involved. Hence, our results in general prove that the nitrate ion does not merely reduce the threshold, but—and this is the essential new finding of our research—it also can somehow modify the events of the latent period and increase the contractile strength of each excited fiber.

It is significant that the effect of nitrate on the mechanical behavior, both of the latent period and of the peak tension development of the contraction period, occurs so rapidly after immersion of the muscle in the nitrate-Ringer's solution. This indicates that the nitrate ion could not have had sufficient time to penetrate into the muscle fibers and thus modify the mechanical response by a direct action on the contractile system. We therefore infer that this ion directly affects only the surface—i.e., the excitatory membrane of the fibers—and that this alteration leads, in turn, to the observed changes in the mechanical response by a modification of the mechanism by which excitation of the membrane by the electric shock is coupled to the inner contractile system.

That the membrane is altered is clear. This is proved by the increase in excitability. But it is also demonstrated by the fact that the nitrate ion slightly increases the resting potential (*5*) and considerably enhances the permeability (*8*) of muscle cells. Probably, as Chao suggests (*2, 3*), the increased excitability is a consequence of the greater dispersibility of the membrane under the influence of nitrate, and the possibility that this structural change may underlie the other differences in membrane properties we have mentioned is not excluded.

Our principal interest, however, lies in the influence of nitrate on the mechanism of excitation-contraction (*E-C*) coupling. Our results indicate that this ion has its initial effect on the excitation part of this process. This influence, however, is not on the reactions responsible for the action potential, since the electrical response of the excitatory membrane of each fiber is unaffected. It would therefore seem that some other feature of the excited membrane is altered by the nitrate ion, and that this occurs in such a fashion as to change the subsequent course of the coupling mechanism. The time course of *E-C* coupling extends from the instant of stimulation to the onset of tension change—i.e., it is coincident with the events of the latent period, or at least with the earlier phases of this interval. Hence, it is not surprising that under the influence of nitrate we record latency alterations—the shortening of L_R and L, and the deepening of the latency relaxation. Previous work (*7*) has shown that the *LR* is an expression of the contractile material undergoing activation for its positive tension development. It is therefore evident that these latency mechanical changes may reflect the modified course of the latter portion of *E-C* coupling, during which the chain of events initiated by the action of the stimulus on the membrane becomes linked to the contractile material, and in such manner, enables the fiber-tension output to be so greatly potentiated.

Needless to say, the full elucidation of the mechanisms discussed above requires the performance of much more research. We plan to study the effects of other anions and of a variety of other agents (e.g., of the cholinesterase system) that are believed to play a role in, or to affect, excitability mechanisms. Some insight into the complexity of the processes is indicated in already reported results (*9, 10*) concerned with the effects of the potassium ion. This agent in relatively small concentrations acting for prolonged periods, or in larger concentrations acting for short durations, increases R up to 300% or more, potentiates T about 25%—effects that are irreversible—and reversibly depresses the action potential. Here, as in the NO_3 effects, the various changes are due to the direct action of K on the excitatory membrane. Yet, as can be seen by comparing the noted actions of these two ions, considerable differences exist regarding the quantitative alterations in R and T, the action potential behavior, and the possibility of reversal of the induced changes. It is felt, however, that a systematic study of these phenomena will shed some light on the fundamental mechanisms of excitation-contraction coupling, and, since this process activates contraction, it is hoped that any knowledge gained concerning it may also help in furthering comprehension of the contraction process itself.

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The Mechanism of Action of Organic Mercury Compounds on Cytochrome Oxidase

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Several organic mercury compounds (phenylmercuric nitrate [PMN], phenylmercuric hydroxide [PMOH], and *p*-chloromercuribenzoic acid [benzoate]), known to react with the –SH group, have been shown to inhibit the cytochrome c–cytochrome oxidase system at the oxidase portion of the chain (*1*). Since Barron (*2*) and others have considered this oxidase not to be sulfhydryl-dependent, it was suggested that the inhibition of the oxidase (a crude rat heart preparation) was nonspecific and might be due to the particulate nature of the preparation.

Investigations presented in this note, in which a solubilized sodium desoxycholate preparation of cytochrome oxidase (*3*)[1] was used, show that this partially purified enzyme is also inhibited by the mercurials. The in-

[1] The cytochrome oxidase was generously supplied by S. J. Cooperstein, of Western Reserve University.

hibition measured manometrically[2] was of the same degree as with the crude enzyme. When rates of oxidation of reduced cytochrome c by the treated oxidase were determined spectrophotometrically, the inhibition was less than with the crude enzyme (Table 1). This decrease in inhibition was especially marked with the benzoate as inhibitor; higher concentrations of this reagent (oxidase: inhibitor ratio 1:3.04) caused a slight (20%) depression in the rate of oxidation.

TABLE 1

Mg ratios* Oxidase : Inhibitor	Average % depression			
	Manometer		Spectrophotometer	
	Crude	Purified	Crude	Purified
1 : 1.80 PMN	100	100		
1 : 0.04 PMN	50	52.5	51.0	40.6
1 : 1.26 PMOH	100	100		
1 : 0.04 PMOH	50	56.4	46.5	34.9
1 : 2.10 Benzoate	100	100		
1 : 0.16 "	50	44.2	67.3	0

* In terms of crude enzyme. Purified enzyme was approximately 5 times as active, and corresponding adjustment in oxidase : inhibitor ratio was made.

The mechanism of action of the mercurials was studied by means of spectral analysis. After treating the oxidase with the mercurials in mg ratios of oxidase to mercurials which gave nearly 100% depression manometrically, the shifts in the visible spectral bands shown in Table 2 were observed. In concentrations of mercurial giving only 50% depression of the oxidase, the shift in the reduced bands was not as great (cf. visible spectra in [*4*]).

TABLE 2

	Oxidized bands mμ	Reduced bands mμ	
Untreated enzyme	417–419	443	601–603
Treated "	415–417	433–435	593–599

The effect of the mercurials on the ultraviolet spectrum is to increase the extinction throughout the region examined, 235–320 mμ (Fig. 1). The observed maximum at 277–278 mμ and minimum at 250–52 mμ were not shifted significantly, nor did any new bands appear. The extinction was particularly increased over the bands and showed least rise in the region between 285–300 mμ. In mixtures containing higher concentrations of –SH reagents (those giving nearly 100% depression), a clouding of the solution, which could not be removed by centrifugation or Seitz filtration, caused a high nonspecific absorption which masked both the maximum and minimum (Curve 4, Fig. 1). No clouding was visible macroscopically when concentrations of mercurial giving 50% depression were used. However, the rise in extinction which was also observed in these experiments indicates that an increase in density did occur.

[2] See reference (*1*) for manometric and spectrophotometric procedures.

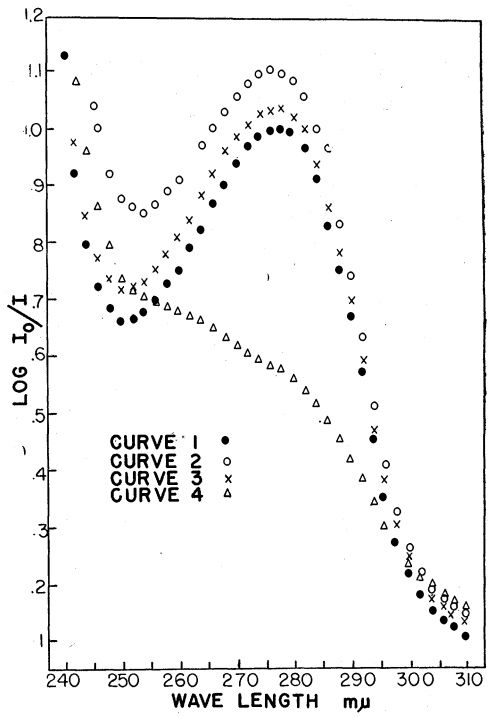

FIG. 1. Effect of chemical and physical treatment on the ultraviolet spectrum of cytochrome oxidase. Curve 1, control; Curve 2, oxidase-PMOH in mg ratio of 1 : 0.05; Curve 3, heat-denatured oxidase, 56° C for 15 min; Curve 4, oxidase-PMN in mg ratio of 1 : 0.5, solution diluted in 1 : 2. Concentration of oxidase in Curves 1, 2, and 3 is 10 mg/ml; in Curve 4, 3.3 mg/ml.

Study of chemical inactivation by the mercurials was paralleled by a similar study of physical inactivation by heat. The rate of oxidation of cytochrome c by oxidase heated for various lengths of time at 56° C was measured. Under the conditions of our experiments, heating for 30 min at 56° C caused complete inactivation of the enzyme. No macroscopically visible clouding occurred.

Spectral studies on this heat-denatured enzyme showed the same type of increase in extinction in the ultraviolet, although the percentage of increase was considerably less than that caused by the mercurials. Likewise, the same shifts were observed in the reduced spectrum in the visible region. In the oxidized spectrum no shift occurred.

Although the effect of the mercurials is more drastic, the type of inactivation in the two instances appears to be similar—a denaturation of the protein moiety. As a result of the alteration of its protein component, the bands in the visible region characteristic of the prosthetic group in the intact enzyme undergo a shift, and the specific absorption attributed to the native molecule is greatly decreased with an increase in nonspecific absorption.

Phenylmercuric hydroxide and, especially, *p*-chloromercuribenzoate have been employed as specific reagents for the sulfhydryl group in the protein molecule (*5*). The present studies show that in concentrations commonly used for this purpose these reagents can inactivate an

enzyme generally believed to require no –SH group for its function. Slater (*6*) has also noted that *p*-chloromercuribenzoate "has some effect on cytochrome oxidase." It has been shown that phenylmercuric nitrate can inactivate other nonsulfhydryl enzymes (*7*). It should be emphasized that, although it is possible to protect cytochrome oxidase against mercurial inhibition by means of cysteine or other sulfhydryl compounds, it is not possible to reverse the inhibition by such means (*1, 8*). When enzyme inhibition by mercurials is due to combination with essential –SH groups, such reversal can normally be accomplished (*5*).

Details of these investigations and additional data concerning the mechanism of inactivation will be published elsewhere.

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Differentiation of Bacterial Species and Variation within Species by Means of 2,3,5-Triphenyltetrazolium Chloride in Culture Medium[1]

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When different species or varieties of bacteria are grown as separate colonies on a clear agar medium, such as tryptose agar, containing 0.01% or less of triphenyltetrazolium chloride, the colonies of each will develop either a different shade of red coloring or a similar shade of red along with a different pastel color in the borders of the colonies. The latter colors, often the most pronounced differences seen, are tints of green, blue, yellow, or red.

The following procedures are recommended for use in the detection of colonial differences in bacteria: A 1% solution of tetrazolium chloride is prepared in distilled water and sterilized at 115° C for 15 min. One ml of this solution is added to 100 ml of melted tryptose agar and mixed well just before pouring on Petri plates. The depth of the agar in the plates should be approximately 0.5 cm. Since a solution of the salt is colorless in the oxidized form, the agar, after pouring, will remain colorless. The poured plates should be incubated at 37° C for about 20 hr to evaporate the excess moisture from their surfaces.

[1] Journal article No. 1129 n.s., Michigan Agricultural Experiment Station.

The surface of the agar plate should be inoculated in such a way that the colony population that arises will not be crowded. The bacterial suspension should be prepared in sufficient dilution so that when 1 cm is spread over the surface of the agar, from 200 to 400 colonies will arise on incubation. The period of incubation at 37° C before examination for differences in color will depend upon the rapidity of growth of the microorganisms under study. Those falling in the genera *Salmonella, Shigella, Escherichia, Alcaligenes,* and *Streptoccus* should be incubated for 48 hr. Agar plates inoculated with *Brucella* should be incubated for 4 days. For the differentiation of *Staphylococci* and certain aerobic spore-bearing *Bacilli,* the concentration of the salt in the agar medium should be reduced to 0.0025%, as higher concentrations inhibit their growth.

The color patterns and differences in color patterns of bacterial colonies can best be seen by examining the growth on agar Petri plates under a low power (×12) dissecting microscope, illuminated by reflected, oblique light from the new Spencer microscope lamp. The light is focused at an angle of 40° on a small concave mirror placed in front of the microscope. The rays are reflected at an oblique angle from the mirror and strike the under surface of the Petri plate resting on the glass stage.

Thus far 15 different species or varieties of bacteria of 7 different genera have been studied. The bacterial cultures examined were from a culture collection. Each had been previously identified as to its genus, species, and variety by standard laboratory methods. The colonial variants of the species of *Brucella* used were from a large collection previously separated and identified by the senior author. With the exception of *Brucella,* no previous attempt had been made to bring about dissociation of the cultures studied.

All the varieties of *Salmonella* cultures examined, with the exception of *S. typhimurium,* showed two or more easily distinguishable colonial growth phases. Each phase could be distinguished from the other by differences in size, shape, the shade of red in the central area, and pronounced differences in delicate pastel tints in the border. The culture of *S. typhimurium,* showed only one growth phase. The color of the colonies was a light shade of red extending from the center to the border. Each of two cultures of *E. coli* showed two growth phases, differing in shades of red in the central area and pastel tints in the borders of the colonies. One culture of *S. sonnei* showed two distinct colonial growth phases, one of which was larger and more irregular in shape than the other. The larger colonies contained irregular deposits of deep red color superimposed on a lighter shade of red; the border was tinted light yellow. The smaller colony was more circular; the central area was colored light red and the border a light yellow-green.

Colonies of the smooth phase and closely related phases of the 3 *Brucella* species can be distinguished from each other more easily on agar medium containing tetrazolium chloride than on regular tryptose agar. Colonies of the 3 species and all growth phases of each are circular in shape. The central circular area of S phase colonies of *Br. suis* is stained a darker (almost black) shade of red