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Embryonic Cell Respiration

The Effect of Methylene Blue and 2-4 Dinitrophenol on Embryonic Cell Respiration *

By JOSEPH HALL BODINE AND LAURENCE ROCKWELL FITZGERALD

ABSTRACT

The effects of M.b. and 2-4 D.N.P. on the oxygen uptake of mitotically active and blocked grasshopper embryos have been worked out over wide concentration ranges. For both reagents an increase in stimulating effects occurs with increasing concentrations up to a maximum. For M.b. this maximum stimulation continues over a very wide range of concentrations and little injury for exposures of 1 to 2 hours results. With 2-4 D.N.P. rather marked toxic effects occur after the maximum stimulating doses are increased. The relative efficiency of the two reagents is such that 2-4 D.N.P. exerts greater stimulating effects than M.b. at similar concentrations. No evidence has been obtained to indicate any additive effects of the reagents on the oxygen uptake when applied to the cell. Once maximum stimulation has been produced by either compound further additions of stimulating doses of either reagent do not change the result.

That methylene blue and related dyes stimulate respiration of various types of cells and tissues seems well established (1 to 7). Explanations of the mechanisms involved in such action, however, vary to an extreme degree. The stimulating effects of 2-4-dinitrophenol on oxygen consumption have also been demonstrated for many organisms (7 to 13). Inasmuch as both methylene blue and 2-4 dinitrophenol show marked stimulating effects upon cellular respiration in such a strikingly similar fashion it becomes of some interest to compare them and to learn further concerning the details of such action upon the respiration of a relatively simple and rigidly standardized biological material. To this end a rather intensive quantitative study has been made of the effects of these reagents upon the respiration of mitotically active and blocked embryos of the grasshopper, *Melanoplus differentialis*. Concentration effects, toxicity and possible antagonistic actions of these drugs have been especially noted. A semi-quantitative standard for the expression of relative degrees of stimulation produced by methylene blue and 2-4 dinitrophenol has been established and will serve as a basis for further comparative studies on inhibitors of cellular respiration.

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GENERAL PROCEDURE

Embryos, mitotically active and blocked, were dissected from eggs of the grasshopper in a manner essentially similar to that previously indicated (13). One hundred were suspended in Ringer solution (pH 6.8) and oxygen determinations carried out by the standard Warburg techniques using respiration flasks of 5 ml. capacity at 25°C. All reagents were made up in Ringer and added to the flasks from the sidearm so that concentrations given in the text are final ones in the respiration flasks. For all determinations a minimum of 18 manometers were used and averages plotted represent many runs for each concentration of reagent. Methylene blue employed was from the National Aniline and Chemical Company, Inc., while the 2-4 dinitrophenol was from Eastman Kodak Company. These compounds will be designated *M.b.* and 2-4 *D.N.P.* throughout this paper. Experimental respiration periods ran from 1 to 2 hours.

In all experiments no significant qualitative differences in re-

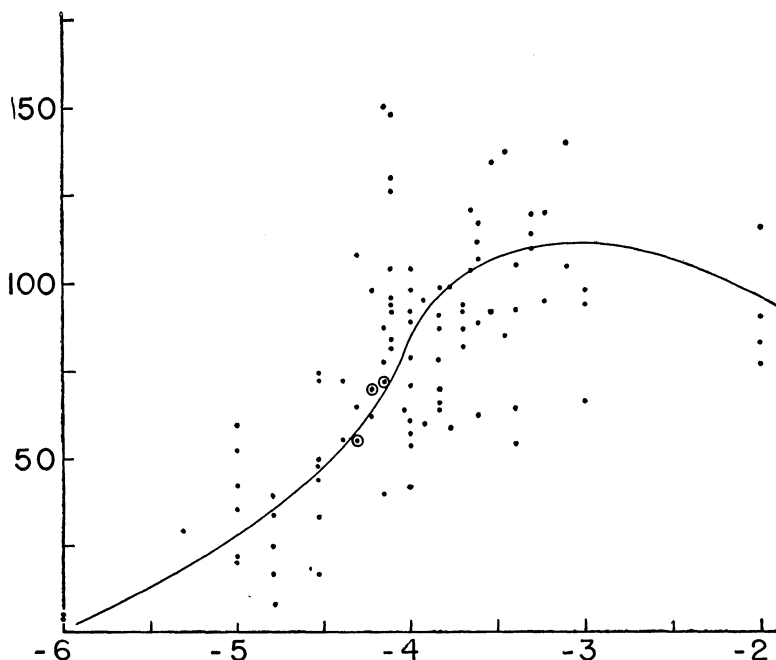


Figure 1 Shows effect of different concentrations of *M.b.* on oxygen consumption of embryos. Abscissa, negative log molar concentration of *M.b.* in Ringer. Ordinate, per cent increase in oxygen uptake of 100 embryos. All points are averages for 1 hour exposure periods. (Results for all figures are for 1-day postdiapause embryos.)

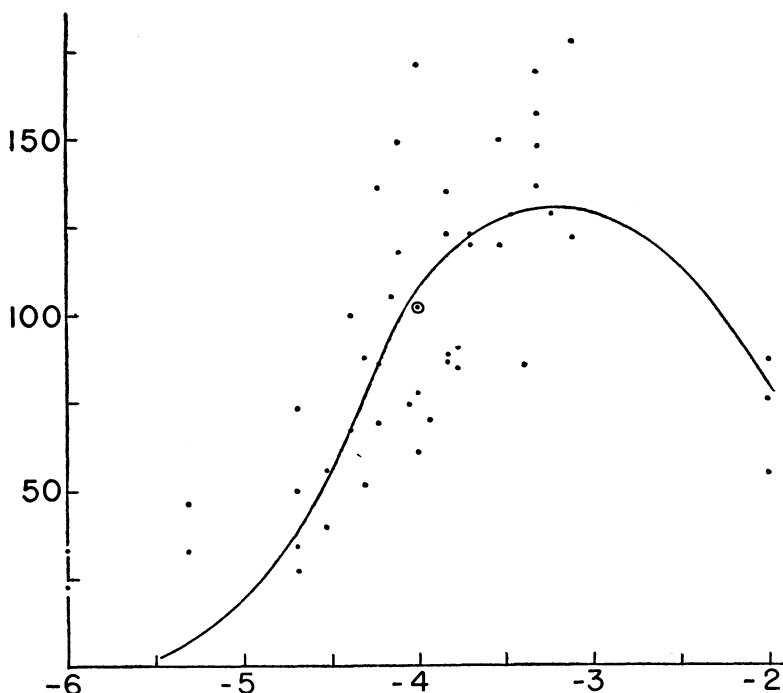


Figure 2 Same as figure 1, except for second hour of exposure to M.b.

sponse of active and blocked embryos were noted. Blocked embryos tended to be more stimulated than active ones (5).

METHYLENE BLUE

The effect of M.b. on the oxygen uptake of embryos was carried out over very wide concentration ranges in order to test both its stimulating as well as possible toxic action. Summarized results are graphically shown in figures 1 and 2. Most striking is the marked variation shown in the response to the reagent by embryos in similar morphological and physiological stages. In general, the stimulating action of M.b. increases with concentration in a fairly uniform manner and then reaches a plateau at which it remains over a wide concentration range. No marked toxic effects of the dye are noted until very high concentrations are employed. As a matter of fact the embryos become intensely stained in the higher concentrations and when returned from exposures of 2 hours and washed in Ringer, show rates of oxygen uptake but slightly different from controls (Fig. 3). Exposures to the dye for 2-hour periods are essentially similar in response to those for 1 hour and recovery is *practically identical* (Figs. 2 and 3). Curves have been drawn

through the points to indicate and approximate concentrations of dye necessary to produce different degrees of stimulation (Figs. 1 and 2). It is to be especially noted that, within limits, once maximum stimulation is reached, addition of dye produces no further response (Figs. 1 and 4).

2-4 DINITROPHENOL

The action of 2-4 D.N.P. on the oxygen uptake of embryos closely parallels that for M.b. in that marked variations in response

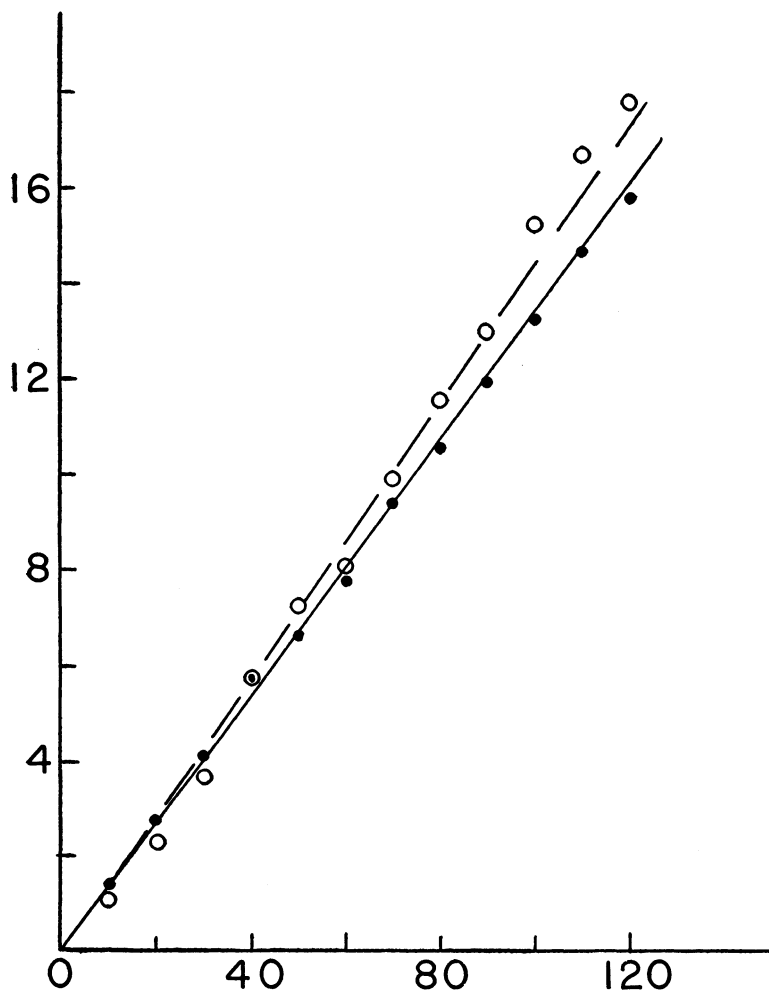


Figure 3 Shows recovery of oxygen uptake after exposure of 100 embryos to $25. \times 10^{-5}$ M. M.b. for 2 hours. Abscissa, time in minutes. Ordinate, (mm.)³ oxygen per 100 embryos. Solid circles, control; open circles, after 2 hours exposure to M.b., washed in Ringer and then suspended in Ringer.

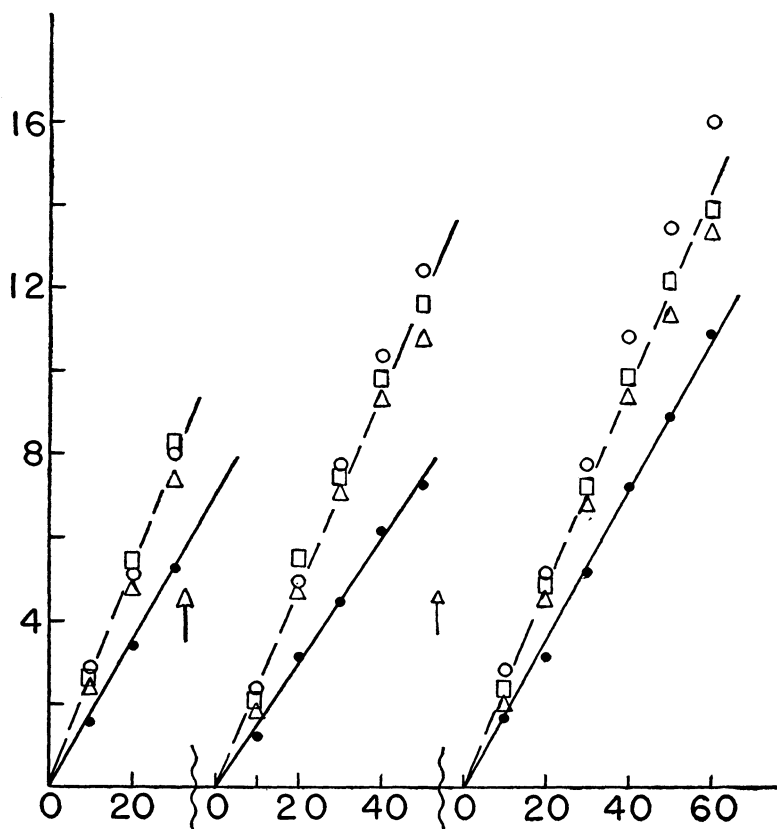


Figure 4 Shows effect of adding successive amounts of M.b. from two side arms of cups. Otherwise as in Fig. 1. Solid circles, Ringer then Ringer; open circles, $25. \times 10^{-5}$ M.b., then $25. \times 10^{-5}$ M.b., then $25. \times 10^{-5}$ M.b.; triangles, $25. \times 10^{-5}$ M.b., then 12.5×10^{-5} M.b., then Ringer; squares, $25. \times 10^{-5}$ M.b., then Ringer, then Ringer.

are noted for the stimulating concentrations employed (Fig. 5). A striking difference in the case of 2-4 D.N.P. is its notable toxicity once a maximum concentration is reached. A gradual increase in stimulation effects with concentration up to a maximum occurs but a definite toxicity effect then follows. A rough curve drawn through the points indicates the relative effects produced by the various concentrations of the drug. By comparison with M.b., it apparently takes a much lower concentration of 2-4 D.N.P. to produce similar stimulating effects.

COMBINATIONS OF M.B. AND 2-4 D.N.P.

The effects of both M.b. and 2-4 D.N.P. on the oxygen uptake are quite similar insofar as they both produce marked stimulation

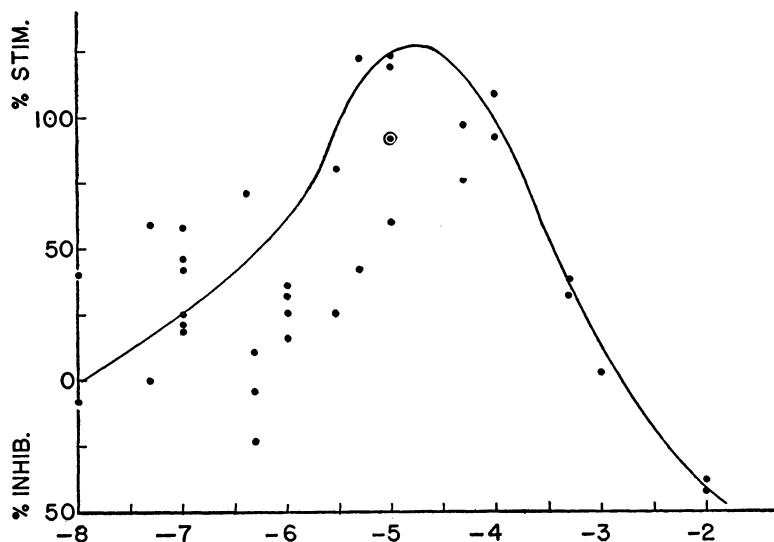


Figure 5 Same as figure 1 except for 2-4 D.N.P.

of the respiration of the active and blocked embryos. It becomes of some interest, therefore, to compare the action of combinations of the 2 compounds in order to see if additive or antagonistic effects are apparent. Results of such experiments might well serve as a basis for an analysis of the action of the 2 compounds on the respiratory mechanism of the cell.

When equi-potential doses (e.g., those producing 50% stimulation) of M.b. plus 2-4 D.N.P. are added to embryos no additive effects are noted and stimulation is still approximately 50% as for either compound when added separately (Figs. 6 and 7). Similar results are produced for all combinations employed. Once maximum stimulation is produced by either reagent further addition of the same, or of the other compound, causes no further changes. No indication of additive or antagonistic actions of the 2 compounds on the oxygen uptake have thus far been detected for stimulating doses. Perhaps one may reasonably assume that either two separate parts of a single respiratory system are affected by the drugs or that two quite different systems making up the complete respiratory mechanism of the cell are affected. Data to be presented in a future paper seem to indicate the latter of the two assumptions to be correct.

The order of addition of the reagents to the embryos apparently makes no difference in the final results for the oxygen uptake.

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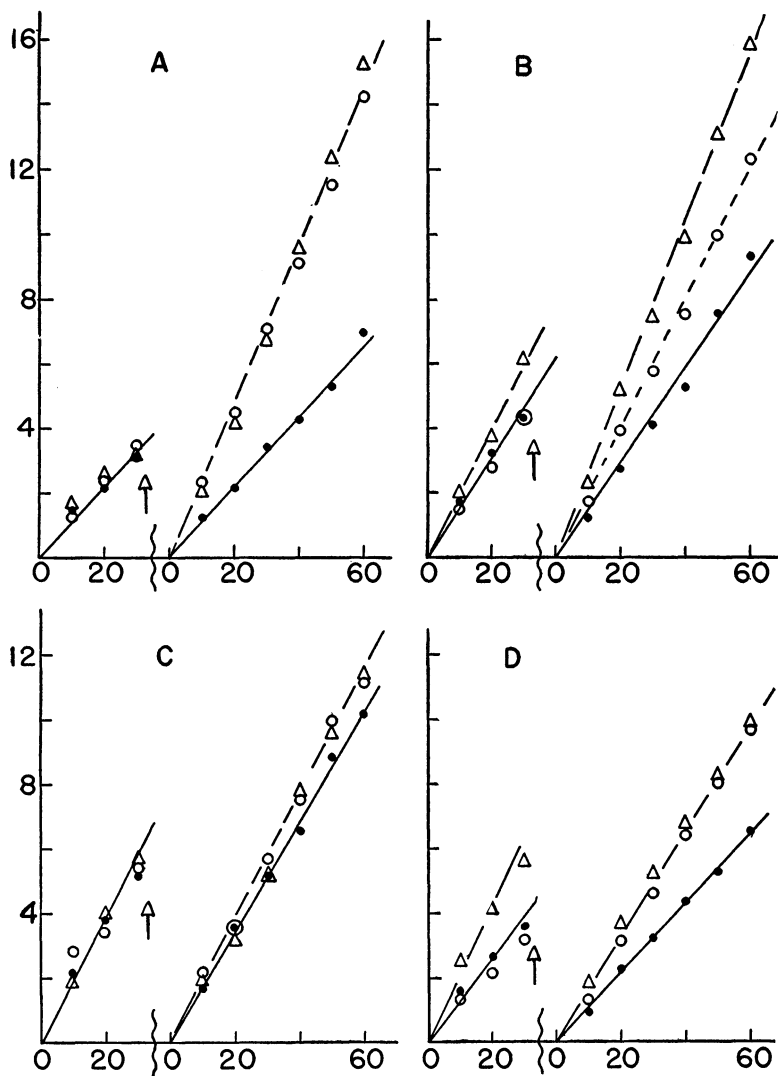


Figure 6 A Shows effect of 2-4 D.N.P. alone and when added with M.b. on the oxygen uptake of embryos. Abscissa, time in minutes. Ordinate, (mm.)³ oxygen per 100 embryos. Solid circle, Ringer; open circle, Ringer then 1×10^{-5} 2-4 D.N.P.; triangles, Ringer then 1×10^{-5} 2-4 D.N.P. plus 3×10^{-5} M.b. (all concentrations are final concentrations in respiration flask). Arrow, indicates time of emptying content of side arm into respiration vessel.

B Same as A except for slightly different procedure. Solid circles, Ringer; open circles, Ringer then 3×10^{-5} M.b.; triangles, 3×10^{-5} M.b. then 1×10^{-5} 2-4 D.N.P.

C Same as B. Solid circles, Ringer; open circles Ringer then 3×10^{-6} 2-4 D.N.P.; triangles, 3×10^{-6} 2-4 D.N.P. then 3×10^{-5} M.b.

D Same as B. Solid circles, Ringer; open circles, Ringer then 3×10^{-5} M.b.; triangles, 3×10^{-6} M.b. then 3×10^{-6} 2-4 D.N.P.

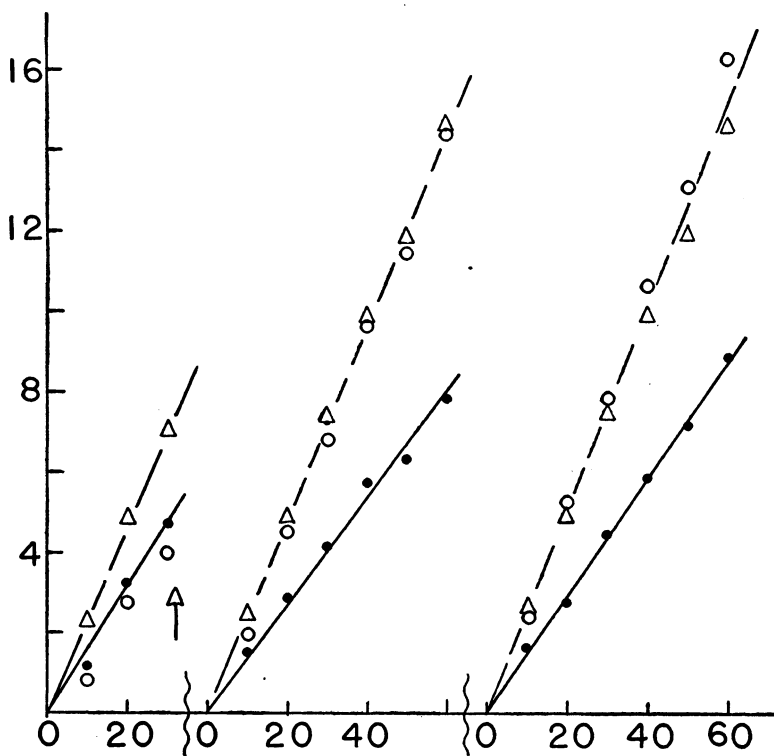


Figure 7 Shows effect of adding M.b. and 2-4 D.N.P. for 2-hour runs. Otherwise same as Fig. 1. Solid circles, Ringer; open circles, Ringer then 1×10^{-5} 2-4 D.N.P.; triangles, 1×10^{-5} 2-4 D.N.P. then 3×10^{-5} M.b.

DISCUSSION

Interest in the action of M.b. and 2-4 D.N.P. centers about the fact that these two compounds of quite different chemical natures produce rather strikingly similar effects upon respiration of a cell. The manner by which such stimulating action is brought about becomes of some importance since an understanding of such phenomena might possibly aid in the further elucidation of the respiratory mechanisms of the cell. The action of reagents such as narcotics or inhibitors of cellular respiration upon cells with increased oxygen uptake rates produced by these stimulating compounds may well afford clues as to many basic properties of both the cell and the action of the various chemicals involved in such reactions.

The effects of M.b. and related dyes on cells have been variously explained. Barron and others (3,4) have assumed such action to belong to the type of oxidative dehydrogenations and that their

catalytic roles are due to their reversibility and spontaneous oxidability by molecular oxygen without a catalyst. Brooks (14) considers the action to be based largely upon the redox properties of the dyes and their relations to similar properties of the cell. That redox dyes do stimulate the oxygen uptake of cells seems well established from the results of many investigators (2). The fact that cyanides reduce the increased oxygen consumption produced by M.b. has been demonstrated for the grasshopper embryo (6). However, in the case of the sea urchin egg, red blood cells and other biological materials, quite the reverse seems to be the case (4). M.b. has been shown by Brooks (14) to completely counteract the injurious effect of CO for a variety of biological forms. Grasshopper embryos behave in this respect much like the sea urchin egg (6). In general, it is usually concluded by all workers that M.b. and related compounds act as H acceptors or carriers in the Warburg system of cellular respiration.

The apparent lack of intensive, systematic studies on the effects of M.b. *per se* on cellular respiration prompted the present investigation. It seemed essential first to have a rather complete concentration-effect curve using a fairly standardized biological form before making comparative studies on the basic effects of the dye and related compounds. The outstanding variations noted in the responses to the dye by grasshopper embryos, practically identical morphologically and physiologically, are indeed of interest and illustrate the necessity of reserve in arriving at conclusions on the effects of such reagents without intensive supporting experimental background. Stimulation of respiration by such reagents is apparently conditioned by many factors inherent in experimental materials not at present well understood.

The effects of 2-4 D.N.P. on the respiratory activity of cells seem almost as variable as those for M.b. That the drug is more toxic is readily apparent. The exact mechanism of the action of this compound upon cellular metabolism seems less clear than for M.b. From existing data it seems that 2-4 D.N.P. does produce a stimulating as well as a decided toxic action on cells (12).

With the establishment of the effects of M.b. and 2-4 D.N.P. on the oxygen consumption of the cell we have available a background with which an analysis of the effects of narcotics, inhibitors, etc. can better be understood. It now seems reasonably certain that M.b. and 2-4 D.N.P. act quite independently and in no way can be considered additive or antagonistic in their effects upon the respira-

tory mechanism of the cell. When the respiratory system is stimulated by the one or both reagents to its maximum capacity, further addition of either reagent (within limits) in no way interferes with the original reaction. No evidence has thus far been obtained indicating any additive effects. One naturally assumes, therefore, that M.b. and 2-4 D.N.P., although producing similar final results on the oxygen consumption of the cell, must do so by either acting similarly on the same respiratory mechanism or through an entirely different set of reactions. In a subsequent report evidence will be presented indicating that perhaps the latter of these two assumptions is correct.

Results of experiments carried out on toad muscle by Ungar (15) indicate additive effects of M.b. and 2-4 D.N.P. on oxygen consumption but the material and methods are so different from those used in the present experiments that valid comparison cannot be made at this time.

CONCLUSION

The effects of M.b. and 2-4 D.N.P. on the oxygen uptake of mitotically active and blocked grasshopper embryos have been worked out over wide concentration ranges. For both reagents an increase in stimulating effects occurs with increasing concentrations up to a maximum. For M.b. this maximum stimulation continues over a very wide range of concentrations and little injury for exposures of 1 to 2 hours results. With 2-4 D.N.P. rather marked toxic effects occur after the maximum stimulating doses are increased. The relative efficiency of the two reagents is such that 2-4 D.N.P. exerts greater stimulating effects than M.b. at similar concentrations. No evidence has been obtained to indicate any additive effects of the reagents on the oxygen uptake when applied to the cell. Once maximum stimulation has been produced by either compound further additions of stimulating doses of either reagent do not change the result.

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