if phenethyl alcohol was also added, the characteristic synergism was again observed (10^4 survivors after 5 minutes), whereas phenethyl alcohol itself offered some protection against "thymineless death."

We conclude from these experiments that the synergistic effect of phenethyl alcohol is not a consequence of inhibition of DNA synthesis. Recent reports discuss two other effects of phenethyl alcohol which cannot be consequences of the inhibition of DNA synthesis. These are the inhibition of sporulation and germination of *Bacillus megaterium* (*10*) and the inhibition of replication of a RNA bacteriophage (*11*).

The administration of cyanide to a culture of *E. coli* establishes a reducing environment within the cells by inhibiting the transfer of electrons to oxygen. Consequently, the strong synergistic action of cyanide with both streptonigrin and mitomycin C is probably due to more rapid reduction of the quinone ring in these compounds. The similarity between the effects of cyanide and phenethyl alcohol in our experiments suggests that phenethyl alcohol also may facilitate reduction, possibly by interfering with electron transport. The following results of work in our laboratory support this notion (*12*): (i) Phenethyl alcohol greatly facilitates the reduction of the tetrazolium compound MTT [3-(4,5-dimethylthiozalyl-2)-2,5-diphenylmonotetrazolium bromide] in a growing culture. (ii) The synergism of phenethyl alcohol with mitomycin C is substantially less if MTT is also added to the culture. These two facts suggest that MTT competes with the antibiotic for a source of electrons which is provided by the action of phenethyl alcohol. Although the point of interaction of MTT with the respiratory chain of *E. coli* has not been reported, in rat liver suspensions MTT is apparently reduced at two points of the respiratory chain (*13*).

The effect of dinitrophenol on streptonigrin inhibition is obviously different from the action of cyanide or phenethyl alcohol. All three synergists may facilitate the reduction of mitomycin C at various points in the system of electron transport; but, if so, dinitrophenol evidently affects a point at which streptonigrin does not readily receive electrons (*14*).

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Intracellular Perfusion of Chilean Giant Squid Axons

Abstract. *Axons of the Chilean giant squid were subjected to continuous intracellular perfusion with either potassium- or sodium-rich salt solutions. In axons immersed in natural sea water and internally perfused with potassium glutamate solution, action potentials which exceeded 160 millivolts were often observed. The resting potential did not vary appreciably with the internal concentration of potassium glutamate. With perfusing solutions containing sodium aspartate or sodium glutamate, action potentials were observed which exceeded the value calculated from the Nernst equation applied to sodium-ion activity. Nervous conduction could be maintained more than 1 hour with equal concentrations of sodium inside and outside the axon.*

South American squid, *Dosidicus gigas*, readily available at the Marine Biological Station, Montemar, Chile, usually weigh 5 to 10 kg and measure more than 1 m in body length. Each stellate nerve of these squid contains three to four giant axons ranging from 0.6 to 1.5 mm in diameter. These axons have been used for studies of the chemical properties of the axoplasm (*1*) and of the effect of intracellular injection of trypsin on nervous conduction (*2*).

In this report we describe the results of an electrophysiological study carried out at the Chilean station. Our object was to examine the nature of the resting and action potentials of the Chilean giant squid axon when subjected to continuous intracellular perfusion; the method of perfusion (*3, 4*) permitted more direct electrochemical measurements on axons than are possible by other techniques.

The technique of intracellular perfusion was described previously (*3*). The perfusing fluid was introduced through a glass pipette (0.22 to 0.25 mm in diameter) inserted at one end of the axon. The fluid was drained through a large pipette, approximately 0.5 mm in diameter, at the other end. In one stage of this investigation we used a simplified arrangement without a drainage pipette. The axoplasm in the perfused zone of the axon was removed beforehand by suction.

The length of the perfused zone was approximately 15 mm in one series of the experiments (records *A* and *B* in Fig. 1). In these instances the unperfused portion of the axon on each side of the zone under study was separated by vaseline partitions and was rendered inexcitable by external application of 0.6*M* $MgSO_4$ solution. In later experiments (record *C*), almost the entire length (35 to 40 mm) of the axon in the fluid medium was directly exposed to the internal perfusion fluid. The resting and action potentials in the perfused zone were recorded with a long glass pipette electrode (0.1 mm in diameter). Stimulating electric currents were applied to the axon through a pair of external electrodes placed near the end of the perfused zone.

Solutions used for intracellular perfusion were prepared by mixing 0.6*M* potassium aspartate (or glutamate), 0.6*M* sodium aspartate (or glutamate), and glycerol solution, 12.5 percent by

volume; the *p*H was adjusted to between 7.2 and 7.3 with phosphate buffer. The flow rate of the fluid through the axon was usually 40 to 50 μl/min. The extracellular fluid medium was either natural sea water or a solution containing NaCl, $MgSO_4$ or $MgCl_2$, $CaCl_2$, and sucrose (to maintain isotonicity).

In the first series of experiments we re-examined the relation between the intracellular concentration of potassium salt and the membrane potential. When axons immersed in natural sea water were intracellularly perfused with a sodium-free mixture of potassium glutamate and glycerol solution, the amplitude of action potential rapidly increased. When the potassium concentration was between 0.1 and 0.5*M*, action potentials with amplitudes of 160 mv or more were observed in eight different axons (see record *A* in Fig. 1). Excitability of the axon could be maintained for more than 3 hours under continuous perfusion. As in *Loligo* axons, the potential of the resting membrane of *Dosidicus* axons was very insensitive to large changes in the internal concentrations of potassium salt; the potential was between −40 and −50 mv when the concentration ranged between 0.1 and 0.5*M*. The duration of the action potential was always between 1 and 1.5 msec in these observations at 16° to 18°C.

In the second series of experiments, an extensive study was made of the effect of a high internal concentration of sodium on the membrane potentials. Axons were intracellularly perfused with solutions containing 0.3*M* sodium and 0.2*M* potassium; both aspartate and glutamate salts were used. To prevent repetitive firing of nerve impulses and subsequent depolarization, axons to be perfused were immersed in a medium with a low sodium concentration (0.3*M*) and high divalent cation concentrations (50 m*M* magnesium and 35m*M* calcium).

Results obtained were consistent with those reported previously (*5*): *Dosidicus* axons in which the concentration of sodium ions inside was the same as the concentration in the outside medium were capable of developing action potentials with a large overshoot (the amplitude of the action potential minus the amplitude of the resting potential).

Records B_1 and B_2 in Fig. 1 illustrate our frequent finding that the amplitude of the action potential gradually increased during continuous perfusion with a solution rich in sodium ions. This gradual increase was absent in injured axons. The increase in the amplitude was characterized by an increased sharpness at the peak of the action potential. After this gradual 10 to 15 percent increase in the amplitude of the response, a stage was reached during which the amplitude remained unaltered for 20 to 40 minutes. In this series of experiments, 11 axons maintained their ability to develop action potential exceeding 70 mv in amplitude for more than 60 minutes under perfusion with equal concentrations of sodium inside and outside the axon.

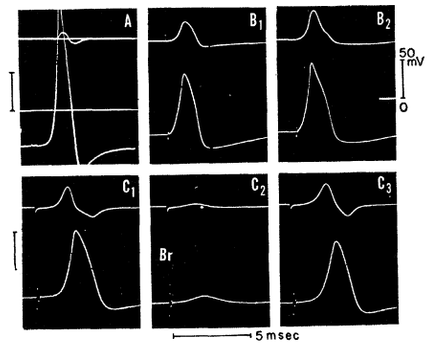

Fig. 1. Action potentials of Chilean squid axons intracellularly perfused with potassium-rich (record *A*) and sodium-rich (*B* and *C*) solutions. The top and bottom traces represent extra- and intracellularly recorded responses, respectively. The concentration of potassium in the perfusion fluid was 400 m*M* in *A*, 200 m*M* in *B* and *C*; the concentration of sodium was 50 m*M* in *A* and 300 m*M* in *B* and *C*. The external medium contained approximately 500 m*M* Na^+ in *A* and 300 m*M* Na^+ in *B* and *C*. Record C_2 was taken 20 seconds after the asparate ions inside were replaced with bromide ions. Recording sensitivity and time base are the same for all records.

The resting potential across the membrane was measured by withdrawing the recording electrode from the interior of the axon and placing it in the surrounding fluid medium; it was approximately 40 mv, inside negative. The overshoot of the action potential was then between 30 and 55 mv under the experimental conditions. An immediate corollary of this observation is that the overshoot is not determined by the Nernst equation applied to sodium ions.

Direct experimental evidence indicates that no appreciable chemical binding exists between the sodium ion and the glutamate or aspartate radicals in the perfusion fluid. Measurement of the activity of the sodium ions using a sodium-ion glass electrode (Beckman 39278) showed that the "sodium equilibrium potential" was only about +6 mv—that is, one-fifth to one-tenth of the observed overshoot. A previous measurement of electric conductivity of the perfusion fluid (*5*) indicated that the major portion of the sodium in the solution was free.

It is interesting that the Nernst equation fails to account for the overshoot of action potentials in axons perfused with solutions rich in potassium. Record *A* in Fig. 1 shows results obtained with 50 m*M* sodium inside. Since the axon under study was immersed in sea water, the ratio of the sodium concentrations was approximately 10, giving a potential difference of 50 mv according to the Nernst equation. The observed value of the overshoot was 100 to 140 mv.

Records C_1 to C_3 are presented to illustrate that the layer of axoplasm between the axonal membrane and the perfusion fluid does not constitute a significant barrier for diffusing ions. When the aspartate ions in the perfusion fluid were replaced with bromide ions, there was a rapid (within 30 seconds) decrease in the amplitude of the action potential, followed by complete loss of excitability. When aspartate ions were readmitted in the perfusion fluid, there was, as a rule, a perfect restoration of the action potential within 1 minute. Since halogen ions have to reach the membrane in order to produce effects, it can be safely concluded that the intervening layer of axoplasm is highly permeable to these halogen ions and to at least one other ion species. This conclusion is borne out by previous measurements of fluxes of various ionized radioactive tracers across surface membranes of intracellularly perfused axons (*6*).

Effects on the membrane potentials of various cations and anions applied to the inner side of the axonal membrane can be arranged in an order similar to the lyotropic series in colloid science (see, for example, Bungenberg de Jong, *7*).

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Genetic Studies on the Mixed Leukocyte Reaction

Abstract. *When leukocytes from pairs of unrelated human subjects are mixed and cultured for several days, blast-like cells appear that are capable of DNA synthesis and mitosis. This reaction can be estimated quantitatively by measuring the uptake of tritiated thymidine in the cultures. In experiments with 15 sibling pairs, the leukocytes of most individuals reacted less strongly with those of their siblings than with those of an unrelated subject.*

When leukocytes from two unrelated individuals are cultured together in the absence of phytohemagglutinin, some of the cells become transformed to immature basophilic cells capable of mitosis (*1, 2*). Only these immature cells synthesize DNA, so the intensity of the reaction between the leukocytes from a particular pair of subjects can be estimated by measuring the uptake of tritiated thymidine at the end of the culture period. The blast-like mitotic cells in cultures containing phytohemagglutinin are derived from small lymphocytes (*3*), so the immature cells in leukocyte mixtures, which are indistinguishable from those seen in phytohemagglutinin cultures, probably are also of lymphocytic origin. A similar transformation has been noted when certain antigens are added to leukocytes from single individuals (4).

These findings suggested that the mixed leukocyte reaction may be a response to foreign antigens, possibly the "individual-specific" antigens that are present in most tissues, and which are responsible for homograft rejection (*2*). Subsequently, other workers (*5*) have concluded from their data that the mixed leukocyte reaction may be helpful in the selection of homograft donors.

In our previous studies, there was no reaction in leukocyte mixtures when the two subjects were identical twins. The results with fraternal twins were variable: two pairs reacted and two pairs did not react. It was decided, therefore, to investigate mixtures of leukocytes from sibling pairs who were not twins. In this way, the effect of genetic relationship on the mixed leukocyte reaction could be studied without the results being influenced by the blood chimerism and resulting tolerance to skin grafts, which, although extremely rare, has been reported to occur between human fraternal twins (*6*).

Fifteen normal sibling pairs were studied. The culture method and quantitative estimation of the reaction by means of tritiated thymidine autoradiographs have been described previously (*1, 2*). In addition, a technique was devised for measuring the uptake of tritiated thymidine in whole cultures with a liquid scintillation counter, based on Chen's method for measuring H^3 and C^{14} in serum (*7*). In each experiment, leukocytes from the two members of the sibling pair were mixed together, and at the same time leukocytes from each member of the pair were mixed with those from a third, unrelated, individual. Unmixed control cultures were also prepared. Their H^3-thymidine uptake was always very low compared with mixtures from unrelated individuals.

The results are shown in Fig. 1. The two methods gave very similar results and correlated well, particularly within individual experiments. The mixed leukocyte reaction was diminished when the two subjects were closely related.

The experiments were performed over a period of several months, during which time minor technical changes were made to increase the sensitivity of the method. These changes contributed to the variability of the results, the liquid scintillation counts probably being affected more than the autoradiographs. Thus, it was important to compare the results within each experiment. When this was done, it was found that only five of the 30 individuals composing the 15 sibling pairs reacted more strongly with their siblings than with an unrelated subject. All of the results were analyzed by means of the *t*-test, and the mean difference between sibling-unrelated subject mixtures and the corresponding sibling-sibling mixtures was significantly greater than zero ($p < 0.01$). Whether the subjects were of the same or different sexes did not appear to make any difference.

Five of the sibling pairs, when compared with unmixed controls, showed no reaction that could be detected either by autoradiographs or by liquid scintillation counting. Some of these pairs were studied twice, and no reaction was seen on either occasion. Similarly, other sibling pairs consistently showed a positive reaction. All of these individuals

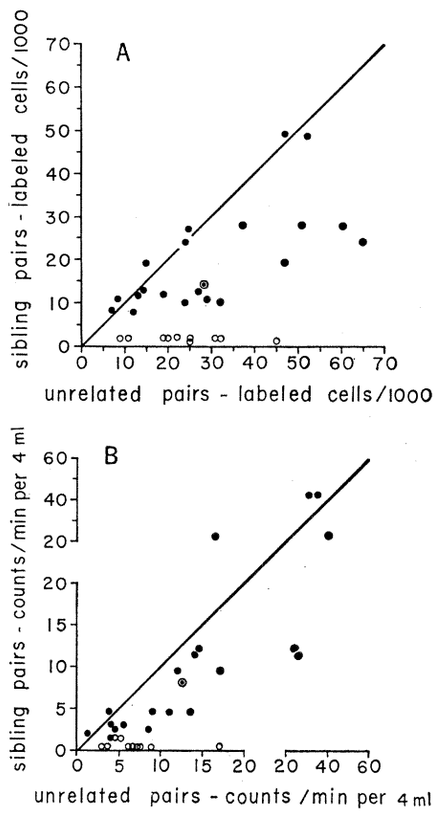

Fig. 1. Tritiated thymidine uptake in leukocyte mixtures. (*A*) Autoradiographs. (*B*) Liquid scintillation counts. For each point, the ordinate is the reaction between an individual and his sibling, and the abscissa is the reaction between the same individual and an unrelated subject. Each sibling pair is thus represented by two points on the graph. Symbols: closed circles, experiments where sibling pair showed positive reaction; open circles, experiments where sibling pair showed no detectable reaction; open circle with center point, mean value of mixtures from all sibling pairs versus mean value of mixtures from each sibling and a third, unrelated individual. The diagonal line shows where the points would lie if sibling pairs and unrelated pairs reacted equally.