron was found to be ferrous by this phenanthroline "test."

If it is allowed that Klotz and Klotz have established a case for mixed valence states in the oxygen-containing proteins, then some of the structures proposed by them can be considered. At this point, however, the authors confuse two models. One is of the type discussed by McConnell and Davidson (*2*) in the mixed valency complexes, such as $Cu(II) \cdot Cl^- \cdot Cu(I)$ in solutions of which both cuprous and cupric ions could be detected, and the other is the no-bond complexes in which also charge-transfer forces play a part, for example, $Cu(I) \cdot O_2 \cdot Cu(I)$. Contribution to the stability of the latter type of complex is made by the structure $Cu(II) \cdot O_2^- \cdot Cu(I)$, but cupric ions are not detectable by chemical tests. These charge-transfer structures for oxygen-carrying proteins are contained in Pauling's and Mulliken's descriptions of double bonding in the transition metal complexes with unsaturated ligands and have been discussed with regard to hemocyanin (*3*). The evidence cited by Klotz and Klotz does not elucidate these matters.

Finally, turning to the absorption spectra of the oxygen-carrying proteins, the following observations are worth note. Ferrous and cuprous ions form complexes with aromatic diimines, which have very similar absorption spectra. The characteristics of these spectra have been explained on the assumption that the excitation involves the partial transfer of electrons from the cation to the ligand (*4*). Very similar absorption bands are found in certain cobaltous complexes. All three cations in other complexes appear to be able to carry oxygen, but the ferrous and cobaltous complexes do so only if, on the uptake of oxygen, there is a change in paramagnetic moment. Simultaneously with the change in paramagnetic moment, there is a large change in the absorption spectra of the complexes. This change in absorption is so like that described by Klotz and Klotz in hemerythrin on oxygen uptake that it is tempting to conclude that hemerythrin is a ferrous protein and paramagnetic but that it becomes diamagnetic on uptake of oxygen. It is also a mistake, on the evidence available, to assume that the absorption spectrum of oxyhemocyanin is not that of a cuprous protein (*1*) and (*3*). Many cuprous complexes do absorb strongly in the near ultraviolet, and many others have absorption bands in the visible. The final word on the electronic states of the oxygen-carrying proteins has not been said.

R. J. P. WILLIAMS
Inorganic Chemistry Laboratory, Merton College, Oxford, England

References

1. I. M. and T. A. Klotz, *Science* 121, 477 (1955).
2. H. McConnell and N. Davidson, *J. Am. Chem. Soc.* 72, 3168 (1950).
3. R. J. P. Williams, *Biol. Revs. Cambridge Phil. Soc.* 28, 381 (1953).
4. ———, *J. Chem. Soc.* 1955, 137 (1955).

27 May 1955

The heart of R. J. P. Williams' criticisms of our paper is based on an incorrect reading of our actual experimental procedure in carrying out chemical tests for the valence state of copper in hemocyanin and of iron in hemerythrin.

Let us consider (oxy)hemocyanin first. The actual procedure consists of adding a solution of biquinoline in glacial acetic acid, *the acid serving as an agent for the release of protein-bound copper,* to a small portion of oxyhemocyanin. A pink color develops instantly, although there are small changes over a period of a few minutes. The intensity of light absorption is measured. Thereafter a reducing agent, hydroxylamine or ascorbic acid, is added *to the same test tube.* The intensity of the pink color increases immediately, and a second absorption measurement is made. If, as Williams claims, the first reading is low because not all of the copper has been released from the protein, why is the reading increased approximately twofold when a minute amount of hydroxylamine is added? From this experiment alone it seems difficult to believe that the biquinoline fails to complex all the cuprous ion present. Furthermore, as a letter of inquiry would have established, we used higher biquinoline concentrations also and obtained the same answers. Finally, as an examination of the literature would have shown, the copper of hemocyanin becomes dialyzably free in acid solution.

Turning to hemerythrin, essentially the same answer applies. In this case we added a solution of *o*-phenanthroline in dilute *sulfuric acid* to a small portion of oxyhemerythrin. Essentially no color developed (if a small amount of fluoride ion was added to complex ferric ion, no color at all appeared). Thereafter hydroxylamine (a reducing agent) was added *to the same test tube.* A deep orange color developed immediately, although it changed more slowly toward its asymptotic final reading. If, as Williams claims, the first reading is low because not all the iron is released from the protein, why is the reading increased to full value when a minute amount of hydroxylamine is added? From this experiment alone it seems difficult to believe that the phenanthroline fails to complex any ferrous ion present. Furthermore, as an additional test of complete removal of iron from the protein, we carried out a number of experiments at different acidities and found no effect over an appreciable *p*H range. Finally, as an examination of the literature would have shown, the iron of hemerythrin becomes dialyzably free in dilute acid solutions.

There are also certain secondary points raised by Williams' communication. With regard to the state of the released oxygen in hemocyanin, there is no reason for his stating categorically that in strong acid molecular oxygen goes off. As was clearly implied in our paper, it might be released as a hydroperoxide ion, O_2^-, or as HO_2. Some experiments in this direction would be more in order, and we have carried them out. With regard to the fraction of Cu(I) in oxyhemocyanin, the appropriate figures to take from Table 1 of our paper, for reasons listed there, are $2.9 \times 10^{-4}M$ and $6.5 \times 10^{-4}M$, which lead to a fraction of 45 percent, instead of the 39 percent cited by Williams. Within the precision of the analyses, 45 percent seems sufficiently close to justify the "approximately one-half" that we stated.

The statement that we are confusing two models in connection with our Fig. 3 is unwarranted. We have proposed as an analogy only the mixed valency complexes $Cu(II) \cdot Cl^- \cdot Cu(I)$. The insertion of charge-transfer forces is Williams' contribution.

Finally, the analogy between the ferrous (or cuprous) complexes with aromatic diimines and hemerythrin (or hemocyanin) may or may not be useful. If one starts from the premise, as Williams has, that these proteins contain no-bond complexes, then it becomes desirable to point to excited states involving charge-transfer complexes. However, in the light of our chemical data, it is difficult to see how one can maintain such a premise. In any event, any model of these oxygen-carrying proteins must account for the mixture of valence states of the metal ions released from the oxygenated or nonoxygenated form. Our model does so; the no-bond model does not.

I. M. KLOTZ
T. A. KLOTZ

Northwestern University, Evanston, Illinois

1 August 1955

Effect of Insulinase-Inhibitor on Hypoglycemic Action of Insulin

Utilizing the release of iodine-131 from I^{131}-labeled insulin as an index of insulin degradation, it has been demonstrated that exogenous insulin is destroyed by the intact animal (*1*), just as it is by homogenates, extracts, and slices of liver and other tissues (*2–4*). This destruction appears to be due to the action of an enzyme system, insulinase, that is relatively specific in catalyzing the hydrolysis of insulin (*5*). The subcutaneous or intraperitoneal injection of a nonprotein liver fraction that inhibits the *in vitro* inactivation of insulin by homogenates (*6*) and slices (*3*) of liver is effective also in inhibiting the degradation of I^{131}-labeled insulin by the intact animal (*7*). The active component of the liver fraction is tentatively referred to as "insulinase-inhibitor."

Table 1. Effect of liver insulinase-inhibitor on fasting blood glucose concentration (expressed as mean ± standard error)

Injection	Before (mg/100 ml)	After 1 hr (mg/100 ml)
Saline	81.7 ± 2.1	78.7 ± 1.8
Inhibitor	76.2 ± 2.2	77.4 ± 1.2

In order to establish that the inhibition of insulin degradation *in vitro* is associated with an increase in the biological activity of insulin, the effect of a crude preparation of liver insulinase-inhibitor on the hypoglycemic action of exogenous insulin was determined in rats and rabbits (*8*).

The crude insulinase-inhibitor was prepared as follows: 100 g of fresh beef liver was homogenized with 1000 ml of water, and the *p*H was adjusted to 4.8 with hydrochloric acid. The homogenate was boiled for 10 minutes and was filtered, and the filtrate was dried by lyophilization. The lyophilized product was extracted with 20 vol of glacial acetic acid, and the acetic acid filtrate was precipitated with 3 vol of cold acetone. The precipitate was washed with acetone and dried with ethyl ether. The dried precipitate was dissolved in water and adjusted to *p*H 7.0. This preparation inhibited the action of insulinase *in vitro* and *in vivo,* as measured by the inhibition of the release of I^{131} from labeled insulin.

Four groups of 12 male rats of the Carworth strain were used after an overnight fast. The rats weighed from 200 to 275 g. Blood samples were taken from the cut tail, and the concentration of glucose was determined by the Nelson procedure (*9*). After a preliminary blood sample had been taken, two groups of rats were given a subcutaneous injection of 6 ml of a 10-percent solution of the liver preparation per 100 g of body weight, and the other two groups were given a similar volume of saline. One hour after the subcutaneous injections, a second blood sample was taken. Then one control group and one experimental group of animals were given an intraperitoneal injection of 0.5 unit of insulin per kilogram of body weight, and the other control and experimental groups were given 1.0 unit of insulin per kilogram of body weight. Thereafter, blood samples were taken at hourly intervals for 4 hours.

The subcutaneous injection of the liver insulinase-inhibitor preparation produced no significant change in the concentration of the blood glucose (Table 1).