EPF. Do they also differ chemically?

These data also emphasize another problem: namely the differing time response of hypoxic EPF and anemic EPF. The former shows a quick rise and a quick reversion to normal. The latter goes up quickly after a significant anemia is established and remains up for long periods of time. We have recently assayed plasma from severely iron-deficient rats that have been anemic for 3 months(13). High values of EPF were found without exception. Does this differing time response likewise imply a different source and/or a different identity of hypoxic and anemic EPF? Detailed studies of the chemical properties of EPF produced by these various stimuli are in progress in our laboratory.

Summary. Rabbits have been placed in a low O_2 atmosphere for varying periods of time and plasma erythropoietin content determined after set intervals of hypoxia. EPF was found to rise significantly at 8 hours, reach peak levels at 24 and 48 hours, and return to normal at 72 hours.

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A Microbioassay for Acetylcholine.* (26384)

WILLIAM L. NASTUK AND LEONARD LEVINE† (Introduced by Walter S. Root)
Department of Physiology, College of Physicians and Surgeons, Columbia University, New York

Many classical researches reported in literature are concerned with the action of acetylcholine (ach). In such work there has often been a need to determine low concentrations of ach in small volumes of fluid or perfusate. Direct chemical analyses by methods such as the one described by Hestrin(1) are ordinarily too insensitive for these applications and one must turn to a bioassay. Minz(2) has summarized the most frequently employed bioassay procedures and has pointed out the advantages and disadvantages of each. There is, for example, an approach in which one measures the contraction of the dorsal muscle of the leech, a preparation which will respond to ach at a concentration of 5×10^{-8} M. This preparation is suspended in a bath whose volume is usually about 10 ml and therefore if a 0.1 ml sample of solution is added to the bath, this sample, if it is to be successfully assayed, must contain ach at a minimal concentration of 5×10^{-6} M.

We wished to conduct certain experiments

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† Present address: Dept. of Biophysics, University College, London.

wherein one must be able to determine low concentrations of ach in very small volumes of fluid. To achieve this goal we have combined several technics previously employed in this laboratory and thereby have devised a reliable method by which one may detect ach in concentrations as low as 2.5×10^{-8} M in samples whose minimum volume approaches 0.15 μl.

In this assay we have taken advantage of the well-known fact that the chronically denervated frog sartorius muscle becomes very sensitive to diffusely applied ach(3,4). In our method, the denervated endplate area of a muscle fiber is observed under a microscope while this site is microperfused with a solution whose ach concentration is to be determined. The microperfusion is accomplished using a glass micropipette of tip diameter 20-40 μ. Such pipettes are manufactured from 1.5 mm O. D. Pyrex tubing using only the weak pull of the micropipette puller designed in this laboratory(5). A small volume of the sample solution is drawn into the pipette whose shank is then thrust into a short length of plastic tubing, the other end of which is slipped over the tapered end of a glass tube. This assembly is mounted at a 45° angle in a micromanipulator. To the upper end of the glass tube is connected another length of plastic tubing to which is applied a pulse of air pressure sufficient to eject the sample. For this purpose it is convenient to use a pressure bottle, an air filled hypodermic syringe or one may apply pressure by mouth.

At the start it is best to become familiar with the technic by using normal frog sartorius muscle. The dissected normal muscle is mounted flat in a chamber filled with Ringer's solution and this assembly is placed on a microscope stage in order that the transilluminated muscle can be observed at 100-150 $\times$ magnification(6,7). Thereafter the Ringer's bath solution is drawn off and replaced at 20 min intervals. It is essential to use an optical system in which the objective and condenser have an N.A. of at least 0.25 in order to obtain the necessary resolving power (cross striations should be clearly visible). On scanning the medial surface of the muscle one may easily locate main nerve branches and these are followed to their various terminations where groups of endplates are located. The tip of the loaded perfusion pipette is placed immediately above but not touching a single endplate. If the threshold concentration of ach is present in the sample, then shortly after the perfusion begins, a brief easily observed tetanus will be evoked in the perfused muscle fiber. At higher concentrations of ach, the contractile response in this fiber will be briefly sustained after the perfusion is terminated and, adjacent muscle fibers, whose endplates lie nearby, will also respond. At the lower concentration of ach at which a reproducible contractile response may be obtained (ach threshold), the latency of the response averages about 1 sec and rarely exceeds 5 sec. We have found the ach threshold to lie in the concentration range $1.1\text{-}2.2 \times 10^{-5}$ M. At an endplate whose ach threshold is 2.2×10^{-5} M one cannot obtain the contractile response when ach at 2.09×10^{-5} M is applied. If determinations at a single endplate are repeated at 10 to 15 min intervals in order to permit recovery from receptor desensitization due to applied ach(8), then the ach threshold can be shown to remain constant over at least a 5 hr period. We have not attempted an endurance test for normal muscle but it is likely that such preparations when properly treated, remain useable for even longer periods (see below).

When a chronically denervated sartorius muscle is used, the assay sensitivity increases approximately 1000 fold. The procedure in this case is as follows. Under urethane anesthesia the hind leg of a frog is denervated by severing spinal nerves 7-9. The nerves are approached from the dorsal body surface, about 1 cm of nerve is removed, and the central stump is sutured to the skin thereby hindering reinnervation. Such denervated frogs are maintained for 2 months or more in a tank with running tap water at 15°C. Survival time is increased and the size of the muscle fibers is better maintained by force-feeding the frogs every second or third day with small pieces of liver(9).

At an appropriate time, the chronically denervated muscle is dissected and mounted as described previously. Identification of the endplate areas in this preparation is more difficult but with some experience and a good optical system one can locate these sites by following the old nerve pathways which remain marked by the surviving connective tissue elements. If this guide is unsuccesful, a trial and error testing with ach perfusion will reveal sites of greatest sensitivity. After 2 months denervation, the average ach threshold at endplate sites is 2.8×10^{-8} M (range 0.55-16.5 $\times$ 10^{-8} M). Rate of development of this chemosensitization depends on the temperature at which the frogs are kept. One may take advantage of the range in sensitivity of a group of endplates to obtain a quantitative estimate of the concentration of ach in an unknown solution by applying it to several sites of different ach sensitivity. It may, of course, be necessary to dilute the unknown solution to place its final ach concentration within the range of the ach thresholds.

The ach threshold for each endplate site remains stable and reproducible for many hours. It is likely that the same muscle can be used for even longer periods because we have been able to obtain stable ach thresholds for muscles tested daily over periods of 2 to 4 days. These muscles were stored at 4°C during idle periods and at these times the Ringer bath was unchanged.

In trying to determine the maximum sensitivity of this method, we were able, using chronically denervated muscle, to obtain a contractile response when an ach containing solution was ejected from a typical micropipette (I. D. 27 μ) at the rate of 0.15 μl/sec. The amount of ach delivered, in the one second period required to produce a response, amounted to approximately 10^{-15} moles. One can see therefore that this method of delivering ach to the receptor site is relatively wasteful since by the iontophoretic method a single twitch can be evoked by applying only 10^{-15} to 10^{-16} moles of ach to a *normal* endplate(10,11). It would therefore appear that our assay could be increased in sensitivity by further refinements, but perhaps we are limited by having already approached the minimal *concentration* of ach which must be applied in order to produce a contractile response.

Some further increase in assay sensitivity could have been obtained by using an internal microelectrode to measure, as an index, a subthreshold reduction in transmembrane potential produced during the ach perfusion. This advantage may be outweighed because the technic is more laborious, technically demanding, requires more equipment, necessitates determination of a dose response curve for each area tested, and since the membrane must be punctured, there is a limit on the repeated use of each endplate. We may also add that addition of physostigmine sulfate (1.55×10^{-5} M) to the Ringer's solution bathing the chronically denervated muscle increases its sensitivity to ach only by a factor of 1.5 (avg). This is not surprising since there is evidence that the concentration of cholinesterase at the site of the denervated endplate in amphibian sartorius muscle decreases markedly by the 30th day after motor nerve section(12).

In this assay, the production of a muscle twitch involves reduction of the transmembrane potential to the critical value required for initiation of a propagated action potential. A sample may yield a positive response because it contains ach but such a response may also be obtained because, for example, the sample contains a high concentration of potassium ions. A spurious result of this type can be distinguished by adding d-tubocurarine to the Ringer's solution bath and to the sample. d-Tubocurarine will prevent depolarization by ach but not by potassium ions. Another means of differentiating between ach and potassium ions is to make use of the fact that ach has a greatly reduced depolarizing power at non-endplate sites while potassium ions are equally effective at all regions of the muscle fiber. Therefore one should test the sample both at the endplate and at an endplate free site such as may be found in the proximal portion of the pelvic end of the sartorius muscle.

Axelsson and Thesleff(13) have reported that one to 2 weeks post denervation, mammalian muscle becomes uniformly sensitive over its entire length to ach applied iontophoretically. Such preparations seemed to offer opportunity to improve our assay since they would eliminate the prolonged period of denervation and the need to identify endplate areas. Therefore we extended the study of the assay procedure to the denervated tenuissimus muscle of the cat, testing it 22 days after nerve section. Using our method of ach application we were able to initiate a contractile response at any site on the denervated muscle fiber but the ach concentration required to do this was 0.55-1.1 $\times$ 10^{-5} M, (a value close to the ach threshold for the endplate of an innervated fiber). As the concentration of ach solution applied was reduced, the region of the denervated tenuissimus fiber from which a response could be initiated became progressively smaller. The regions of greatest sensitivity (presumably corresponding to the endplate areas) had an ach threshold about 100 times less than that of the least sensitive regions. The highest sensitivity that we observed in a muscle denervated 22 days corresponded to an ach threshold of 5.5 $\times$ 10^{-8} M.

Kuffler(14) reported earlier that some amphibian muscle fibers which he examined several months after denervation seemed to be sensitive everywhere to ach applied in the form of small droplets. Recently Miledi, using the frog sartorius muscle(15) has made a detailed study of the progressive enlargement of the ach sensitive area with time after nerve section. Work done in this laboratory(9) confirms the fact that after prolonged periods of denervation the frog sartorius muscle becomes sensitive everywhere to ach, and as also reported by Kuffler and by Miledi, that a differential sensitivity between endplate and more remote areas persists for at least several months.

Summary. A microbioassay for acetylcholine is described in which a solution containing acetylcholine is microperfused onto the endplate area of a chronically denervated skeletal muscle fiber. The endpoint, a brief tetanus, can be produced by application of as little as 0.15 μl of a solution which contains acetylcholine chloride at a concentration of 2.5 $\times$ 10^{-8} moles/l.

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