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).

However, the hypoglycemic action of the insulin injected 1 hour after the liver preparation was markedly increased. This statistically significant ($P < 0.001$ at 2, 3, and 4 hours after 0.5 unit/kg of body weight and $P < 0.05$ at 3 hours and $P < 0.01$ at 4 hours after 1.0 unit/kg of body weight) effect is illustrated in Fig. 1, where the blood glucose concentration is expressed as a percentage of the preinjection level.

Whereas the insulinase-inhibitor preparation was nontoxic for rats even at a dosage of 10 g/kg of body weight, it was toxic in rabbits at dosage levels as low as 1 g/kg of body weight. Occasional rabbits survived the subcutaneous injection of 1 or 2 g of the preparation per kilogram long enough to permit the determination of the hypoglycemic response to the intravenous injection of 0.1 unit/kg of body weight. Figure 2 illustrates the hypoglycemic response of a rabbit that lived about 18 hours after the subcutaneous injection of a solution containing 2 g of the preparation per kilogram of body weight.

In this experiment, the hypoglycemic response to the intravenous injection of insulin was tested in two rabbits. After a preliminary test in which both rabbits gave essentially the same response, one animal was given a subcutaneous injection of saline and the other an injection of the liver extract. One hour later, the hypoglycemic response to the intravenous injection of insulin was determined again. It is apparent that the injection of the insulinase-inhibitor preparation 1 hour before the insulin resulted in a marked increase in the biological effectiveness of the insulin. Similar results have been obtained with other rabbits that survived the injection of the liver preparation.

The data reported here reveal that a liver insulinase-inhibitor preparation that

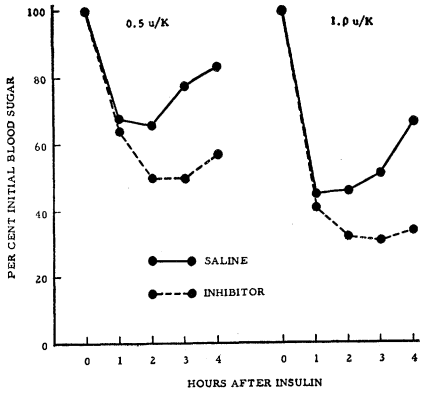

Fig. 1. Effect of insulinase-inhibitor on hypoglycemic action of insulin in rats. Response expressed as percentage of the blood sugar concentration immediately before the intraperitoneal injection of insulin.

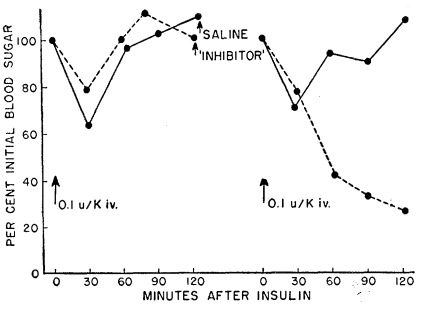

Fig. 2. Effect of insulinase-inhibitor on hypoglycemic action of insulin in rabbits. Two rabbits were given an intravenous injection of insulin at zero time. A second injection of insulin was given 1 hour after the subcutaneous injection of either saline or liver preparation. Response expressed as percentage of the blood sugar concentration immediately before each intravenous injection of insulin.

effectively inhibits the destruction of insulin *in vitro* and *in vivo* is effective also in increasing the biological activity of insulin in rats and rabbits.

I. Arthur Mirsky
Gladys Perisutti

Department of Clinical Science, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania

References and Notes

1. I. A. Mirsky, G. Perisutti, R. Jinks, *Endocrinology* 56, 484 (1955).
2. I. A. Mirsky and R. H. Broh-Kahn, *Arch. Biochem. and Biophys.* 20, 1 (1949).
3. I. A. Mirsky and G. Perisutti, *Endocrinology* 52, 698 (1953).
4. I. A. Mirsky, G. Perisutti, F. Dixon, *Proc. Soc. Exptl. Biol. Med.* 36, 228 (1954).
5. ———, *J. Biol. Chem.* (May 1955).
6. I. A. Mirsky, B. Simkin, R. H. Broh-Kahn, *Arch. Biochem. and Biophys.* 28, 415 (1950).
7. I. A. Mirsky, G. Perisutti, D. Diengott, *Proc. Soc. Exptl. Biol. Med.* 88, 76 (1955).
8. This work was aided by a grant from the Eli Lilly Co. and the Foundations' Fund for Research in Psychiatry.
9. N. Nelson, *J. Biol. Chem.* 153, 375 (1944).

16 May 1955

Prenatal Ingestion of Fluorides and Their Transfer to the Fetus

McKay and Black have pointed out that factors influencing the integrity and structure of tooth enamel are effective only during the calcification period (*1*). It is logical then to assume that any beneficial effects from fluorides would be derived only while the teeth are in developmental stages when the matrix is being formed and the enamel is undergoing calcification or maturation.

On this premise, a long-range study was instituted in 1948 to determine the value of fluorides in preventing caries. The fluorides are given to the expectant mother during pregnancy and are also administered to the child until permanent tooth calcification has occurred (*2*). A final report of this work will not be made until the teeth of the offspring can be evaluated for their resistance to decay. This paper, a phase of the study, presents the results of an investigation to determine the relationship between maternal ingestion of fluorides, placental storage, transplacental passage, and fetal cord blood levels.

Past studies have mentioned the transfer of fluorides from mother to fetus (*3*) and have shown a positive correlation between fluoride supplementation and the fluoride content of the placenta (*4*). However, there is no report in the literature of an attempt to correlate the fluoride concentration of fetal blood and placental tissue in a study using fluoride tablets during the pregnancy.

Four groups of patients were used: (i) patients given one tablet of calcium fluoride (each 2 mg) (*5*) or sodium fluoride (each, 2.2 mg) per day. Treatment was initiated at various stages of pregnancy; (ii) controls, from the same locale, who had no known supplemental fluorides; (iii) individuals who drank artificially fluoridated water throughout their pregnancy; and (iv) controls from a nearby area that did not have a fluoridated water supply.

Sections of approximately 25 g of tissue were taken from the periphery of the placenta, and about 25 to 50 ml of blood was expressed from the umbilical cord after it had been severed. The fluorides were then separated by the Willard-Winter distillation (*6*) process and their concentration was determined by the William's titration method as modified by Smith and Gardner (*7*). Every possible precaution was taken to rule out any laboratory error. A constant and a percentage correction factor, as well as the standard deviation of 2.4 percent of the technique, were considered before the results given in Table 1 were reached.

The average fetal blood fluoride concentration in the tablet study group was 41 μg/100 ml; in the control, 17 μg/100 ml. The average placental fluoride concentration in the tablet study group was 111 μg/100 g; in the control, 101 μg/100 g.

In the fluoridated water supply study group, the average cord blood concentration was 38 μg/100 ml; in the control, 22 μg/100 ml. The average placental concentration was 85 μg/100 g in the study cases; 68 μg/100 g in the control.

In both study groups the average cord blood fluoride concentration was higher than it was in the respective controls. The concentration in the group that took fluorides by tablet was 250 percent higher than it was in the control; in the group that took fluoridated water, the concentration was 175 percent higher. Twenty percent of the tablet study group had a