Fluorometric Measurement of Pyridine Nucleotide Reduction in the Giant Axon of the Squid

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ABSTRACT By monitoring the fluorescence of the isolated giant axon of the squid *Loligo pealei*, it was possible to follow changes in its oxidation-reduction state as caused by the action of anoxia, cyanide, Amytal, and azide. The response to oxygen depletion was very rapid, the NAD within the axon being 90% reduced within 1–2 min. Cyanide and Amytal gave essentially similar results, although somewhat longer periods of time elapsed during their onset and washout periods. The extent of NAD reduction was essentially the same under conditions of anoxia and treatment with cyanide and Amytal. Azide was less effective in this respect, and at comparatively high levels of concentration (25–50 mM) gave values of 40% or less of the reduction observed with the other inhibitors. The application of ouabain and strophanthidin gave no observable NAD reduction. Variations in the time required to consume given quantities of dissolved oxygen before and after stimulation indicated an increase of 10–20% in oxygen uptake rate associated with activity, although this figure appeared to be a function of the surface-to-volume ratio of the axon. A biochemical analysis of axoplasm for oxidized and reduced pyridine nucleotide was made. Fluorometric examination of centrifuged axoplasm indicated that the NAD-NADH was largely confined to the mitochondria of the axon.

INTRODUCTION

The giant axon of the squid has proven to be a valuable experimental preparation for studies of conduction properties of nerve and investigation of ion transport characteristics of the cellular membrane. The physical fact of the large size of the nerve and the comparative ease in isolating the axon and in its preparation for experimentation have been the major reasons for the popularity of this tissue. Despite the abundance of reports in the literature concerning studies utilizing the giant axon of the squid, investigations of the ion transport and electrical properties of the preparation make up the bulk of published information. Relatively little work has been reported concerning

the metabolism of squid nerve in contrast with the large amount of published data pertaining to conduction and ion transport characteristics so that information relating to the respiratory-linked oxidative metabolism of the axon may be of interest. To this end, the fluorometric method was utilized to follow the kinetics of the changes in the oxidation-reduction state of pyridine nucleotide within the axon, as affected by anoxia and the presence of several metabolic inhibitors.

The success of the microfluorometric technique in investigating intracellular redox states is well documented in the literature. The method depends upon the fluorescent properties of nicotinamide adenine dinucleotide (NAD, DPN, diphosphopyridine nucleotide) as described by Warburg (1948). It is only in its reduced form (NADH, DPNH) that the nucleotide exhibits its strong fluorescence. This property was employed by Theorell (1948) and Boyer and Theorell (1956) in studies of the liver alcohol dehydrogenase system. Duysens and Amesz (1957) and Duysens and Kronenberg (1957) reported that the emission peak of the NADH was shifted toward shorter wavelengths as compared to a solution of the nucleotide. It was suggested that the emission spectrum shift was due to binding of NADH. The magnitude of the shift is regarded as an indication of the degree of binding of the reduced nucleotide. In 1958, Chance and Baltscheffsky utilized the fluorometric technique to study the kinetics of pyridine nucleotide reduction in isolated rat liver mitochondria; in this case, the large amount of spectral shift indicated a high degree of binding of the NADH. Shortly thereafter, Chance and Legallais (1959) and Chance, Cohen, Jöbsis, and Schoener (1962) observed the redox changes of pyridine nucleotide in cells and tissues, in which it had been earlier demonstrated that the blue fluorescence which had long been associated with living tissues was due to a large extent to the presence of NADH (Chance and Jöbsis, 1959). The triphosphonucleotide, NADPH (nicotinamide adenine dinucleotide phosphate, TPNH, triphosphopyridine nucleotide), also has a fluorescence emission spectrum identical to that of the NADH, but in most cases, its concentration in cells is significantly lower than the NADH so that its interference can be neglected.

The position of NAD-NADH on the main pathway of oxidative transfer in the respiratory chain allows measurements of its redox state to serve as an accurate indicator of intracellular redox conditions. Recent studies by Chance, Schoener, and Schindler (1964) and Chance (1965) suggest that the change of fluorescence of tissues and cells is perhaps the most sensitive and meaningful indicator of true intracellular oxidation states, having more sensitivity than oxygen electrode techniques or hemoglobin dissociation methods.

METHODS

The purpose of the experimental apparatus was to measure and record the fluorescence emission intensity of isolated squid giant axons and indicate the changes in

fluorescence intensity which accompany the application of various metabolic inhibitors to an axon. The principle of the measurement is the alteration of the intensity of NADH fluorescence as it is made to undergo oxidation-reduction cycles, with a shift in the equilibrium between NAD and NADH.

Microfluorometer Instrumentation A Leitz-type SM microscope was modified, with all substage components and mountings removed. A General Electric type AH-6 1000 w water-cooled mercury arc lamp was located beneath the microscope stage, with a filter-condenser system between the lamp and the stage. The filters served to isolate the 366 mμ line of mercury, and consisted of two Corning type 7-39 heat-resistant glass filters and a single narrow-bandpass (8 mμ transmission half-width) filter supplied by Baird Atomic, Inc., Cambridge, Mass. This latter filter was of the interference type, with its peak transmission of 47% centered at 366 mμ. The lamp, housing, condenser-filter system, and microscope frame were held in rigid alignment on a heavy metal plate. The axon preparation was contained within a Pyrex glass chamber, as described below. Immediately above the axon chamber, and in contact with it, was the main barrier filter. This filter, also a glass-based interference type made by Baird-Atomic Co., excluded all ultraviolet-exciting radiation from the microscope and detection system, but allowed the fluorescence emission wavelengths to pass in a rather broad band between 410 and 510 mμ. The fluorescence emission was peaked at 466 mμ, in the center of this band. It was found that placement of the barrier filter between the microscope objective and the axon significantly lowered the background light level caused by the slight fluorescence of the microscope optics. The degradation of the microscope image was negligible for the purposes at hand. By excluding all ultraviolet radiation from the microscope, the background fluorescence level was significantly reduced.

The microscope objective was a × 10, dry, Leitz, with a nominal field of view of approximately 550 μ at a working distance of 7 mm. The usual barrel on the microscope was replaced by a trinocular type, the binocular portion used for viewing purposes, and the third aperture for the photomultiplier detector. A sliding prism within the barrel allowed the image to be shifted from the viewing apertures to the photomultiplier position. A second filter, Wratten type A2, was positioned immediately before the photomultiplier, and blocked transmission of wavelengths below 410 mμ. This gave additional insurance that all 366 mμ radiation was excluded from the detector.

The photomultiplier was an RCA type 1P28, with a nominal S-4 spectral sensitivity function, peaked near 400 mμ wavelength. High voltage DC was supplied to the detector by a Fluke Model 412A power supply (Fluke Manufacturing Co., Inc., Seattle, Wash.); a typical dynode supply voltage was between 750–850 v. The low-current signals generated by the photomultiplier were fed into a Keithley Model 603 electrometer amplifier (Keithley Instruments, Cleveland, Ohio). The sensitivity of the amplifier was such that detector currents of 0.5 μamp gave full-scale deflection of the recording system. A Heath strip-chart recorder was used to obtain permanent records of the fluorescence changes. In order to reduce the small, rapid fluctuations introduced by thermal and shot noise of the detector, a shunt capacitance of 0.005 μf was used; this gave an effective time constant of response for the system of 0.5 sec. A schematic outline of the experimental system is shown in Fig. 1. The use of an

opaque mask in the backfocus of the microscope objective served to exclude all portions of the image which did not correspond to the axon itself. In this manner, all fluorescence as seen by the detector originated from the axon.

Axon Chamber The axon was contained within a clear Pyrex glass block. It was necessary to construct the chamber of glass rather than more workable materials such as Plexiglass or Lucite because of the inherent fluorescence of such plastics. This block was ¼ inch thick, ¾ inch wide, and 2¾ inches in length. A flat-bottomed groove, or trough, was cut into the block, this being 1 mm wide and slightly less than this in

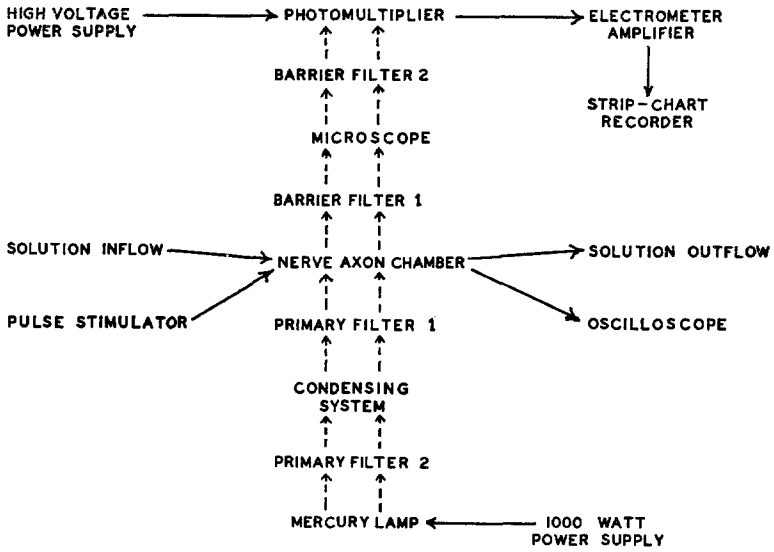

FIGURE 1. Relationship of components of fluorometer. Excitation of fluorescence by 1000 w mercury arc lamp, with isolation of 366 mμ wavelength line by primary filters 1 and 2. Axon is held in glass chamber on microscope stage, with ultraviolet radiation incident from below. Fluorescence emission band centered near 465 mμ, passing upward through barrier filter 1, microscope objective, and barrier filter 2, to the photomultiplier detector. Output signal from detector is amplified and recorded.

depth, extending to within ⅜ inch of each end of the block. A small hole was drilled into each end of the block, below and parallel to the surface, so as to intercept the ends of the trough, as shown in Fig. 2. Stainless steel tubing was cemented into these holes, which allowed for the passage of solution into one tube, down the length of the trough, and out the other tube, the fluid never contacting the surface of the block. Platinum wire electrodes were inserted into similar holes in one side of the block, these being parallel to the surface and 1 mm beneath it, intercepting the trough near each end and perpendicular to the long dimension of the trough. These electrodes contacted the axon, which lay upon them in the groove, and in this manner the nerve could be stimulated at any time during the course of an experiment while it was sealed in the chamber. The transmitted impulse was picked up by the pair of record-

ing electrodes and displayed on an oscilloscope. A Tektronix waveform generator and pulse generator were used for purposes of stimulation.

After the axon was placed into the trough, the top surface of the chamber was lightly greased and a large rectangular cover glass placed over the entire block. This thin glass completely covered the trough and sealed it from the air and from fluid leakage. Syringe pumps were used to force solutions through the chamber, the flow being from one end of the trough to the other. The usual flow rate was between 0.8

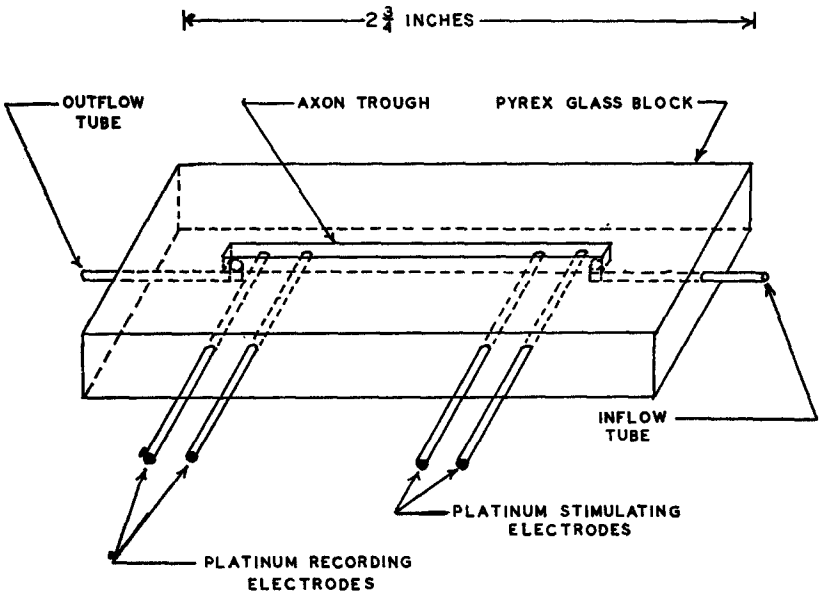

FIGURE 2. Details of axon chamber. Constructed of a single Pyrex glass block. Flat-bottomed trough in center of block, 1 mm square in cross-section, with inflow-outflow connection at ends of trough. Platinum electrodes contact bottom of trough, for stimulation and recording of nerve activity.

and 1.0 ml/min. The total volume of the trough was slightly more than 50 μl, giving nearly 40 volume changes per min. The entire axon chamber could be positioned by the microscope stage manipulator in such a manner as to place a selected length of the axon in the field of view of the instrument. This allowed the choice of large, well-cleaned areas of the axon to be used, and also provided for the comparison of one area of the axon with another. Since the entire chamber was constructed of a single block of glass, any point along the 2 inch length of axon in the trough was accessible for measurement, except the four points where the opacity of the electrodes interfered with the excitation radiation.

The microfluorometer was very sensitive and quite stable over short periods of time. There was, however, a constant and gradual decrease in base line photomultiplier current, which became evident over longer periods of measurement. This took the form of a linear decrease in signal level, over periods of 30 min or more; this was

thought to be the result of a gradual discoloration of the quartz lamp envelope and aging of the lamp. The linearity of the fluorescence emission intensity as a function of known concentrations of NADH in solution was very good for levels of NADH below 135 μM. It was found, however, that the intensity vs. concentration function, while linear, extrapolated back to a NADH concentration less than zero (see Fig. 3). This may have been brought about by the high absorbance of NADH to the excitation wavelength of 366 mμ. Thus, as the concentration of NADH was increased, the absolute fluorescence intensity increased also, but not proportionately, due to the increased absorption of the exciting radiation at the higher concentrations. Less of the ultraviolet reached the upper layers of solution, which was the very region most effective in emitting fluorescence to the detector. These measurements of NADH

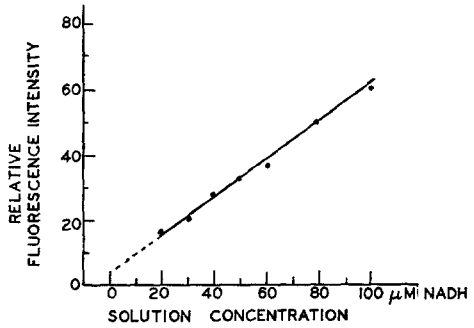

FIGURE 3. Fluorescence emission intensity as a function of known concentration of NADH in solution. The solutions were contained in glass capillary tubes of approximately the same internal diameter as a squid axon, and measurements taken with the microfluorometer as used with actual axons.

solutions were made using a glass capillary 520 μ in inside diameter and filled with the various concentrations of NADH solution. In this manner, the cylinder of solution within the capillary was approximately the same size and configuration as an actual squid axon, and therefore closely represented the conditions of actual usage of the instrument during an experiment. In the case of an actual axon, there is the additional effect of scattering of light by the tissue components, contributing to an additional loss of fluorescence emission intensity.

Squid Axon Preparation The nerve preparation used throughout the whole of the experiments to be described was taken from fresh, live squid of the species *Loligo pealei*. The animal was decapitated and the pair of giant axons removed immediately from the mantle under flowing fresh seawater. The axons were carefully cleaned of all observable connective tissue, using a low-powered dissecting microscope. The total operation required approximately 30–40 min per axon. The average axon size was between 450–550 μ in diameter and 7–10 cm in over-all length. After cleaning, the axon consisted essentially of a cylinder, with the viscous, gel-like axoplasm filling the interior. The plasma membrane and its associated Schwann cell layer made up the walls of the cylinder, along with a thin layer of connective tissue which inevitably

remains after cleaning. The axons were kept in cold seawater until the time of actual use, and were checked for excitability before placing them in the trough of the axon chamber.

The ends of the axon were tied with fine thread to facilitate handling and prevent the loss of axoplasm and entry of seawater, although effects of the latter would have been minimal even without the tie. Once within the chamber, the cover glass was sealed over it and the flow of solution through the chamber was initiated. All solutions were made up in artificial seawater, since this had a considerably lower fluorescence level than natural seawater, even after filtering. The formula for the artificial seawater was the standard type used at the Marine Biological Laboratory. All solutions were used at room temperature (21–23°C). The solutions were forced through the chamber by several motor-driven syringe pumps. Each pump had its syringe of solution connected to a common manifold through a length of small-bore plastic tubing. This manifold was located as close as possible to the chamber, and had a single outflow tube connecting with the axon chamber. This short length of tubing common to all solutions minimized the "dead space" between changes of solution concentration or composition.

It was found important to purge the entire "plumbing" of air bubbles before starting an experiment. This prevented the bubbles from being trapped in the system and later entering the chamber, with rather drastic effects upon the observed fluorescence level as they entered the field of view of the microscope. Solution changes were readily brought about by activating the appropriate syringe pump, and in this manner the system was always kept sealed against the entry of air. When the solution flow was started, the excitability of the axon was again checked, and if satisfactory, the experiment was initiated.

Biochemical Assay for NAD, NADH, NADP, and NADPH The oxidized and reduced forms of pyridine nucleotide are in a state of dynamic equilibrium within the cell, the ratio between the two forms being a function of the respiratory state. Under normal respiring conditions, the equilibrium between reduced and oxidized forms invariably favors the oxidized form of NAD. There are no values in the literature for the pyridine nucleotide levels in squid nerve, and since the fluorometric method is not ideally suited for absolute concentration measurements, it was necessary to make an analysis of the axoplasm for the pyridine nucleotides present. It was expected that the concentrations of NAD and NADH would be quite low, as indicated by the rather small oxygen uptake rate of the axon, and the sparse distribution of mitochondria throughout the axoplasm.

The analysis is complicated by the low concentrations involved and particularly by the small sample volumes with which one has to work. A typical sample of axoplasm from a pair of axons would be on the order of 15 μl. The axoplasm was drawn up into small glass capillaries after extrusion, and kept at −80°C until the time of the analysis. At that time, the axoplasm was blown into a small tube of liquid nitrogen and pulverized using a glass rod. The method of extraction for the nucleotides closely followed the procedure of Klingenberg (1965). After independent extraction of NAD, NADH, NADP, and NADPH samples, an enzymatic cycling technique was used for

the analysis, as described by Lowry et al. (1961). The method is based upon the cycling of the nucleotides between their oxidized and reduced forms.

RESULTS

The Effects of Anoxia In order to induce anoxia in the squid axon, high-purity nitrogen gas was bubbled through artificial seawater to exchange for the dissolved oxygen in the water. The nitrogenating flask was sealed to the air and the gas forced through the seawater and out of the flask by 20 lb/

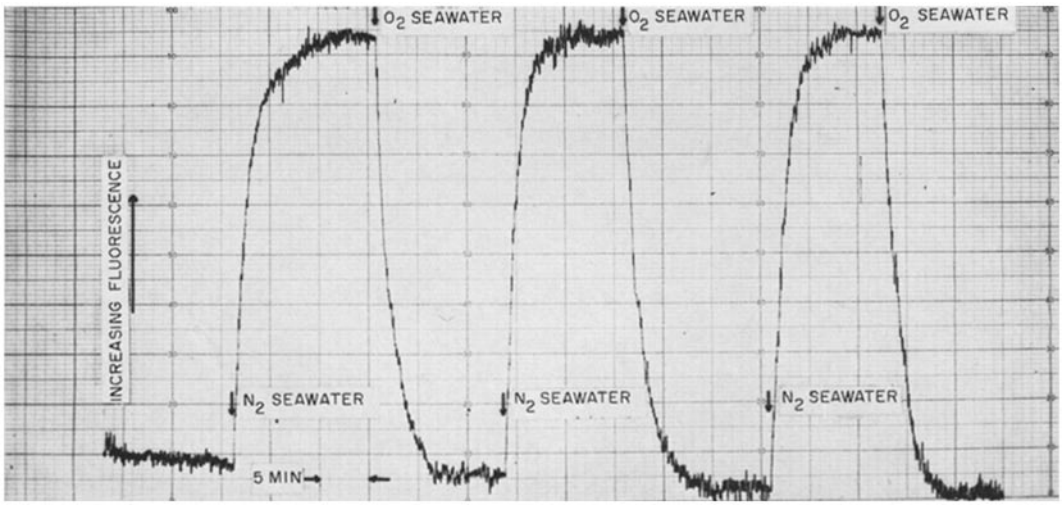

FIGURE 4. Fluorescence increase due to anoxia. At the points indicated, nitrogenated seawater was substituted for normal, oxygen-saturated seawater, the solution flowing at all times. After stabilization of fluorescence level, oxygenated seawater was resumed, with a drop in fluorescence intensity to near original level. Three such oxygen-nitrogen-oxygen cycles are shown.

inch^2 positive pressure. The outlet from the flask was restricted by a clamp. As expected, virtually all oxygen had to be removed from the seawater in order to see a response of NAD reduction. The "high-purity" grade of nitrogen had a nominal purity of 99.9%, but still contained sufficient residual oxygen to cause difficulty in eliciting an immediate anoxic effect. It was found necessary to use "prepurified grade" nitrogen, which had an oxygen content not exceeding eight ppm. Several days of nitrogen treatment usually served to reduce the partial pressure of oxygen in the water to adequately low levels.

The response to the deoxygenated seawater was rapid, the initial response usually being evident within a few seconds, and reaching a peak in 5–7 min. The rise in fluorescence was of an exponential form, with a time constant of approximately 2.5 min. A typical response is shown in Fig. 4, which is a recorder tracing of the fluorescence increase of the axon as the nitrogenated

solution reached the nerve. The time to peak and the time course of the last stages of NAD reduction were somewhat variable according to the size of the axon, its "age" from dissection, and presumably, the amount of residual oxygen remaining in the water. It was always possible to demonstrate an indefinite number of oxygen-anoxic cycles, three of which are shown in Fig. 4. Upon readmission of normal, oxygenated seawater, the NADH becomes rapidly oxidized, reaching the initial state within 5–6 min.

The rate of oxygen consumption by the squid axon has been reported by Connelly (1952) and Connelly and Cranefield (1953) as being 70 μl/g/hr for the resting nerve. In terms of a typical axon as used here, weighing 10 mg, this corresponds to 0.012 μl/min. A water solution in air can be expected to contain about 0.6% oxygen by volume at saturation. The axon chamber volume was somewhat less than 60 μl, giving the oxygen volume in a saturated solution within the chamber as 0.36 μl. An axon consuming 0.012 μl/min would then require a minimum of 30 min to exhaust the oxygen within the chamber, assuming that the oxygen consumption rate is linear all the way to zero concentration.

The immediate response to oxygen-free conditions has been shown in Fig. 4. A second type of response was observed when the oxygen level in the solution was greatly reduced from saturation, but of sufficient amount to support the respiration of the axon as long as the partially deoxygenated solution was kept flowing through the chamber. As soon as the flow was stopped, a rise in fluorescence was observed, indicating that the level of oxygen in the water was close to the critical concentration at which it becomes rate-limiting. When the solution was stopped, no oxygen was being brought into the chamber by fresh solution, and the axon rapidly exhausted that which was present. An example of such a response is shown in Fig. 5 A, where a change in fluorescence intensity is seen only upon interruption of the flow. The oxygen becomes rate-limiting within a few seconds after the flow is stopped.

With higher concentrations of oxygen in the water (as obtained with the less pure grade of nitrogen) a response is seen similar to that in the last case, but the lag time from flow interruption until the initial fluorescence response is considerably longer. Delay times between 3 and 8 min were seen; a typical case is shown in Fig. 5 B. The differences in lag time clearly represent the times required to consume the residual oxygen in the water, and reflect the variation in oxygen concentrations and in respiration rate between axons.

The Effect of Stimulation It has been reported by Connelly (1952, 1962) that the oxygen uptake rate of nerve is increased by stimulation, this increase being on the order of 10% or more of the resting rate. Several experiments were undertaken to determine whether such an effect could be observed in terms of NAD reduction. Clearly, the additional oxygen uptake associated with stimulation would not have a significant effect upon an axon bathed in a

highly oxygenated solution, since the increased "load" upon the system is not a large percentage of the normal respiratory rate. If, however, most of the oxygen is removed from the solution beforehand, under stopped-flow conditions there should be a measurable difference in the time required to consume the remaining oxygen in the sealed chamber. The results of such an experiment are shown in Fig. 6. It is seen that under conditions of stopped flow,

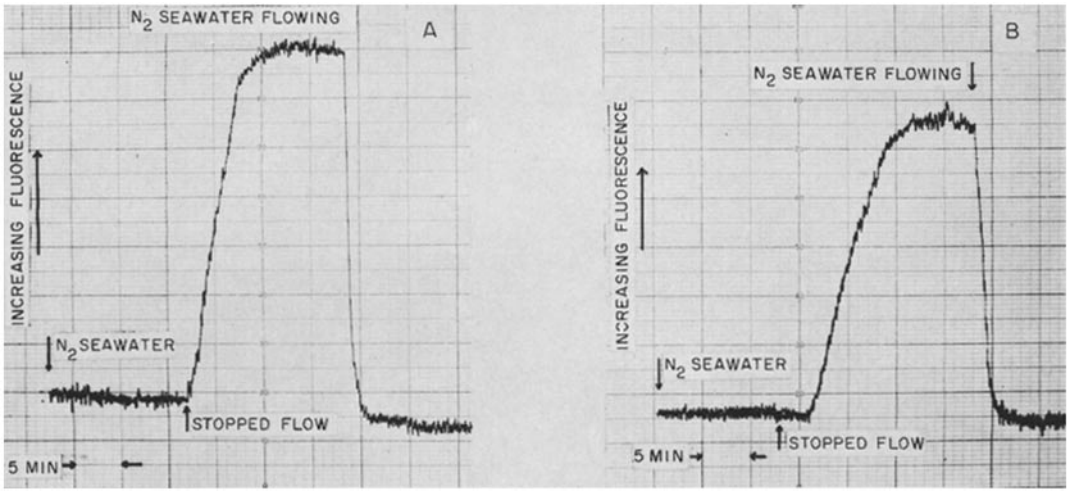

FIGURE 5 A. Fluorescence increase under stopped-flow conditions. In this case, the oxygen concentration in the flowing seawater bathing the axon was near the rate-limiting value for normal respiration. Within seconds of stoppage of flow, the consumption of the remaining oxygen by the respiration of the nerve causes a transfer to conditions of anoxia. Upon resumption of flow, the new supply of oxygen returns the respiratory state of the axon to its original conditions.

FIGURE 5 B. As in 5 A, but with a slightly higher concentration of oxygen in the seawater. Upon stopped-flow, several minutes are required for the nerve to consume the residual oxygen in the water.

with no stimulation, the axon required approximately 14 min until NAD reduction became evident. The seawater was then set flowing again, until the base line fluorescence level was the same as at the start of the experiment. At that time, the axon was stimulated for a period of 5 min at a rate of 200 impulses/sec. Concurrent with the start of stimulation, the flow was interrupted. Under conditions of stimulation, the lag time between flow stoppage and the start of NAD reduction was seen to be 11 min, or approximately 20% faster than was the case before stimulation. To ensure that this was not due to a change in the respiratory capability of the axon, the first cycle was repeated, with no stimulation. There was a slight increase in lag time, to about 15 min, probably due to deterioration of the nerve after nearly 2 hr in the chamber. While absolute oxygen consumption rates were not determined, the

increase in respiration brought about by stimulation, as indicated by decrease in time required to consume the oxygen in a given solution, was always between 15–20% for the several experiments of this type which were performed. This is quite consistent with the data of Connelly (1952) and Connelly and Cranefield (1953).

The Effect of Cyanide Solutions of cyanide seawater were flowed through the axon chamber and their effect upon NAD reduction recorded. Fig. 7 shows such a recorder tracing, where the responses to anoxia and to cyanide

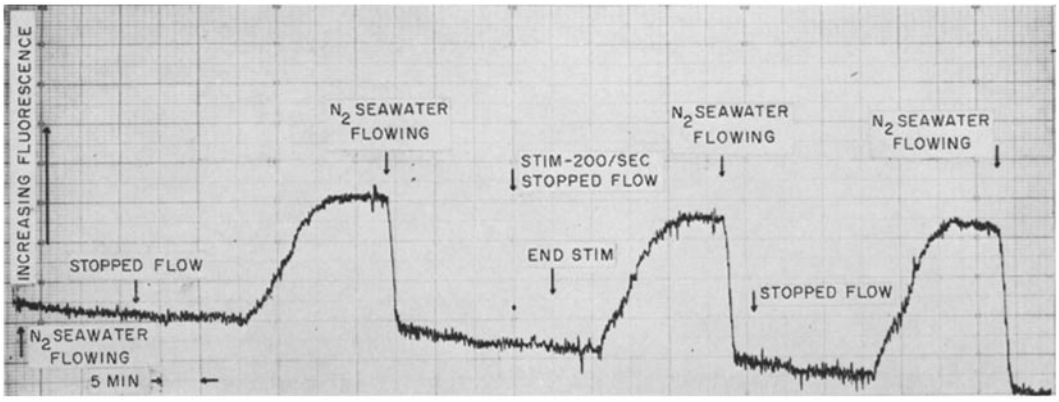

FIGURE 6. Stimulation effects upon respiration rate. Partially deoxygenated seawater was flowed through the axon chamber, then stopped at the point indicated on the left portion of the trace. After 14 min, the remaining oxygen was consumed, resulting in a rise in NADH fluorescence as the axon became anoxic. Resumption of solution flow returned the axon to its initial redox state. The flow was again stopped, and the axon stimulated at 200/sec for 5 min. The time to consume the dissolved oxygen was shortened to 11 min in this case. A repeat of the cycle without stimulation gave a time of 15 min for oxygen consumption, a lengthening of time of 1 min over the initial rate, probably due to slight decrease in respiratory ability of the axon over the 2.5 hr experimental period.

are compared on the same axon. The first peak in fluorescence is that due to anoxia; the second peak is the response to cyanide treatment, at a concentration of 1 mM. A period of normal, oxygen-saturated solution separates the two peaks. The inhibition of respiration by cyanide, as reflected by the reduction of NAD, is completely reversible upon flushing the cyanide solution from the chamber with fresh seawater. Many such cycles were carried out, with apparent complete recovery of respiratory ability. The responses to anoxia and cyanide were essentially identical, with a slightly slower recovery from cyanide in its later stages.

A series of experiments was performed to determine the concentration-dependence of cyanide inhibition of NADH oxidation. Although cyanide concentrations on the order of 2 mM are commonly used in blocking respira-

tory systems, its effect can readily be seen at much lower levels. A series of five concentrations of cyanide seawater was used, each on the same axon, with washout periods of normal seawater between each cyanide application. The relative fluorescence peak height, representing NAD reduction, as a function of cyanide concentration, is given in Table I. The inhibitory effect

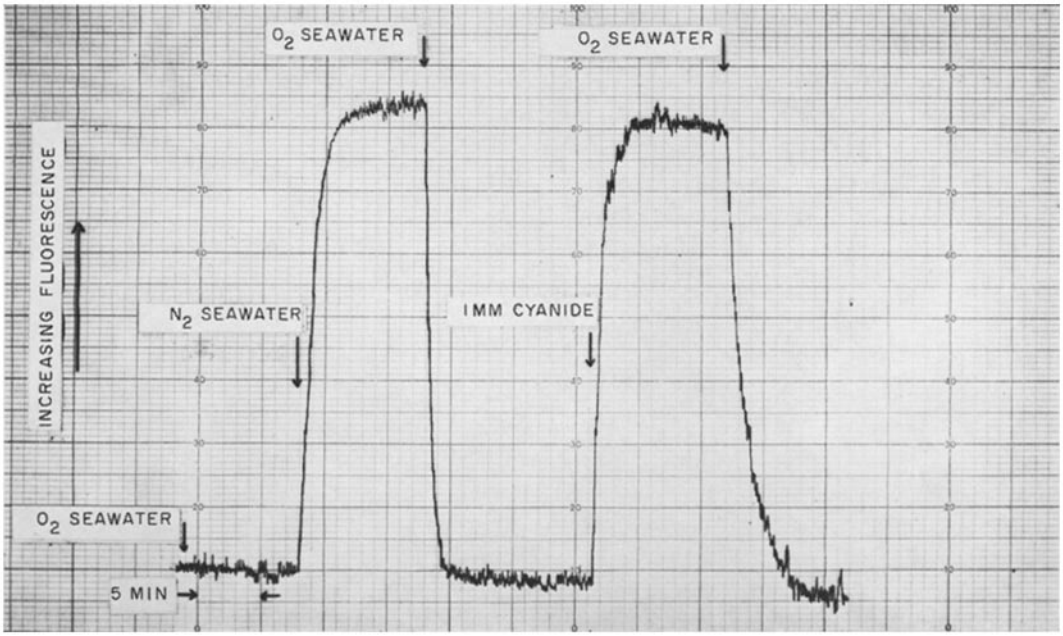

FIGURE 7. Comparison of anoxia- and cyanide-induced NAD reduction. The fluorescence peak on the left is due to NAD reduction in the axon upon transition to nitrogenated seawater, and represents essentially complete anoxia. After returning to normal oxygen-saturated seawater, a 1 mM cyanide seawater solution was flowed through the chamber, resulting in the fluorescence peak on the right. The degree of NAD reduction in each case is the same.

is largely complete at 100 μM, with slight further action at 0.5 mM, by which time saturation of effect occurs.

The Effect of Amytal Amytal (amobarbital) is known to be an effective inhibitor of NAD-linked respiration (Eiler and McEwan, 1949; Ernster et al., 1955), apparently blocking oxidation of NADH by flavoprotein in a phosphorylating system (Jowett and Quastel, 1937; Jalling et al., 1955). In the present study, Amytal-seawater solutions were flowed through the axon chamber and the changes in NAD reduction recorded, as before. A quick response was seen (within 10 sec), very similar to the action of cyanide, the most notable difference being a consistently longer period of washout required for reversibility. Complete recovery from cyanide occurred within 4–6

min, with half-complete recovery in 1.5 min; recovery from Amytal usually required 6–8 min, with recovery half-complete within 2 min. A concentration-dependence determination was made, as described previously for cyanide, and the results are summarized in Table I. A concentration of 5 mM Amytal gave a saturation of effect, considerably higher than that required for cyanide (100 μM).

The Effect of Azide Sodium azide is known to have a variety of actions upon respiring and phosphorylating systems. It was applied to the axon in the same manner as the inhibitors already described, and the concentration-dependence of its reduction of NAD is shown in Table I. It is seen that com-

TABLE I

CONCENTRATION-DEPENDENCE OF INHIBITOR-INDUCED NAD REDUCTION

Inhibitor concentration	Relative NAD reduction*		
	Cyanide	Amytal	Azide
mM			
0.05	0.56		
0.10	0.94		
0.50	1.0	0.22	
1.0	1.0	0.72	0.10
5.0	0.98	0.94	0.37
10.0		1.0	0.39
25.0		0.99	0.40

* Arbitrary units of NAD reduction taken as 1.00 unit = extent of reduction under total anoxia.

paratively high levels of azide (near 10 mM) were required to achieve saturation of effect. Also, the washout times were between 9–13 min. This may reflect a stronger binding of azide to its site of action, since the longer reversal time cannot be explained on the basis of molecular size and expected diffusion times.

The action of azide as an inhibitor of respiring systems is known to be highly pH-dependent. Keilin (1936) observed that in an active yeast system, azide shows a much greater effect in acidic solutions than at higher pH values. The presumed reason for this is that it is in its undissociated form that the drug readily penetrates biological membranes, and this is favored at low pH. In seawater, with a pH near 7.8–8.0, the azide is highly ionized as N_3^-, and thus the effective concentration of its permeable form is greatly reduced. This may to some extent explain the significantly higher concentrations of azide which are required to observe an effect. The relative slowness of washout of azide may also depend in part upon ionization factors, although in the axoplasm's pH of 7.1 (Caldwell, 1958) there will be less ionization than in the external

seawater solution. Nevertheless, with a pK of 4.6 for hydrazoic acid, the drug will remain in a largely ionized form.

Relative Effects of Cyanide, Amytal, and Azide Of the three inhibitors discussed above, the most significant difference was in the degree of NAD reduction brought about by azide as compared to cyanide and Amytal. The

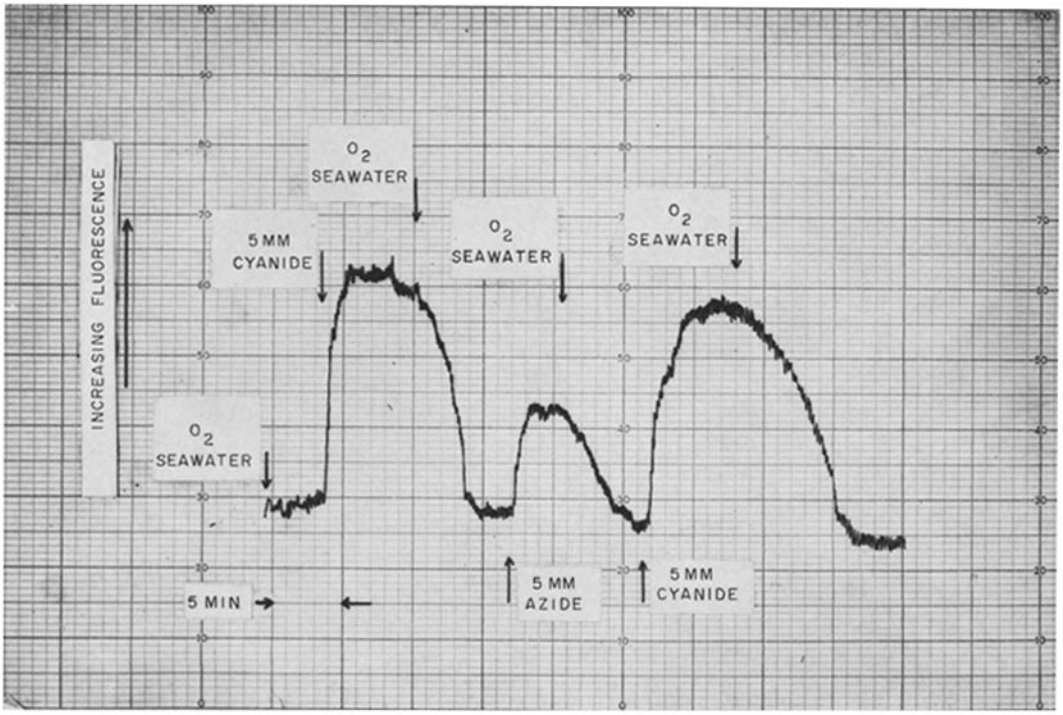

FIGURE 8. Comparison of cyanide- and azide-induced NAD reduction. First and last fluorescence peaks due to action of 5 mM cyanide solution upon NAD reduction. Center peak due to 5 mM azide. Effect of azide upon NAD reduction approximately 35–40% that of cyanide, even at much higher concentrations.

latter drugs were essentially identical in the degree of fluorescence response induced, and much the same as anoxia in this respect. Azide, however, consistently caused much less reduction of NAD, usually between 35–40% of the value for the other inhibitors. A comparison of the effect of azide and cyanide upon NAD reduction is shown in Fig. 8. The concentration of both drugs was 5 mM. The cyanide was applied first, washed out with seawater, and azide then introduced into the chamber. After the azide cycle, cyanide was again applied to check on the repeatability of the response. Both cyanide responses are much the same height (allowing for the gradual drop in fluorescence base line) while the effect of azide was only about 40% as great. This

indicates a far from complete blockage of respiration, and this fact was true even at concentrations as high as 50 mM.

The Effects of Ouabain and Strophanthidin The cardiac glycosides, ouabain and strophanthidin, are known to interfere with the sodium pump of the membrane, presumably by blocking the hydrolysis of the ATP which supports this energy-requiring process. Such interruption of the "transport ATPase" brings about a rapid decrease in sodium transport upon application to the outside of the axon; no effect is observed upon injection of the glycosides within the axoplasm of the nerve (Caldwell and Keynes, 1959). Several experiments were undertaken to determine what effect these inhibitors would have upon the NAD-NADH equilibrium of the axon. In every case, no significant change in NAD redox state was seen, indicating that the change in respiratory rate brought about by the decrease in load due to pump inactivation was insufficient to change the NADH oxidation rate.

The Effect of Succinate The reduction of NAD through the action of succinate in a respiring system is well-established. In a succinate-rich environment, succinate is preferentially oxidized over substrates requiring a pyridine-linked dehydrogenase (Krebs et al., 1961; Kulka et al., 1961). Also, reversed electron transport, from succinate to NAD, at the expense of ATP, has been reported by many investigators (Chance, 1960; Klingenberg et al., 1959). Since succinate does not readily penetrate the axon membrane, it was necessary to microinject the material directly into the axon. The injection was made down the central axis of the axon from one end, using the procedure of Brinley and Mullins (1965). A centimeter or so of axon length was injected, with careful note being made of the location of the injected region. The fluorescence intensity of the succinate-injected region was then compared to that of the axoplasm on either side of the succinate location. The resulting fluorometer trace is given in Fig. 9. A definite reduction of NAD takes place in the succinate region. These measurements were made with no respiratory inhibitor present in the axon.

Localization of Fluorescence It was not possible to localize the fluorescence emission of intact axons to any of the subcellular compartments, these being well beyond the resolution of the microfluorometer. In an effort to ascertain whether the emission was associated with the cytoplasmic or particulate portion of the axon, samples of axoplasm were carefully extruded from the axon sheath, using the method described previously by Brinley and Mullins (1965). In this manner, it is possible to obtain samples of nerve axoplasm which are free from membrane components and other contaminants. A typical axon extrusion would net about 6–7 μl of the rather viscous axoplasm. This was drawn up into a small glass capillary tube and the end sealed. The interior bore of the capillary was such that 6.6 μl of axoplasm was con-

tained in 1 cm of its length. By combining the "squeezes" from a pair of axons, nearly 2 cm length of filled capillary could be obtained. This was then centrifuged at 30,000–50,000 *g* for 4–6 hr, at 5°C. At the end of that time, the sample of axoplasm was sharply divided into two portions, the major one being in the form of a clear supernatant. The remainder was a cloudy sediment at the tip of the capillary, and represented perhaps 5–8% of the total volume.

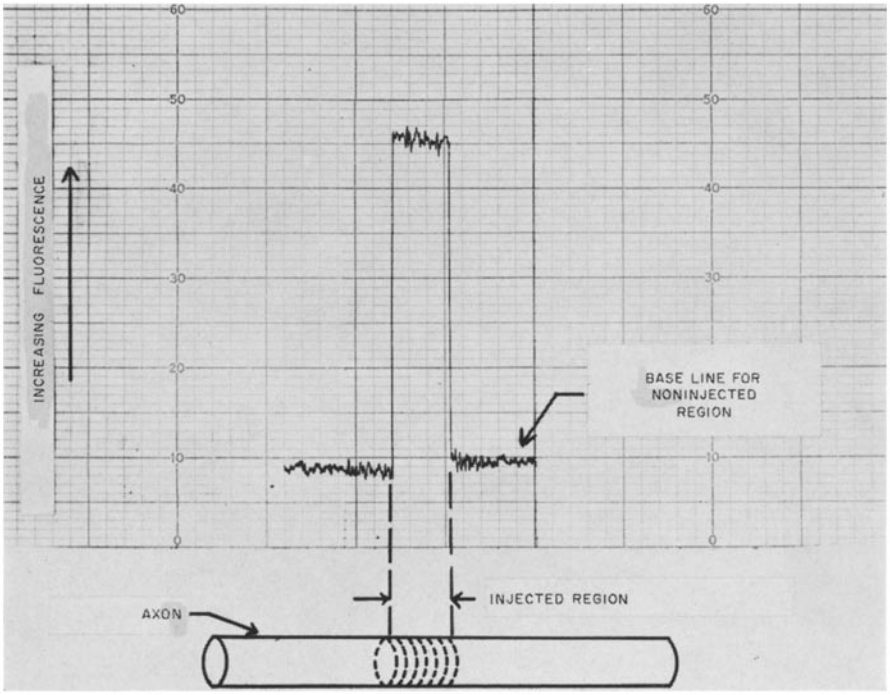

FIGURE 9. Effect of succinate upon NAD reduction. Succinate was injected into center region of axon, with consequent increase in fluorescence of the injected area, due to NAD reduction. Injection was made through a microcapillary inserted down the center of the axon from one end. Recorder tracing compares fluorescence of axon regions on each side of injected portion of the nerve with that in center of succinate-injected region.

Electron micrographs of the sediment and supernatant confirmed that all the mitochondria and other particulate portions of the axoplasm were present in the sediment. Many of the mitochondria were partially broken or damaged by the procedure, probably by the fixing method employed. Since virtually all the axon's mitochondria were present in the sediment, a measure of the fluorescence of this material as compared with the supernatant offers an indication of the relative bound NADH concentrations in the mitochondrial and cytoplasmic compartments, respectively. A measurement of fluorescence intensity as a function of the distance from the bottom of the centrifuged capil-

lary was made. Fig. 10 illustrates that nearly all the fluorescence emission is from the sediment, indicating that most of the bound NADH within the axon is associated with the particulate portion, presumably the mitochondria. This fact is important when considering the relative roles of glycolytic and aerobic metabolism in the nerve.

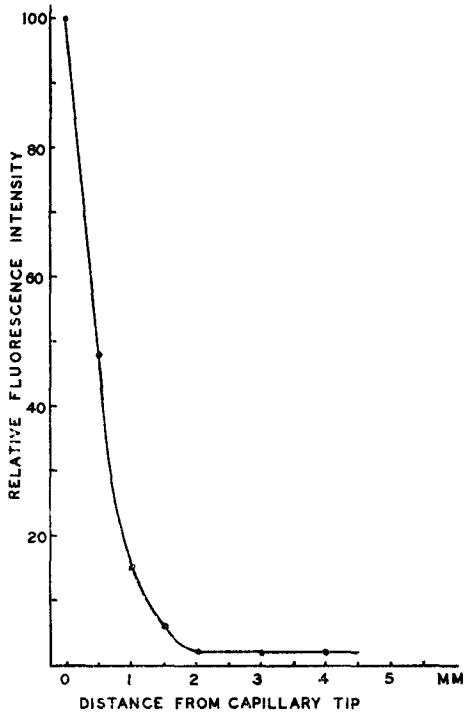

FIGURE 10. Fluorescence of centrifuged axoplasm. Squid axoplasm was extruded from an axon and drawn up into a microcapillary. This was centrifuged and the result was a sharp division between sediment and clear supernatant. The fluorescence intensity as a function of distance from the bottom of the sealed capillary is shown, indicating that nearly all the NADH is present in the sediment at the bottom of the capillary. Field of view of fluorometer was 0.55 mm of capillary length.

The much greater fluorescence emission of the sediment of the axoplasm (and therefore presumably from the mitochondria) than from the supernatant (cytoplasm) does not imply a corresponding distribution of NADH concentration. Mitochondrial NADH is known to show a greater degree of fluorescence enhancement than NADH in cytoplasm or aqueous solution. The magnitude of this enhancement has been given as between 5- and 15-fold (Estabrook, 1962; Avi-Dor et al., 1962). In a recent study, Jöbsis and Duffield (1967) have reported that in toad muscle, the fluorescence of mitochondrial NADH is enhanced about tenfold in comparison with cytoplasmic NADH.

Due to this fluorescence enhancement difference between cytoplasmic and mitochondrial NADH, quantitative values cannot be determined from fluorescence measurements alone. It appears safe to say, however, that the major portion of the NADH in axoplasm is associated with the mitochondria. Assuming a fluorescence enhancement factor of 10, with the fluorescence of the sedimented axoplasm seen to be more than 50 times that of the cytoplasm, a considerable excess of the reduced nucleotide in the mitochondria is indicated even taking relative fluorescence enhancement factors into account. A biochemical analysis of the upper and lower portions of the centrifuged axoplasm would be useful, but was not performed at the time.

Fluorescence Emission Spectra Studies by many workers have shown that the binding of NADH to other substances causes a shift in the location of the spectral emission peak (Boyer and Theorell, 1956; Chance and Baltscheffsky, 1958; Chance et al., 1962; Duysens and Amesz, 1957; Duysens and Kronenberg, 1957). The shift is always toward shorter wavelengths, as compared to unbound NADH in solution. Chance and Baltscheffsky (1958) have suggested that the binding of NADH in biological systems may be with (*a*) the dehydrogenases of the Krebs cycle, which supplies the NAD-linked substrates; (*b*) the dehydrogenases of the flavins associated with the reduction of NAD; or (*c*) a cofactor of oxidative phosphorylation involved, perhaps, with the respiratory control function. Often, the extent of the spectral shift is taken as an indication of the degree of binding present in the tissue. In a yeast-alcohol dehydrogenase system, a spectral shift of 19 mμ was observed (Duysens and Amesz, 1957; Duysens and Kronenberg, 1957) and all or most of the NADH is thought to be bound. In beef heart mitochondria, a spectral shift of 15 mμ was seen, as compared with the emission from the whole tissue (Chance and Legallais, 1959) indicating that there is a large quantity of unbound NADH present in the cytoplasm, with a smaller amount bound within the mitochondria. In general, it has been found that the NADH within mitochondria is in the bound form, while that in the cytoplasm is unbound. The latter gives little, if any, shift in emission peak location.

In past studies which have been made on whole tissue, fluorescence measurements have been complicated by the strong fluorescence emission found in connective tissue which is usually present in the sample. The use of extruded axoplasm in the present work insures freedom from such contaminants, and offers a reliable indication of binding of NADH without the necessity of taking difference spectra.

The present study with the giant axon of the squid indicates that most of the NADH within the axon is in the bound form, and within the mitochondria. The sedimented axoplasm described in the previous section gave a fluorescence emission peak at approximately 466 mμ. The spectrum for the whole axon was essentially the same, although reduced in intensity, due to

the dilution of the mitochondria in the axon as compared with the sediment. These measurements were made with an Aminco-Bowman spectrofluorometer (American Instrument Co., Silver Spring, Md.), with the excitation at 366 mμ. A specially constructed sample holder was used to hold the

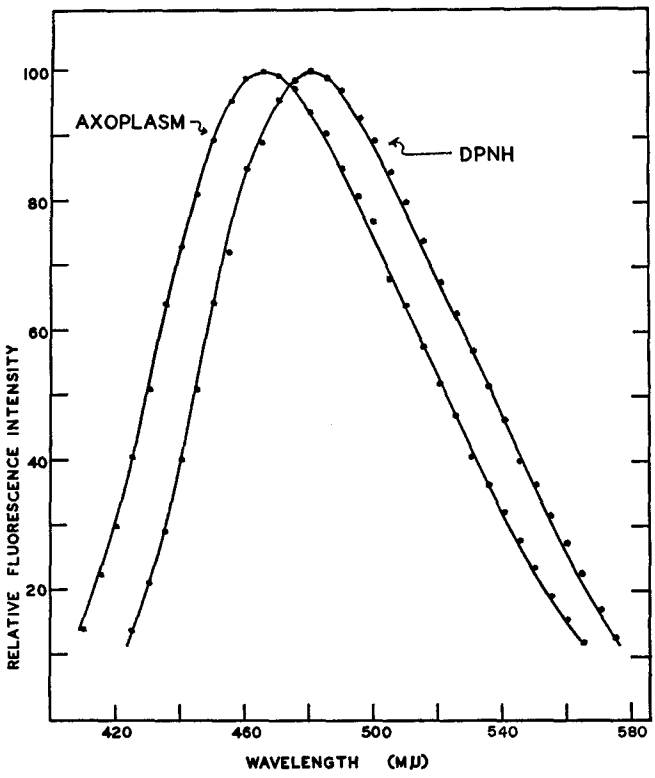

FIGURE 11. Fluorescence emission spectrum of axoplasm and NADH solution. Extruded axoplasm showed a fluorescence emission peak near 466 mμ. A solution of 100 μM NADH had an emission peak near 480 mμ. The measurements were made using an Aminco-Bowman spectrofluorometer. Estimated uncertainty in location of peaks is ±4 mμ; peak values normalized to 100 units. The 14 mμ shift in axoplasm NADH fluorescence peak indicates considerable binding of the nucleotide within the axoplasm.

microcapillaries of axoplasm which were described earlier. Due to the greatly reduced sample volume as compared with that which the instrument was designed to accept (1 cm square cuvettes), the magnitude of the fluorescence signal was very small, with a consequently reduced accuracy in the determination of the wavelength location of the fluorescence peak. The spectra reported here are the average of four different axons, with an uncertainty of peak location of ±4 mμ.

A solution of NADH showed a peak emission near 480 mμ; that of the

sedimented axoplasm occurred near 466 mμ. The shift of 14 mμ suggests that the NADH within the axon is quite highly bound. The emission spectra for the axoplasm and for a NADH solution are given in Fig. 11.

Pyridine Nucleotide Concentrations in Squid Axoplasm As described previously, biochemical analyses were performed on squid axoplasm, using the procedures of Lowry et al. (1961). In this manner, the absolute concentrations of NAD, NADH, NADP, and NADPH were determined. A summary of the results is given in Table II. For purposes of comparison, values for brain as reported on an over-all weight basis by Lowry et al. (1961) and on a protein basis by Garcia-Buneul et al. (1962), are included.

TABLE II
PYRIDINE NUCLEOTIDES IN SQUID AXOPLASM AND RAT BRAIN TISSUE

Nucleotide	Axoplasm			Brain*	
	μM/liter	μM/kg	μM/kg prot*	μM/kg	μM/kg protein
NAD	50.6	46.1	1565	322	2370
NADH	8.8	8.0	248	95	380
Total NAD	59.4	54.1	1813	417	2750
NAD/NADH	5.75			3.4	6.2
NADP	1.2	1.1	38.4	7.2	160
NADPH	5.9	5.4	181	22.3	100
Total NADP	7.1	6.5	219.4	29.5	260
NADP/NADPH	0.21			0.32	1.6

* The protein in squid axoplasm is taken to be 3240 ± 120 mg % as reported by Roberts et al. (1958). Values quoted for rat brain are from Lowry et al. (1961) and Garcia-Buneul et al. (1962).

The figures given for squid axoplasm are the average of two determinations on axons from different squid. These values differed by less than 10% total NAD. As might be anticipated, the pyridine nucleotide content of the axoplasm was relatively low, and 85% of the total NAD was in the oxidized state. Only 17% of the NADP was in the oxidized form, comparing quite favorably with reported values in other tissues. In the case of brain, 77% of the NAD is oxidized and 41% of the NADP.

It is of interest to calculate the actual amount of NADH seen by the microfluorometer. If one assumes an average axon diameter of 500 μ, and a field of view of 550 μ, then the volume of axon in the field is 1.10 μl. From the biochemical analysis of axoplasm, the NAD concentration is approximately 50 μM/liter, giving the amount of NAD in the field of view of the fluorometer as 55×10^{-12} moles. Since this would represent total NAD reduction, or full peak height, it was easily possible to detect $\frac{1}{20}$th of this amount, or approximately 2.7 pmoles. Thus, under the conditions of use, it was possible

to detect changes of NADH within the axon of amounts at least as small as 2.7×10^{-12} moles. It was quite possible to increase the sensitivity of the electrometer amplifier by an order of magnitude, which gave an increase in noise level but still resulted in a usable signal. However, such sensitivities were not required in the present study.

DISCUSSION

The control of respiratory rate is dependent upon a supply of substrate at one end of the respiratory chain and the availability of oxygen at the other. Under conditions of adequate concentrations of substrate and oxygen, the rate of cellular respiration is largely controlled by the levels of inorganic phosphate (Pi), phosphate acceptor (ADP), and the end product of phosphorylation, ATP (Chance, 1961; Chance and Hollunger, 1961 *a*, 1961 *b*). A rise in ADP or Pi concentration, accompanied in most cases by a decrease in ATP level, will act to stimulate respiration with a consequent increase in substrate oxidation. This increase in activity will continue until the relative levels of ATP, ADP, and Pi are restored to their initial "normal" amounts, or until substrate or oxygen becomes rate-limiting. In the presence of adequate oxygen, the kinetics of oxygen uptake are independent of the actual oxygen concentration. In the case in which the oxygen level is insufficient to maintain the respiratory rate normally associated with the existing ATP, ADP, and Pi concentrations, mitochondrial respiration is determined by oxygen availability, and represents state 5 of Chance (Chance and Williams, 1955). In this state, oxygen is the rate-limiting component. As the oxygen concentration is lowered below the minimum level supporting normal respiratory rates, large gradients of oxygen concentrations are formed within the tissue (Chance, 1965). Cytochrome oxidase requires oxygen for its oxidation (except in the case of reversed electron transport, requiring the expenditure of ATP or a high-energy intermediate thereof), and an ever increasing proportion of the oxidase remains in the reduced state.

Although the terminal oxidase undergoes rather large changes in its redox state as oxygen becomes rate-limiting, the multienzyme respiratory system tends to have a "cushioning effect" (Chance, 1965), maintaining a relatively constant rate of electron flow. This tends to maintain a stable redox equilibrium in the more remote portions of the respiratory chain, until the oxygen level is so low that the oxidation of cytochrome oxidase is near its final point. This fact demonstrates the importance of monitoring the steady state of the respiratory component nearest the substrate end of the respiratory chain rather than the redox state near the terminal oxidase. Thus, the redox changes of NAD-NADH are more meaningful indicators of true intracellular anoxia than the cytochromes themselves. For this reason, the fluorometric method of monitoring NAD-NADH equilibrium offers an accurate technique for observing the changes in the redox state of the cell.

Certain types of tissues lend themselves to this kind of analysis more readily than others. Tissues such as muscle have large energy reserves and high glycolytic activity, and therefore can undergo long periods of anoxia before a significant drop in ATP level is observed. Thus, the role of respiratory processes is masked by the presence of such energy stores, and the dependence of metabolism upon mitochondrial energy production is difficult to assess. In contrast, squid nerve appears to be a suitable material, due to its low (relatively) energy reserves and metabolic rate. Also, glycolytic activity appears to be very small in axoplasm. In addition, the ease with which its electrical activity can be monitored allows stimulation effects to be readily observed.

The results of anoxia illustrate that the oxygen concentration must be very low before true intracellular oxygen starvation becomes apparent. For instance, nitrogen of 99.9% purity was not sufficient to induce a response, and it was necessary to use nitrogen with less than 8 ppm oxygen contamination. The experiments showing the increase in oxygen consumption rate with stimulation confirmed the ability of the fluorometric technique to accurately indicate changes in respiratory state. The increase in respiratory rate of 15–20% by stimulation is consistent with the findings of others (Connelly, 1952; Connelly and Cranefield, 1953). The coupling between stimulation and increase in oxygen uptake rate is an indirect one. It is now generally accepted that the normally high concentration of internal potassium and of low internal sodium found in actively metabolizing cells is generated and maintained at the expense of metabolically derived energy sources. Most notable is the hydrolysis of ATP to ADP and Pi, although there are strong indications that nonphosphorylated high-energy intermediates of oxidative phosphorylation also play an important role in certain active processes. The mechanism by which this energy is utilized to maintain ionic gradients against a chemical and electrical potential has been widely investigated and while numerous models have been proposed to explain its operation, the details of its functioning remain largely a matter of conjecture.

The breakdown of ATP to supply energy for the active extrusion of sodium (coupled with an influx of potassium in squid axons) is buffered to a considerable extent by other high-energy compounds, particularly creatine phosphate in vertebrates and arginine phosphate in invertebrates, including the squid. However, any significant drop in the ATP level, with an associated rise in ADP and Pi, causes a drop in the "phosphate potential" stimulating respiration. It is to be expected, therefore, that any circumstances which lead to an increase in pump activity will be reflected in a greater rate of oxygen utilization. There is no doubt that the electrical activity of the nerve is normally associated with a net influx of sodium, presumably driven by its concentration gradient. A prolonged volley of impulses results in an accumulation of sodium in the cell (approximately 10^{-12} moles per impulse

for a typical squid axon).[1] A rise in internal sodium acts to increase the pump's ATPase activity. Thus, by the above reasoning, stimulation can be expected to cause an increase in oxygen consumption rate.

Conversely, prolonged oxygen lack prevents the regeneration of ATP by oxidative phosphorylation, and in squid nerve anaerobic mechanisms cannot supply sufficient ATP to drive the pump at its normal rate and also supply the remainder of the metabolic energy requirements. Therefore, even resting nerves, under conditions of prolonged anoxia (on the order of several hours), are observed to undergo progressive depolarization (Furusawa, 1929; Gerard, 1930). A more direct relationship has been demonstrated between oxygen consumption and the operation of an active pump by observing a measurable decrease in sodium extrusion in squid nerve under anoxic conditions (Shanes and Berman, 1955). The effect is undoubtedly quite general, and the rather dissimilar tissue of frog skin shows a close correlation between sodium transport and oxygen consumption (Zerahn, 1956).

Many investigators have reported an increase in oxygen uptake rate during and after stimulation (Brink et al., 1952; Connelly, 1952, 1962; Gerard and Meyerhof, 1927). In the case of squid nerve, a figure of 70 $mm^3/g/hr$ has already been mentioned (Connelly, 1952; Connelly and Cranefield, 1953). After stimulation at 100/sec for 20–30 min, an increase in oxygen uptake of approximately 10% has been reported. This was for axons with diameters between 400–500 μ. Stimulation of the much smaller fibers of the stellar nerves of the squid resulted in a much larger percentage increase in rate of oxygen uptake on a per unit weight basis. The increases observed in these small fibers were sometimes more than 50 times the increase seen for the larger giant axon, per weight, per impulse. This is interpreted as showing that the increase in oxygen uptake rate with stimulation is more linearly correlated with the surface (membrane) area of the nerve than with its weight or volume.

The action of cyanide elicited a fluorescence response nearly identical with that of anoxia. This is not surprising, since in each case it is the terminal oxidase which is being blocked from oxidative turnover. The magnitude of NAD reduction under both conditions was the same, as indicated by the magnitude of the fluorescence emission change. Over 20 axons were tested in this respect, and the results were consistent. The slight increase in the time course of complete reversal of cyanide inhibition by washout procedures is most probably a reflection of the time required for cyanide to diffuse out of the mitochondria and the cell. Since cyanide apparently brings about an essentially complete blockage of respiration, the oxidative production of ATP

[1] Keynes and Lewis (1951) have reported 3.8×10^{-12} moles Na/cm^2/impulse entering a nerve axon of *Sepia*. In the present case, with an axon diameter of 500 μ and a distance between electrodes of 2.5 cm, the area of stimulated membrane is 0.39 cm^2, with a consequent Na entry of 1.5×10^{-12} moles/impulse.

is also terminated, much as is the case under anoxic conditions. However, as mentioned previously, the large stores of arginine phosphate serve to keep the ATP levels at normal or near normal concentrations until they themselves are depleted. Thus, a short-term treatment with cyanide is not identical with a prolonged blockage of respiration in its effect upon ATP levels. After several hours of anoxia or cyanide treatment, the ATP level falls and processes of active ion transport fail. The injection of ATP or arginine phosphate into a cyanide-poisoned axon restores the activity of the sodium pump to a great extent (Caldwell and Keynes, 1959).

The cyanide-induced reduction of NAD was very consistent and was always reversible. In some cases many as seven complete cyanide cycles were undertaken. Even after 45 min in cyanide, axons were usually still excitable, although by that time most of the pump activity would be expected to have stopped due to lack of ATP. Eventually, of course, the gradual drop in the ion concentration gradients would cause failure of nerve function.

Amytal has been widely used as an effective inhibitor of respiration. However, its effect appears to be limited to oxidations through the NAD pathway, with little or no effect upon succinate-linked oxidations (Eiler and McEwan, 1949). Ernster et al. (1955) have reported that in a preparation of isolated rat liver mitochondria, 1.8 mM Amytal caused complete blockage of hydrogen transfer from mitochondrial dehydrogenases to oxygen, but at the same time had no effect upon the aerobic oxidation of succinate or the phosphorylation coupled to it. It has been well-established that in phosphorylating systems, Amytal blocks oxidative transfer between NADH and flavoprotein (Jalling et al., 1955). Thus, Amytal should cause blockage of NADH oxidation to the same extent as cyanide and anoxia. The inhibition becomes saturated between 1 and 5 mM concentration, in general agreement with Ernster's findings for rat mitochondria (1955). This is somewhat higher than that required for cyanide inhibition, and is perhaps a result of the relative affinity and strength of binding of the two inhibitors at their site of action. Amytal was somewhat more sluggish in its kinetics of effect and washout, but was completely reversible through many cycles. The extent of NAD reduction caused by Amytal was the same as that observed for anoxia and cyanide.

Ouabain and strophanthidin are known to interfere with the action of the Na-K–coupled transport processes of the cell membrane. In the low concentrations usually employed, they appear to be limited in their point of action to the transport mechanism itself, with no observable effect upon other processes of the cell. Ouabain has been shown by Connelly (1962) to reduce the respiration of resting frog nerve by 30% and of active nerve by more than 90%, presumably by preventing the breakdown of ATP by the pumping reaction. The percentage of respiration associated with pumping in the resting squid axon would be expected to be much less, however. The portion of res-

piration acting to support active transport mechanisms appears to be proportional to the surface to volume ratio of the cell. On this basis, taking the frog nerve to be $\frac{1}{30}$th as large as the squid axon, the resting respiration utilized for ATP production to supply the pump would be expected to be on the order of 1%. Thus, releasing this load of 1% upon respiration would not be expected to shift the NAD-NADH equilibrium noticeably, and this may explain the lack of fluorescence change in the axon due to the action of ouabain and strophanthidin. In a tissue such as kidney, in which one would expect a significant portion of the energy consumption to be utilized for the active transport of ions, ouabain has been shown to inhibit oxygen uptake by approximately 40% (Whittam and Willis, 1963).

The effect of succinate upon NAD reduction was quite pronounced, the fluorescence increase being localized to the region of succinate injection. There was no inhibitor present in the axon during these measurements. It has been established that succinate and NAD-NADH share the same respiratory pathway from cytochrome *b* to oxygen, and since each depends upon the same cytochromes for its oxidation, there may arise a competition between these substances for the respiratory carriers. As hydride or electrons are supplied to the cytochrome pathway by both succinate and NADH, a preferential availability of succinate for oxidation by the cytochromes may explain the blockage of NADH oxidation on the basis of simple competitive action. The magnitude of the fluorescence response indicates that the reduction of NAD in the succinate-injected region was nearly 70% complete, in comparison with cyanide treatment of other axons.

Azide was, perhaps, the most interesting inhibitor of those used in the present study. Even at high concentrations (50 mM) it was capable of inducing at most a 40% reduction of NAD within the axon, as compared to the effects of cyanide, Amytal, and anoxia.

The action of azide upon respiring systems appears to be a rather complex process, with several types of effect being reported. For instance, it has been shown that azide inhibits oxidation between cytochromes (Keilin, 1936; Horecker and Stannard, 1948); that it acts as an uncoupler of phosphorylation from respiration (Loomis and Lipmann, 1949); that it inhibits ATPase activity (Meyerhof and Ohlmeyer, 1952; Sacktor, 1953); and also stimulates the hydrolysis of ATP (Robertson and Boyer, 1955), with the stimulation preceding the inhibition (Swanson, 1956; Myers and Slater, 1957).

Of particular interest is the reported ability of azide to distinguish between the respiration of stimulated, or "active" systems of nerve and muscle and the lower respiration rate of the "resting" system. Stannard (1939) and Horecker and Stannard (1948) have found that only a portion of the respiration of stimulated frog muscle was azide-sensitive, while the resting respiration was completely insensitive. The increase in oxygen consumption associated with

stimulation was blocked by azide within a few minutes of its application. No effect upon resting frog muscle was observed over a 10,000-fold range of azide concentration. Fischer et al. (1944) have reported that the extra respiration of fertilized sea urchin eggs is inhibited by azide, with the respiration of unfertilized eggs remaining unchanged. Doty and Gerard (1950) found that azide inhibited the extra oxygen consumption associated with the stimulation of frog nerve but had a minimal effect upon the respiration of the resting nerve.

The above data suggest that azide can block the use of oxygen for processes associated with activity (i.e., stimulation), but that it does not interfere appreciably with normal resting cell metabolism. This may indicate that the portion of energy derived from coupled respiration which is utilized by the active transport mechanism of the cellular (and mitochondrial?) membrane is along a different metabolic pathway and perhaps requires a different form of high-energy compound than the resting cell metabolism. Alternatively, there could be a spatially separate pathway for energy transfer in each case, with only one of them readily accessible to azide.

Chance and Williams (1956) reported that respiration in the active state of mitochondria (state 3) is appreciably inhibited by higher concentrations of azide without large effect upon the resting (state 4) respiration. It is suggested that this may be involved in the selective effects of azide upon the respiration associated with active and resting conditions.

Brink (1957) has questioned the likelihood of the differential effect being caused by inhibition of one kind of enzyme in resting nerve and of another in active nerve. He notes that the inhibitory effect of azide in nerve is dependent upon the initial state of the nerve and suggests that the selective effect of azide upon resting and active nerve may be explained as a general property of a complex steady-state system.

It appears likely that the respiration associated with stimulation in nerve is connected with the energy drain of the active transport processes of the cell membrane, and perhaps of the mitochondrial membrane as well. Also involved may be structural changes in the membrane associated with the transport process which would require the expenditure of metabolically derived energy.

Respiratory inhibitors and uncoupling agents of oxidative phosphorylation cause a drop in cellular ATP levels by interfering with its production, and, in the case of uncouplers, there is also a stimulation of ATPase activity. Caldwell and Keynes (1959) have shown that azide causes a drop in cellular ATP levels in squid axoplasm. However, it is quite possible that high-energy intermediates of phosphorylation are still being produced and utilized for many metabolic functions during azide treatment. It has been suggested that azide may act much like oligomycin in its ability to block the phosphorylative step

in ATP production, while still allowing the cellular processes to continue which are capable of utilizing nonphosphorylated high-energy intermediates.

It has been established that a nonphosphorylated intermediate of oxidative phosphorylation supports mitochondrial ion transport and other cellular functions. It has not been firmly established that such intermediates are capable of driving active ion transport across the cell membrane, although it is known that ATP has this capability. It is significant to note that while ouabain and strophanthidin greatly slow the Na-K pump of the cell membrane (presumably by interfering with the ATPase of the transport process), they have no effect at all upon the ion transport of the mitochondria. Thus, it does not appear unreasonable to think that nonphosphorylated high-energy intermediates drive many cellular processes, including mitochondrial ion transport, but ATP is required for the Na-K pump of the cell membrane.

The action of azide could then be explained by postulating its action upon an intermediate step in oxidative phosphorylation, this step being located after the formation of nonphosphorylated intermediate but prior to the formation of ATP, much as in the action of oligomycin. In this manner, ATP production would stop, and the portion of respiration acting to keep the ATP at its normal level would be insensitive to ATP and controlled by the levels of high-energy intermediates. This control may actually be the case under normal conditions as well. The breakdown of ATP by the membrane pump would, of course, continue until the stores of ATP and arginine phosphate were depleted. The continuing respiration would not be supporting the replacement of this ATP, and there would thus be no increase in respiratory rate as a result of pump activity. The normal resting cell metabolism would continue, being supplied by the remaining portion of the respiration.

The localization of axon fluorescence to the mitochondrial portion of the axoplasm implies that most, if not all, of the NAD-NADH is concentrated in these subcellular particles. This, in turn, strongly indicates that the axon relies mainly upon oxidative rather than glycolytic metabolism for its metabolic energy requirements. The large effects of respiratory inhibitors such as cyanide and Amytal confirm that respiratory linked oxidative generation of high-energy compounds plays a dominant role in the energy metabolism, and that glycolysis is rather insignificant in its action.

The ability of NADH fluorescence to act as an indicator of intracellular redox state is a valuable investigative tool, and in the present study offered a sensitive and convenient method for observing the effects of inhibitors upon nerve respiratory metabolism. Although not generally suited for absolute determinations of nucleotide concentrations in cells, due to absorption and fluorescence enhancement effects, when combined with an independent biochemical analysis of nucleotide, it offers a valuable measure of the kinetics and extent of intracellular oxidation-reduction processes.

I am indebted to Dr. L. J. Mullins for his guidance and advice during the course of this investigation. Appreciation is also given to Dr. John J. O'Neill and Dr. T. Sakamoto for the biochemical assay of pyridine nucleotides in squid axoplasm, and to Mr. A. Fitzjarrell for the taking of electron micrographs.

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