The periods of no responding are correlated with the onset of alcohol-induced paralysis, which has a caudorostral progression in the skeletal musculature. The avoidance response consisted in moving in a circle away from the lever on the front legs, rising on the hind legs, and falling on the lever with the front paws to complete the circle. At the end of the 5-ml session, the hind legs were paralyzed, and only the initial, front-leg portion of the response-series occurred. In both of the animals that received the 5-ml dose, complete skeletal paralysis ensued about 2 hours after injection.

The effect of alcohol upon avoidance behavior may be contrasted with the effects of scopolamine (*2*). Both drugs affect the response rate. In the case of scopolamine this may be owing to a disruption of timing behavior, since the number of shocks received increases in spite of an increase in response rates. With alcohol there is a decrease in shocks received, indicating that the drug is not merely disruptive in its effects at lower dose levels. Similar effects may perhaps be produced by other drugs—for example, barbiturates.

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29 February 1960

## Reversible Inhibition of Beef Heart Cytochrome c Oxidase by Polyionic Macromolecules

*Abstract.* The basic proteins protamine (sulfate), histone, lysozyme, and ribonuclease were found to be potent inhibitors of mammalian heart muscle cytochrome *c* oxidase. Their inhibitions were completely reversed in the presence of a strongly anionic polyglucose sulfate. With fresh rat heart muscle homogenates, Keilin and Hartree type of beef heart muscle particulates, and deoxycholate-solubilized oxidase preparations, the reversible nature of the phenomenon was demonstrated with manometric and spectrophotometric assays for cytochrome *c* oxidase.

Subsequent to preliminary observations in this laboratory that cartilage homogenates from certain invertebrate and vertebrate species inhibited mammalian heart muscle cytochrome *c* oxidase, (*1, 2*), a survey of naturally occurring substances was begun in an attempt to find materials capable of inhibiting or of activating this important terminal respiratory enzyme. At the time this work was started, although unknown to us, Smith and Conrad had reported that protamine, a strongly basic, cationic protein, inhibited the oxidase (*3*). Independent of that report we, also, found protamine sulfate to be a very potent inhibitor of the oxidase, thereby confirming the work of Smith and Conrad. We also found that other basic or cationic proteins, such as histone, ribonuclease, and lysozyme, exert potent inhibitory influences upon heart muscle cytochrome *c* oxidase. Of even greater significance, we believe, is our discovery that all the above inhibitions may be completely reversed by a strongly anionic synthetic polyglucose sulfate. The latter compound was brought to our attention recently when Lash (*4*) and Lash and Whitehouse (*5*) identified and isolated it in extracts of the odontophore cartilage of the marine snail, *Busycon canaliculatum*.

To our knowledge, the present paper constitutes the first report of a reversible inhibition of cytochrome *c* oxidase by polyionic macromolecules of opposite charge.

The following cytochrome *c* oxidase preparations were used: (i) fresh rat heart homogenates, (ii) Keilin- and Hartree-type insoluble beef heart-muscle preparations (*6*), and (iii) deoxycholate-solubilized cytochrome *c* oxidase preparations (*7*), made from the Keilin and Hartree insoluble particulates. Beef heart cytochrome *c* (Sigma) was used in both manometric and spectrophotometric assays of cytochrome *c* oxidase activity, as described previously (*2*). Protamine (salmine) sulfate (General Biochemicals), lysozyme (Sigma, twice recrystallized), calf-thymus histone (Mann), and ribonuclease (Worthington) were used as inhibitors. The polyglucose sulfate corresponds to Mora's "preparation H" (*8*).

In Fig. 1, results of a typical series of manometric oxidase assays are shown. Curve *1* illustrates the oxygen uptake of a control deoxycholate-solubilized oxidase preparation, with hydroquinone as substrate. Curve *2* shows that incorporation of 100 $\mu$g of protamine sulfate per milliliter (final concentration) in the above system abolished oxygen uptake completely. In curve *3*, the protamine inhibition was reversed by incorporation of 100 $\mu$g of polyglucose sulfate per milliliter (final concentration) into the system. The restored activity in this experiment equalled 80 percent of the original value. In other experiments, depending upon the ratio of protamine sulfate to polyglucose sulfate, complete restoration of activity was readily accomplished.

In Fig. 2, results of a typical set of spectrophotometric oxidase assays are shown. The curves are direct tracings of continuously recorded absorbancy changes made in the Process and Instruments automatic recording spectrophotometer, model RS3. Curve *A*, a control determination, shows a 3-minute decrease in absorbancy (at 550 m$\mu$) of dithionite-reduced cytochrome *c*, as a result of its oxidation by deoxycholate-solubilized cytochrome *c* oxidase preparation. Curve *B* shows the complete inhibition of the oxidase activity of this preparation produced by addition to it of 33 $\mu$g of protamine sulfate per milliliter (final concentration). During the first 3 minutes there was no oxidation of the cytochrome *c*, and hence no decrease in absorbancy. Instead, a small increase in absorbancy occurred, as a result of a slight turbidity arising from interaction between the protamine sulfate and the enzyme preparation. This turbidity was responsible for the wavering of the absorbancy values of curve *B*. At the end of 3 minutes of complete oxidase inactivation, readings were briefly interrupted for 20 to 30 seconds (arrow) for the addition of 33 $\mu$g of polyglucose sulfate per milliliter (final concentration). In curve *C*

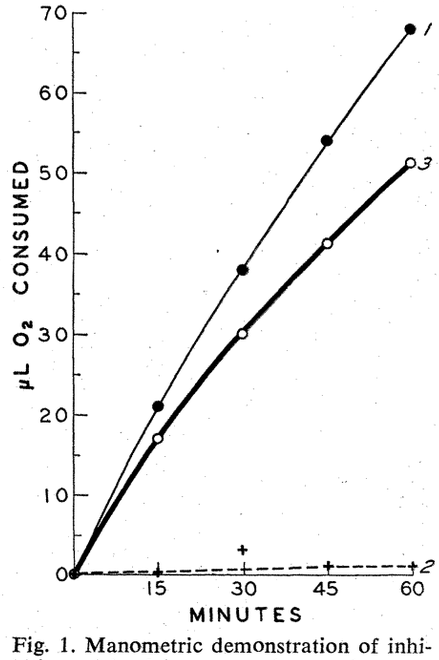


Fig. 1. Manometric demonstration of inhibition of beef heart muscle cytochrome *c* oxidase by protamine sulfate and reversal of protamine inhibition by polyglucose sulfate. Oxygen consumption versus time with hydroquinone as substrate: *T*, 37°C; gas phase, air; final volume, 3.0 ml. All values corrected for auto-oxidation of substrate. Curve *1* (control), $O_2$ uptake of untreated deoxycholate-solubilized oxidase preparation. Curve *2*, complete inhibition of oxidase by incorporation of protamine sulfate (final concentration 100 $\mu$g/ml). Curve *3*, reversal of protamine inhibition by incorporation of polyglucose sulfate (final concentration 100 $\mu$g/ml) into the system.

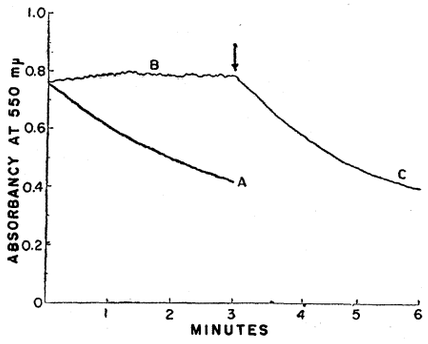


Fig. 2. Spectrophotometric demonstration of inhibition of cytochrome *c* oxidase by protamine sulfate and reversal of protamine inhibition by polyglucose sulfate. Curve *A* (control), oxidation of dithionite-reduced cytochrome *c* by untreated deoxycholate-solubilized oxidase preparation. Curve *B*, complete inhibition of oxidase by incorporation of protamine sulfate (final concentration 33 μg/ml) into the system. Wavering of absorbancy trace was caused by slight turbidity arising from protamine sulfate combination with deoxycholate preparation. Arrow, readings interrupted for 20 to 30 seconds while protamine sulfate (final concentration 33 μg/ml) was added to cuvette with rapid mixing. Curve *C*, reversal of protamine inhibition by polyglucose sulfate addition.

it is shown that not only was the inhibition of the protamine completely reversed by the polyglucose sulfate addition, but, as evidenced by the greater slope of curve *C*, the reaction rate was increased in comparison with that of the untreated control shown in curve *A*. This increase in activity beyond control values resulting from polyglucose sulfate addition is a real phenomenon. It is undoubtedly a result of the fact that in addition to reversal of the protamine inhibition, per se, the amount of polyglucose sulfate used was also sufficient to reactivate some reversibly denatured oxidase in the original enzyme preparation. This finding has also been established by separate heat-denaturation experiments.

Similar reversible inhibitions of cytochrome *c* oxidase have also been demonstrated, using fresh whole rat heart homogenates and the Keilin and Hartree type of insoluble beef heart muscle oxidase preparations. In addition, histone, ribonuclease, and lysozyme were also capable of inhibiting the cytochrome *c* oxidase activity of the above preparations. As with the protamine inhibitions, those produced by the other basic proteins mentioned above could be reversed by polyglucose sulfate.

It is important to stress that the type of reversible inhibition described in this report is in no way specific for cytochrome *c* oxidase. Similar behavior has been established for a number of other enzymes (*9*). However, the fact that cytochrome *c* oxidase may be reversibly inhibited by polyionic macromolecules is of great significance. Until now, the most important reversible inhibitions of cytochrome *c* oxidase have been those accomplished with cyanide, azide, and carbon monoxide. Spectroscopic and spectrophotometric studies of these classic inhibitions have contributed much to our understanding of the enzyme, and in particular, its heme component. It is therefore of great interest that, thus far, spectral changes or shifts during the inhibitions by the cationic macromolecules reported in this paper have not been demonstrable in a large number of experiments. This leads us to suspect that charge-density influences upon the configuration of the protein (and other nonheme components) of the oxidase aggregate may be operating in the present type of inhibitory action. Effects that involve electron transfer through the nonheme moieties of the enzyme are now under consideration.

The disclosure that substances such as protamine, histone, and ribonuclease, which are important intracellular proteins, can exert a reversible inhibition of a major enzymatic component of cell mitochondria also has important implications for the study of nuclear-cytoplasmic metabolic interrelations. Finally, since spectrophotometric evidence, to date, indicates that the heme component of the oxidase is not involved in these inhibitions, we believe that a means is now available for approaching the study of the contributions of the nonheme components of cytochrome *c* oxidase to the electron transfers accomplished by the enzyme.

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8 March 1960

## Trophic Substances in a Blind Cave Crayfish

*Abstract.* The eyestalks, supraesophageal ganglia, and circumesophageal connectives of the blind cave crayfish *Orconectes pellucidus australis* contain a red pigment-concentrating substance and a distal retinal pigment light-adapting one. Assays were performed on the dwarf crayfish, *Cambarellus shufeldti*. The significance of these findings is discussed in relation to endocrine regulation of pigmentary effectors in crayfishes.

The crayfishes *Cambarellus shufeldti* and *Orconectes clypeatus* produce some substances that concentrate chromatophoral pigments and others that cause the distal retinal pigment to migrate toward the fully light-adapted position (*1–4*). No one has determined whether such trophic substances occur in cave crayfishes which lack chromatophores and retinal pigment cells.

Recently we were fortunate to obtain enough specimens of *Orconectes pellucidus australis* (*5*) to learn whether this organism produces a red pigment-concentrating hormone and a distal retinal pigment light-adapting substance. These crayfish were collected in Shelta Cave, Huntsville, Alabama. The specimens of *Cambarellus shufeldti* used as assay

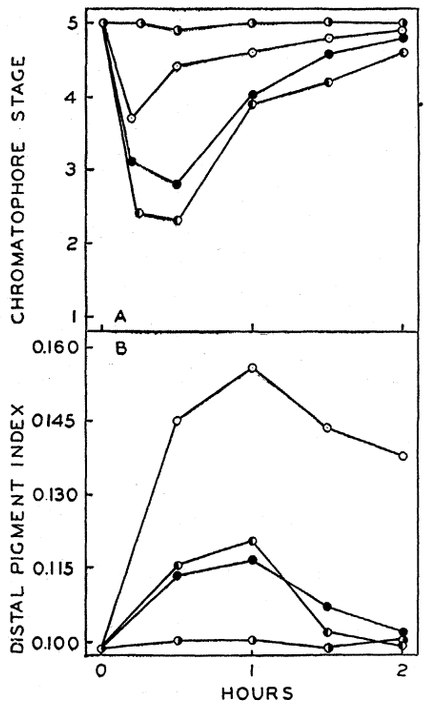


Fig. 1. Responses of (*A*) dark red chromatophores and (*B*) distal retinal pigment of dwarf crayfish to extracts of eyestalks (circles), supraesophageal ganglia (dots), and circumesophageal connectives (circles half-filled on left) of blind cave crayfish. Control, circles half-filled on right.