not dihydroxymaleic acid, as Fenton (*7, 8*) originally thought. The term *dihydroxyfumaric acid* is used in this paper to designate both the keto form and its enediols.

Attempts to demonstrate the enzyme reaction in the reverse direction—that is, reduction of DPN^+ by dihydroxyfumarate—have not been successful. Dye methods are unsatisfactory because of the high reducing power of dihydroxyfumarate in the absence of enzyme. Direct observation of a reduction of DPN^+ by spectrophotometric measurement at 340 mμ is made difficult by the fact that the concentration of dihydroxyfumarate must be kept small in order to avoid appreciable changes in light absorption due to the substrate alone (*5*). Furthermore, such measurements must be made under anaerobic conditions, since dihydroxyfumarate causes an enzyme-dependent oxidation of DPNH when it is added to a reaction mixture in a Beckman cuvette under aerobic conditions. This reaction is dependent on the presence of oxygen and is, thereby, sharply differentiated from the enzymatic oxidation of DPNH by diketosuccinate. The latter reaction proceeds at equal rates under aerobic and anaerobic conditions.

The results of a typical set of experiments illustrating these facts are shown in Fig. 1. The experiments were done with a pea root preparation buffered with phosphate and supplemented with DPNH. The light absorption at 340 mμ due to DPNH is plotted against time. The solid lines show the change that occurred when no precautions were taken to exclude oxygen. A precise duplicate of each reaction mixture was prepared under anaerobic conditions, opened at an appropriate time, and transferred immediately to a cuvette for spectrophotometric reading. The solid points represent the values thus observed. The upper curve shows the slow decline in DPNH in the absence of added substrate. This change is the same anaerobically and aerobically. An anaerobic incubation mixture with added dihydroxyfumarate (0.2 mg) contained as much DPNH after 60 min as the samples without added substrate, whereas the DPNH was almost completely oxidized when the same amount of dihydroxyfumarate was added aerobically. When diketosuccinate was added, on the other hand, the anaerobic and aerobic changes were equal within the limits of experimental error. Two sets of experiments are shown on the graph. Curve *A* was obtained with about 2 mg of diketosuccinate. Curve *B* was obtained with 0.2 mg of diketosuccinate and shows the marked decline in the reaction rate at about 20 min. This decline is believed to be due to the disappearance of the substrate by decarboxylation. The diketosuccinate does not oxidize DPNH in the absence of enzyme and TPNH cannot be substituted for DPNH in the enzyme reaction.

The results obtained with dihydroxyfumarate can be explained by the rapid autoxidation of the compound to diketosuccinate. The autoxidation occurs spontaneously and is also accelerated by an oxidase widely distributed in plants (*10–14*). The diketosuccinate so formed must then be reduced by the DPNH and diketosuccinate reductase. The reductase thus provides a link between any DPN-reducing system and dihydroxyfumaric acid oxidase. The possibility that a system of this nature may function in plant respiration has been suggested by Szent-Györgyi and his collaborators (*11, 12*). Diketosuccinate reductase furnishes the hitherto missing reducing system (*15*) [See (*16*), however].

A full evaluation of the results obtained with dihydroxyfumarate must take into account not only the autoxidations but also the decarboxylation of this substance (*17, 18*). In some plant preparations, for example, the anaerobic decarboxylation product of dihydroxyfumarate oxidizes DPNH enzymatically, and the dependence of the dihydroxyfumarate-DPNH reaction on oxygen is thereby partially obscured. A more detailed description of the nonenzymatic and enzymatic reactions that diketosuccinate and dihydroxyfumarate undergo will be presented elsewhere.

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Stability and Absorption Spectrum of Malononitrile

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The investigation of Hydén and Hartelius (*1*) into the effects of malononitrile on neuronal metabolism and mental disease aroused much interest in its mechanism of action and possible therapeutic value. They stated (*1*):

> It is possible by means of a chemical agent—in this case malononitrile—to stimulate the large nerve cells of the central nervous system to an increased production of nucleic acid and protein substances.

This appears to take place through stimulation of the protein producing system of the nerve cell to increased activity.

This *in vivo* effect of malononitrile as a stimulant to nucleoprotein synthesis has not been substantiated by American investigators. None of the American studies reported spectrophotometric analysis of the malononitrile solutions used. The experiments (*2*) described here show that such measurements are essential.

Materials and methods. Samples of malononitrile in crystalline form were obtained from several sources: Eastman Kodak Company, The Schering Corporation, Nutritional Biochemical Company, and Schwartz Laboratories. In addition, a 5-percent aqueous solution of $CH_2(CN)_2$ specially prepared by the Astra Chemical Company was obtained through the courtesy of Holger Hydén of the faculty of medicine in Göteborg, Sweden. The solution was said to have been "stabilized" by the addition of a simple substance. (This is discussed further in a later section.) It was the same solution used by Hydén and Hartelius in their original experiments.

Samples of the American-made malononitrile were prepared in 0.07*M* concentration for ultraviolet spectrophotometric study and allowed to stand at room temperature in daylight. Readings were taken at various intervals up to 104 days. In the visible region of the spectrum, peak concentrations of 1.5*M* and 0.15*M* were run in addition to the 0.07*M* solutions. Extinction coefficients used in plotting the spectra of these preparations are all based on the original concentrations of the malononitrile in solution.

The spectrum of the malononitrile solution prepared by the Astra Chemical Company was run in 0.01*M* concentration. This preparation was received as a 5-percent aqueous solution (0.75*M*); this was removed from the bottle with a syringe and diluted in a volumetric flask. The recordings were made with the Beckman DU spectrophotometer. The slit widths used for malononitrile solution peak recordings were 0.75 mm at 268 mμ and 0.20 mm at 358 mμ.

Results. Melting-point determinations were run on desiccated samples of malononitrile. The melting point of all samples of American manufacture was found to be 30° ±1°C. The Astra compound was evaporated to dryness in a vacuum desiccator. It showed no sharp melting point, but part of the sample melted at 30° and another part at 70°C.

The *p*H of all samples was determined and found to be on the acid side; a 1.0*M* solution had a *p*H of about 5, and a 0.1*M* solution had a *p*H of 6. There was no significant change in the *p*H of the solutions on standing for 20 days.

The Liebig titrametric and alkaline picrate colorimetric tests for cyanide ion gave indication in all samples tested of 0.1 to 0.3 percent cyanide ion. It is felt, however, that these results may not be reliable in that they could be due to the presence of such substances as isonitriles.

Spectra of the various malononitrile samples were

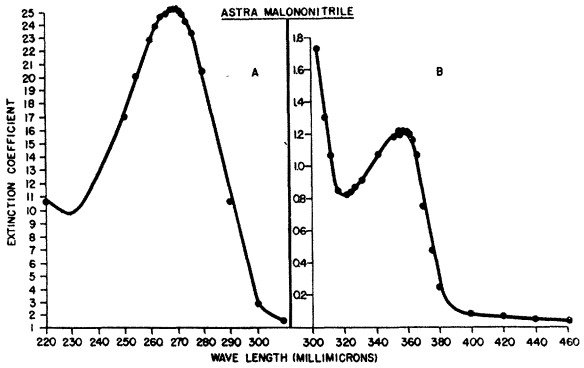

Fig. 1. Spectrum of $CH_2(CN)_2$ prepared by the Astra Chemical Co., Sweden. (*A*) Ultraviolet absorption spectrum, showing the peak at 268 mμ. (*B*) Absorption spectrum in the visible region, showing the peak at approximately 358 mμ.

obtained in both the visible and the ultraviolet. Hydén and Hartelius (1948) had published the ultraviolet absorption spectrum for the Astra malononitrile employed in their experiments. Plotting extinction (which we call absorbance—not extinction coefficient) against wavelength, they reported a peak at 2700 A with an absorption maximum of approximately 0.53. The spectrum obtained in our laboratory for an Astra sample shows a similar peak at 268 mμ with an extinction coefficient of 25 (Fig. 1*A*). Readings taken on this sample in the visible region show a second peak at 358 mμ with a relatively low extinction coefficient of 1.3 (Fig. 1*B*). This region of the spectrum was not reported by Hydén and Hartelius.

When freshly prepared in aqueous solution, none of the American-made products showed any peaks, either in the ultraviolet or visible regions; rather they showed a gradual decrease in absorbance in the direction of the region of longer wavelength. However, when these solutions were allowed to stand at room temperature, with the passage of time, a steady increase in absorbance was noted leading to peak for-

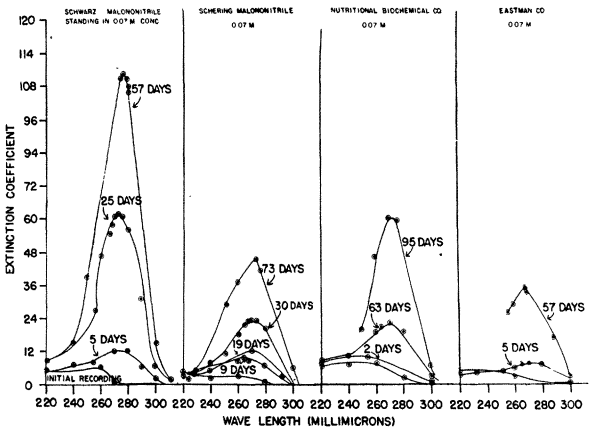

Fig. 2. Absorption spectra of American- and Swedish-made $CH_2(CN)_2$ in aqueous solutions in the ultraviolet and visible regions, showing the changes with time.

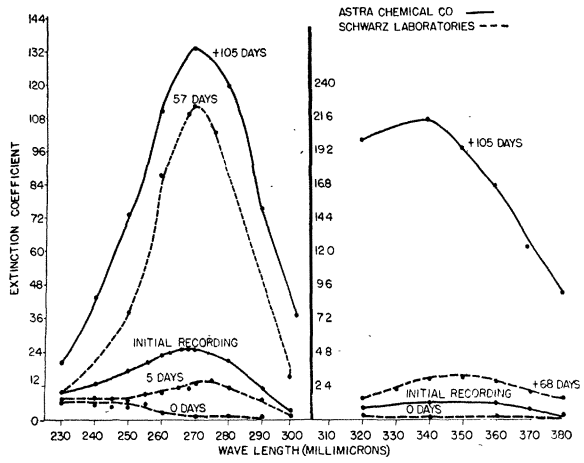

Fig. 3. The effect of the passage of time on the ultraviolet absorption spectra of aqueous solutions of four different samples of American-made $CH_2(CN_2)$. In each case, the curve nearest the abscissa represents the initial recording.

mation at both 268 and 358 mμ. Different rates of change were observed for different samples (Fig. 2). Although little change was evidenced by the Astra solution when stored under refrigeration in the dark, a dilute aqueous solution (0.01*M*) of the Astra product keep at room temperature also showed an increase in extinction coefficient of the peaks at both 268 and 358 mμ (Fig. 3).

Discussion and conclusions. When the malononitrile samples arrived, it became apparent almost at once that they were not stable; that is, some decomposition process was occurring, as evidenced by the formation of a brownish liquid, no matter under what conditions the samples were kept, such as in the dark and under refrigeration. Upon inquiry, additional information relative to this problem was obtained from two sources. The Schering Corporation stated that this substance was unstable, as evidenced by a steady increase in the amount of malononitrile necessary to produce an LD_{50} in rats, an increase that was time dependent—that is, as the malononitrile aged in crystalline form (*3*).

For the remainder of our information in this regard, we are indebted to Holger Hydén. In answer to our query on whether he had observed such decomposition, he informed us that he had. As a result, he had used, henceforth, a "stable" substance prepared for him by the Astra Chemical Company. Something was added by this company to "stabilize" the solution. He promptly sent us a sample of this Swedish preparation. *Our spectrophotometric data indicate that this Astra malononitrile* (as employed by Hydén and Hartelius) *is not the same as the freshly prepared solutions used by the American investigators.* The American preparations, when left standing at room temperature for a long enough period of time, develop the same spectral characteristics as the Astra compound. Different samples of American malononitrile changed at different rates. This may be a result of the presence of different concentrations of impurities that catalyze the reaction. Two conclusions can be drawn from this observation: (i) The Swedish preparation is not malononitrile stabilized in its pure state but appears to be a partially stabilized decomposition or reaction product, and (ii) It is not necessary to add any other substance to the malononitrile (as Astra did) to produce this so-called "stabilized" substance, since it appears in aqueous solutions of the American products merely as a result of the passage of time.

The present experiments showed that, under certain conditions, the Astra product also changes with the passage of time; that is, the absorbance at the 268 and 358 mμ peaks increases, as is the case with the American product. Consequently, in regard to this factor at least, it is evident that the Astra compound is not a truly "stable" one.

Many things could be happening to such malononitrile solutions, such as polymerization, formation of cyclic structures, and so forth; but, until further detailed information is obtained relative to this question, no more can be said than that the malononitrile does undergo a change, by which a second and different substance is formed. Thus, it becomes obvious that the compound used by Hydén and Hartelius was in all likelihood a reaction product of malononitrile or a mixture of malononitrile and its reaction products. *The fresh solutions used by other investigators, since they were not of the same chemical structure, would not be expected to have the same biological activity. On the basis of these findings, it would appear that a reevaluation of the usefulness of malononitrile in the therapy of mental disease must be made.* These data may help to explain why the numerous reports dealing with the role of malononitrile in therapy (*4–6*) of mental disease, as well as other diseases involving nucleoprotein metabolism in the central nervous system (*7–9*), have differed in certain respects from one another and, in general, from that of Hydén and Hartelius.

The question that arises concerns the action of the aged solution. In accordance with the findings of Hydén and Hartelius, it is possible that nucleoprotein stimulation could be part of an initial "excitatory" period following interference with cellular oxidative mechanisms, but it is also possible that these two factors are completely independent of each other. We face, therefore, several complex problems: (i) What is the structure of the active substance? (ii) What is its specific action *in vivo* as compared with the results obtained *in vitro*? (iii) Is there a therapeutic effect of the active substance?

The structure of the active substance is not merely of academic interest. It may be that this is one of that group of substances whose combination with specific cell constituents is dependent on particular molecular structure or reactive groups. It is hoped that investigations of the infrared spectra, which are under way, may provide some information in this regard. If studies of other dinitriles should show this to be so, then

Ferguson's principle could be applied—namely, substances that are present at the same proportional saturation in a given medium have the same degree of biological action—and indication could be obtained regarding the presence or absence of structurally specific or nonspecific action. This principle has already been fruitful in biological investigation; for example, in studies of action of narcotics (*10*). Future experiments are being designed with this in mind.

SUMMARY

1) Spectra in the ultraviolet and visible regions have been obtained on samples of malononitrile under different conditions. The samples were manufactured by several companies in America and Sweden.

2) The spectral analysis has demonstrated that the compound used by Hydén and Hartelius is not the same as that employed by American investigators. If, however, an aqueous solution of the American product is allowed to stand at room temperature for a long enough time, it develops the same spectral characteristics as the Swedish solution.

3) Future studies will concern themselves with attempts to identify the active substance and to examine its effect on neuronal nucleoproteins.

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Stimulus Control of Food- and Shock-Maintained Behavior

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This paper presents a technique for maintaining behavior in a rat that is alternately under the control of shock avoidance and food reinforcement (*1*).

Sidman described a conditioning procedure that maintained a constant rate of bar pressing when each bar press postponed a brief electric shock (*2*). Techniques have also been developed for maintaining lever pressing by following lever-pressing responses intermittently with food. Moment-to-moment rate changes have been generated that are both reproducible and a function of how reinforcement is made contingent upon the bar press (*3–5*). Under both of these techniques, the momentary rate, as well as the day-to-day level of responding, are sensitive base lines for the study of the control of behavior by noxious stimuli and food reinforcement.

Alternate periods of buzzer and no buzzer were used. When the buzzer was off, the Sidman-avoidance procedure was in force. Each lever press postponed a 1/5-sec electric shock by 30 sec. If no bar presses were made, the shock recurred every 30 sec. After 8 min of avoidance procedure, the buzzer was turned on, the shocking circuit was disconnected, and the first lever press occurring after 8 min was followed by a pellet of food. The buzzer was then turned off, the program reverted to the shock-avoidance procedure, and the cycle was repeated. Two cumulative recorders operating in tandem recorded the lever presses that occurred during these alternate periods of buzzer on and buzzer off.

Figures 1 and 2 are cumulative response curves after 120 hr of the avoidance and food behavior. The rat behaved appropriately to the food- and shock-avoidance schedules. When the buzzer was off and the shock-avoidance procedure was operating, the bar pressing occurred at a constant rate of 6 responses/min. When the buzzer was on and the food-reinforcement procedure was operating, the rate of lever pressing was zero for 1 to 2 min after the receipt of a food pellet; during the remainder of the 8-min period, the rate increased gradually to a terminal rate of about 60 responses/min. The diagonal marks on the food record indicate where a food pellet was delivered. Similar curves were recorded for two other rats.

The behaviors under the two schedules show little effect upon each other. The constant rate of emission

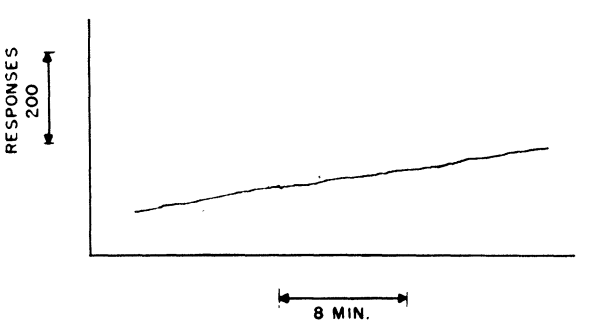

Fig. 1. Each bar press postpones a brief electric shock for 30 sec.

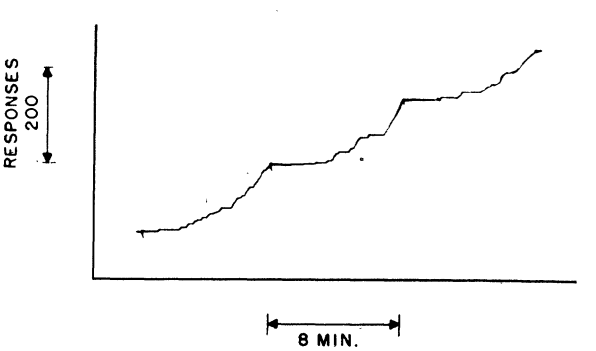

Fig. 2. The first bar press after 8 min produces a pellet.