4. D. M. Kipnis and M. W. Noall, *Biochim. et Biophys. Acta* **28**, 226 (1958).
5. J. L. Kostyo, J. Hotchkiss, E. Knobil, *Science* **130**, 1653 (1959).
6. F. J. Saunders, *Acta Endocrinol.* **26**, 345 (1957).
7. E. Eisenberg and G. S. Gordan, *J. Pharmacol. Exptl. Therap.* **99**, 38 (1950).
8. We express our gratitude to I. C. Winter for generously supplying the synthetic anabolic steroid used in these experiments. This study was supported by grants from the G. D. Searle Co. and the National Institutes of Health (CY 3599).

12 February 1960

Effects of Ethyl Alcohol on Avoidance Behavior

Abstract. Three albino rats, trained to avoid electric shock, were stomach-loaded with from 1 to 5 ml of 32-percent aqueous solution of ethyl alcohol prior to the experimental session. Small doses produced some increases in response rates and a consistent decline in shocks received. Larger doses produced progressive uncoordination, accompanied by lower response rates and an increase in shocks received.

Avoidance behavior may be maintained by allowing each occurrence of a selected response to postpone a brief shock for several seconds (*1*). In the absence of responses, shocks are delivered at regular intervals. The steady rate of responding and the stable frequency of shocks resulting from this procedure may be altered by pharmacological agents—scopolamine, for example (*2*). In the work described in the present report (*3*) the effect of ethyl alcohol on avoidance behavior in the rat was studied.

Three male albino rats (150 days old) were used for 2 hours daily in a sound-attenuating experimental chamber equipped with a lever. Depression of the lever (a response) activated electric counters and automatic programming equipment. The floor of the chamber consisted of 1/12-in. steel bars. In the absence of lever-presses, electric shocks (3 ma) of 0.5-second duration were delivered to the rat's feet through the floor grids every 20 seconds. The polarity of each grid changed rapidly and irregularly during shock administration. Each lever-press postponed the next shock for 20 seconds. After 90 days of this procedure, each rat was deprived of food (but not water) for at least 15 hours prior to each avoidance session. On alternate days, 3 minutes before the start of the session, a dose of ethyl alcohol was introduced directly into the rat's stomach through a rubber tube inserted orally. The doses were 1, 2, 4, and 5 ml of 32-percent aqueous solution of ethyl alcohol. Each animal (each weighing about 350 gm) received these doses in a different, irregular order. The volume of alcohol per unit of weight represented by the 5 ml dose in a 350-gm rat is equaled in a 175-lb man by a dose of about ¾ quart of 100-proof liquor. Rat WM4 was not given the 5-ml dose. On control days its stomach was loaded with 2 or 5 ml of water or nothing. The number of responses and shocks in each 2-hour session were recorded.

Figure 1*a* shows for each rat the total number of lever-presses in a 2-hour session on control days (0) and after doses of 1, 2, 4, and 5 ml of 32-percent ethyl alcohol solution. The responding of each rat declines sharply between doses of 4 and 5 ml. The effect on responding after administration of lower doses is variable: for example, one rat shows a decrease and two rats an increase between doses of 1 and 2 ml.

The number of shocks in a 2-hour session is a more orderly dependent variable (Fig. 1*b*). Each animal receives fewer shocks after a dose of 2 ml of the alcohol solution than after a dose of water. The shock frequency increases above that for water after 4 ml and increases sharply after 5 ml. Low doses of alcohol increase the effectiveness of avoidance responses, although the frequency of responses may not increase (Fig. 1*a*). Higher doses of alcohol decrease the frequency of responding and increase the frequency of shocks.

The relative decrease in response rates following doses of 1 and 2 ml for rat WM5 may be brought about by the decrease in shocks received. This animal's relatively higher rate of responding under control conditions is largely accounted for by bursts of three to four responses per second during and after shocks. A decrease in shock frequency leads to a decrease in the number of such bursts.

An examination of cumulative records of responses during control sessions (water or no dose) reveals a steady rate of responding throughout both hours of the session after a gradual acceleration at the start of the session. A dose of 1 ml produces slight irregularities during the first hour, but the record during the second hour is indistinguishable from the control record. After doses of 2 and 4 ml, responding accelerates markedly and ceases for about 1 minute early in the first hour. The onset of the acceleration is earlier after a dose of 4 ml than after a dose of 2 ml. After 5 ml, responding begins at a rate higher than the control rate.

Extended periods of no responding begin near the end of the second hour after 4 ml and near the end of the first hour after 5 ml. Only one response was made by rat A3 after the end of the first hour with a 5-ml dose, and the experiment was terminated after 27 consecutive shocks had been received. In computing the number of responses emitted and the shocks received (Fig. 1) for rat A3, it was assumed that no further responses would have been emitted and that the shock frequency would have been maximum for the remainder of the second hour of the session. This animal died 12 hours later without recovering from the effects of 5 ml of alcohol.

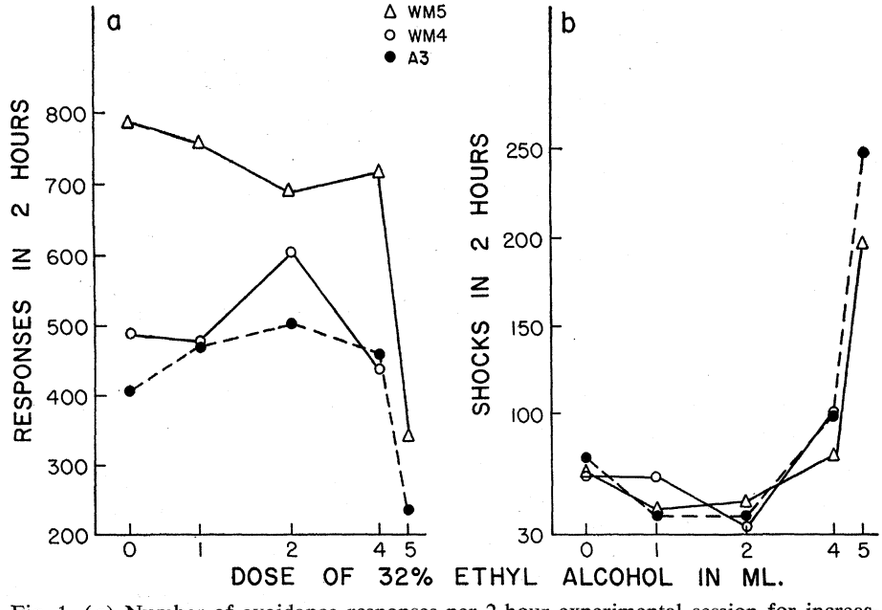

Fig. 1. (*a*) Number of avoidance responses per 2-hour experimental session for increasing doses of alcohol. (*b*) Number of shocks received per 2-hour session. The data for control sessions are shown at 0 on the abscissa. Each point in Fig. 1 is based on from 1 to 5 experimental sessions.

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.

G. S. REYNOLDS
P. VAN SOMMERS
Psychological Laboratories, Harvard University, Cambridge, Massachusetts

References and Notes

1. M. Sidman, *Science* **118**, 157 (1953).
2. R. J. Herrnstein, *J. Exptl. Anal. Behavior* **1**, 351 (1958).
3. This research took place in the Psychological Laboratories, Harvard University, under grant G-6435 from the National Science Foundation.

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 μg 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 μg 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 mμ) 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 μg 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 μg of polyglucose sulfate per milliliter (final concentration). In curve *C*

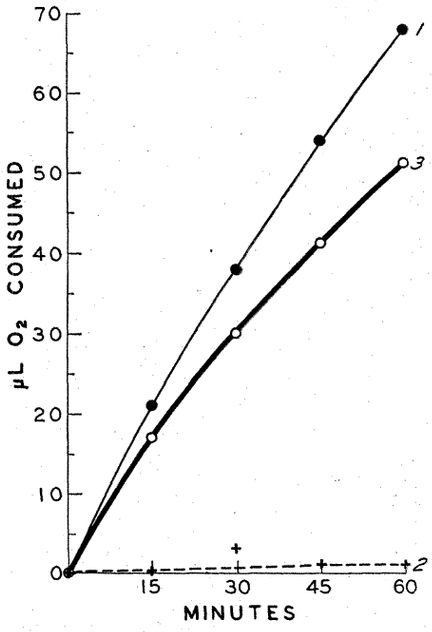

Fig. 1. Manometric demonstration of inhibition of beef heart muscle cytochrome *c* oxidase by protamine sulfate and reversal of protamine inhibition by polyglucose sulfate. Oxygen consumption versus time with hydroquinone as substrate: *T*, 37°C; gas phase, air; final volume, 3.0 ml. All values corrected 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 μg/ml). Curve *3*, reversal of protamine inhibition by incorporation of polyglucose sulfate (final concentration 100 μg/ml) into the system.