and Weisenborn, F. L. (1965), *J. Antibiotics (Tokyo) B18*, 128.
Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.
Pressman, B. C. (1963), Energy Linked Functions of Mitochondria, New York, N. Y., Academic, p 181.
Pressman, B. C. (1965a), *Federation Proc. 24*, 425.
Pressman, B. C. (1965b), *Proc. Natl. Acad. Sci. U. S. 53*, 1076.
Sumner, J. B. (1944), *Science 100*, 413.

Antibiotics As Tools for Metabolic Studies. VI. Damped Oscillatory Swelling of Mitochondria Induced by Nonactin, Monactin, Dinactin, and Trinactin*

Stanley N. Graven, Henry A. Lardy, and Anthony Rutter

ABSTRACT: Nonactin, monactin, dinactin, and trinactin, at concentrations of 10^{-8}–10^{-10} M, alter the light scattering properties of rat liver mitochondria. To induce swelling these antibiotics require an alkali metal cation (Na^+, K^+, Rb^+, or Cs^+), acetate or phosphate, and adenosine triphosphate (ATP) or substrate. At antibiotic concentrations between 10^{-5} and 10^{-6} M in the presence of monovalent cation, a permeant anion, and substrate or ATP, damped oscillations in the light scattering properties of the mitochondria were observed. The swelling of mitochondria induced by the nonactin homologs was dependent on an energy source and recontraction occurred when the energy source was blocked. The oscillations appeared to be the result of cyclic interruptions in the energy required to maintain the mitochondria in the swollen state. It is proposed that the antibiotics act by inducing alterations in the ion translocation system.

The cytotoxic antibiotics nonactin, monactin, dinactin, and trinactin are potent uncouplers of oxidative phosphorylation and inducers of ATP[1] hydrolysis in rat liver mitochondria (Graven *et al.*, 1966). The activity of the antibiotics in these phenomena is influenced by the species and concentration of alkali metal cations in the media. Because of the relationship between adenosine triphosphatase activity and monovalent cation composition of the media, the influence of these antibiotics on mitochondrial swelling was investigated. The following is a report of the changes in the light scattering properties of rat liver mitochondria induced by the nonactin homologs.

Materials and Methods

The mitochondria were prepared by the method of Lardy and Wellman (1952) with the exception that the homogenizing medium contained 0.07 M sucrose and 0.25 M mannitol instead of 0.25 M sucrose. Male, 150–200-g rats were used. The mitochondria were suspended in a volume of sucrose–mannitol medium to make 1 ml/g of original liver, maintained at 0°, and used within 1 hr of preparation. Light scattering by the mitochondria was measured at 180° with a Cary Model 15 recording spectrophotometer at 515 mμ using a 1-cm² cuvet. The reaction was initiated by adding 2.9 ml of reaction mixture (at 23°) to 0.07–0.1 ml of mitochondria in the cuvet. The volume of mitochondria employed was selected to produce an initial apparent optical density reading of 1.8 when suspended in 2.9 ml of 0.25 M sucrose. The reaction mixtures are shown in the figure legends. All studies were conducted at a room temperature of *ca.* 23°.

Doubly distilled water and chemicals of highest purity available were used in the work described. The sources of antibiotics were presented in the preceding paper (Graven *et al.*, 1966).

When added, acetate and formate were neutralized with triethanolamine (TEA). Sodium and potassium salts of ATP were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Tris and diethanolamine salts of ATP were obtained from the Sigma Co., St. Louis, Mo.

Results

The observations on the effect of the antibiotics on the light scattering properties of mitochondria were

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphate; TEA, triethanolamine; DPN, 2,4-dinitrophenol.

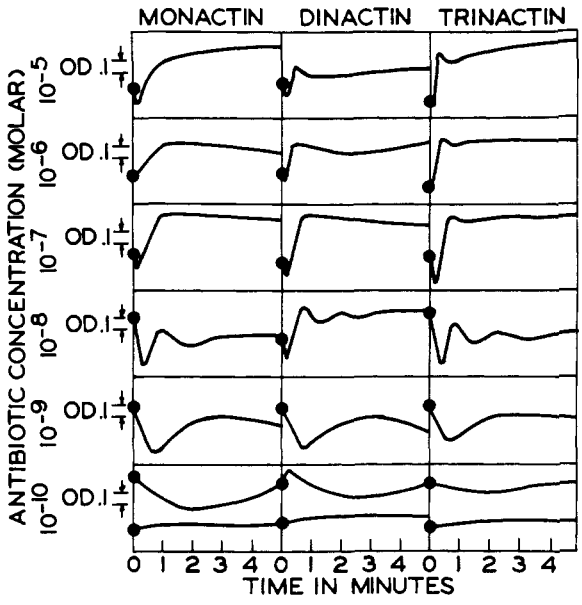

FIGURE 1: The effect of monactin, dinactin, and trinactin on apparent optical density of mitochondria in a potassium-containing medium. The basic reaction medium was: KCl 0.075 M; TEA (Cl) 0.01 M; acetate 0.008 M; ATP 0.004 M; sucrose 0.04 M, at pH 7.4. The volume was 3 ml containing 0.15–0.25 mg of mitochondrial nitrogen. The initial optical density was between 1.5 and 1.9 at 23°. The bottom line is the control without antibiotic.

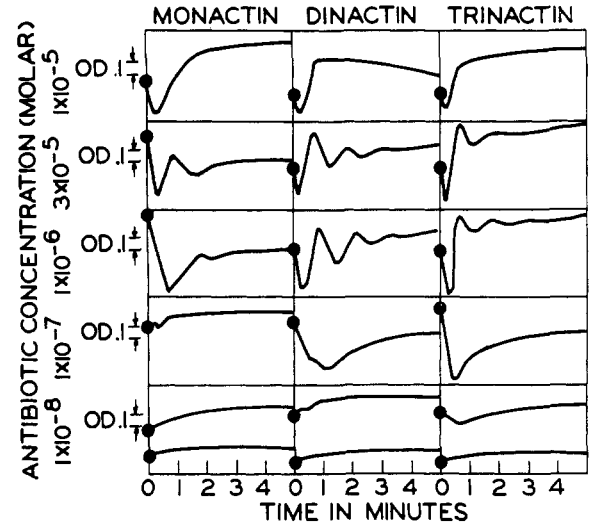

FIGURE 2: The effect of monactin, dinactin, and trinactin on apparent optical density of mitochondria in the presence of sodium ions. The conditions were as in Figure 1 with 0.075 M NaCl in place of KCl.

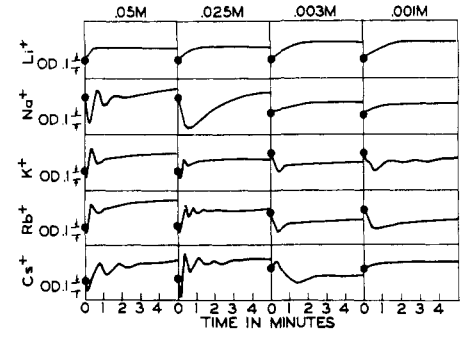

FIGURE 3: The effect of different concentrations of alkali metal cations on the changes in apparent optical density induced by dinactin. The basic medium was: ATP 0.006 M; acetate 0.008 M; sucrose 0.09 M; TEA (Cl) 0.01 M; dinactin 2×10^{-6} M, at pH 7.4 in 3 ml of volume containing 0.15–0.25 mg of mitochondrial nitrogen. The cation concentrations are as shown in the figure.

primarily limited to those effects which occurred during 5–10 min following the addition of the antibiotic to the mitochondria. It was noted from preliminary experiments that the induction of changes in the light scattering properties of mitochondria by the nonactin homologs required a source of energy (ATP or substrate oxidation), an alkali metal cation, and a permeant anion. In all of the data to follow, a decrease in apparent optical density (downward deflection of the trace) is associated with mitochondrial swelling and an increase with contraction.

Antibiotic Concentration. The effect of increasing concentrations of mon-, di-, and trinactin in a potassium-containing medium is shown in Figure 1. Swelling followed by spontaneous contraction was consistently induced by all three antibiotics at a concentration of 10^{-10} M. At antibiotic concentrations of 10^{-8} M the mitochondria alternately swelled and contracted through two to three cycles. At concentrations of 10^{-7} M and above there was a small rapid initial swelling followed by sustained contraction. A similar pattern of responses was observed with Na^+ as the only alkali metal cation added (Figure 2); however, the concentration of antibiotics required was greater by a factor of *ca.* 100. Minimal swelling was observed at antibiotic concentrations of 10^{-8} M, oscillations at 10^{-6} M, and sustained contraction at 10^{-5} M. In the absence of antibiotic there was no significant change in optical density (lower lines of Figure 2).

Alkali Metal Cation. The influence of varying concentrations of different alkali metal cations on swelling induced by 2×10^{-6} M dinactin is presented in Figure 3. No swelling was observed in the presence of Li at any of the concentrations tested. In the presence of Na^+, oscillations were observed at 0.05 M, swelling and contraction at 0.025 M, and no effect at Na^+ concentrations below 0.005 M. The antibiotic-induced swelling

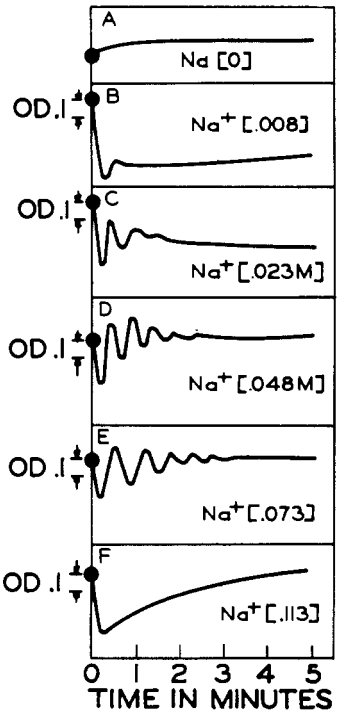

FIGURE 4: The influence of Na^+ concentration on the oscillations induced by trinactin. The basic medium was as in Figure 3 except 0.008 M phosphate replaced acetate and the Na^+ concentrations are as shown in the figure. As the Na^+ concentration increased the sucrose concentration decreased to keep the tonicity comparable; volume, pH, and nitrogen concentration as in Figure 3.

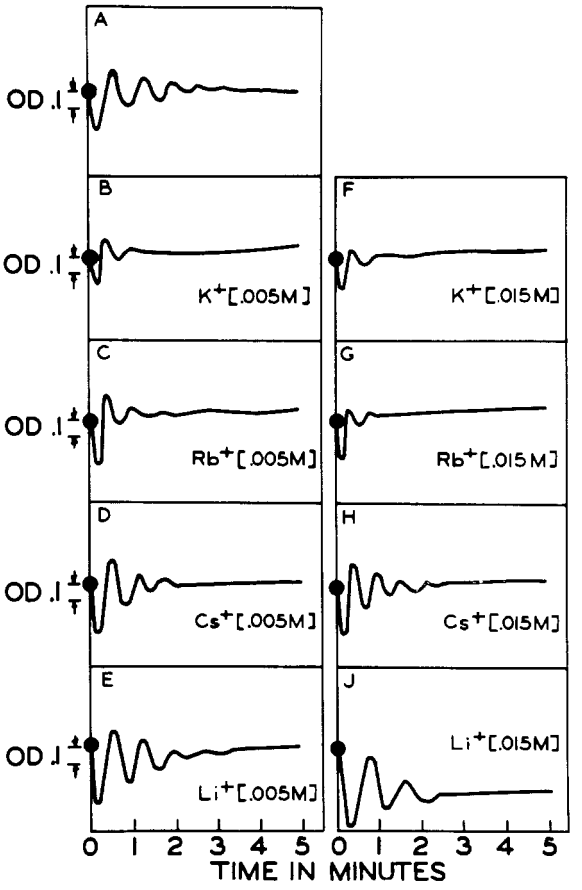

FIGURE 5: The effect of low concentrations of K^+, Rb^+, Cs^+, and Li^+ on the oscillations induced by trinactin in 0.075 M Na^+. The basic medium and conditions were as in Figure 4 with the addition of the cations as indicated with each curve.

at a K^+ concentration of 0.001 M and one oscillation at 0.05 M. The effects of the antibiotic in Rb^+ were similar to the patterns observed in K^+. In the presence of 0.025 M Cs^+, two or three oscillations were observed. No swelling occurred in 0.001 M Cs^+.

The effect of different concentrations of Na^+ on the oscillations induced by trinactin are presented in Figure 4. Five cycles of swelling and contracting occurred in the presence of 0.073 M Na^+. The greatest number of oscillations was observed when the Na^+ concentration was between 0.06 and 0.075 M. Oscillations were not observed at Na^+ concentrations above 0.10 M or below 0.01 M. The effect of other cations on the oscillations induced by trinactin in the presence of 0.07 M Na^+ is presented in Figure 5. Small additions of K^+ or Rb^+ (B, C, F, and G, Figure 5) markedly altered the pattern of oscillations from that observed in the presence of Na^+ alone. Cesium had much less effect on the pattern of oscillations at either 0.005 or 0.015 M concentrations. Li^+ at 0.005 M had no effect on the pattern of oscillation; at 0.015 M the period of the oscillations increased with a gradual swelling of the mitochondria. This effect was observed only with Li^+.

ATP Concentration. The effects of different concentrations of ATP are presented in Figure 6. In the absence of exogenous ATP and with mitochondria treated for 10 min with 2,4-dinitrophenol (10^{-4} M) to remove endogenous ATP, no swelling was induced by dinactin. With untreated mitochondria swelling and contraction were induced by the antibiotics, presumably associated with the hydrolysis of endogenous ATP. Increasing concentrations of ATP gave increasing numbers of oscillations with the maximum number obtained at an ATP concentration between 4×10^{-3} and 10^{-2} M. The addition of phosphocreatine and creatine kinase to convert ADP to ATP did not increase the number or amplitude of the oscillations.

Oligomycin inhibited all except the first oscillation induced by trinactin with ATP as the energy source (Figure 7B). When oligomycin was added to mitochondria 1 min before the trinactin, no swelling or contracting was observed. Aurovertin (Figure 7C) altered the pattern of oscillations by decreasing the amplitude and increasing the period. In the presence of aurovertin only one–two cycles were observed. Dinitrophenol (5×10^{-5} M) caused only a slight decrease in the amplitude of the oscillations (Figure 7D).

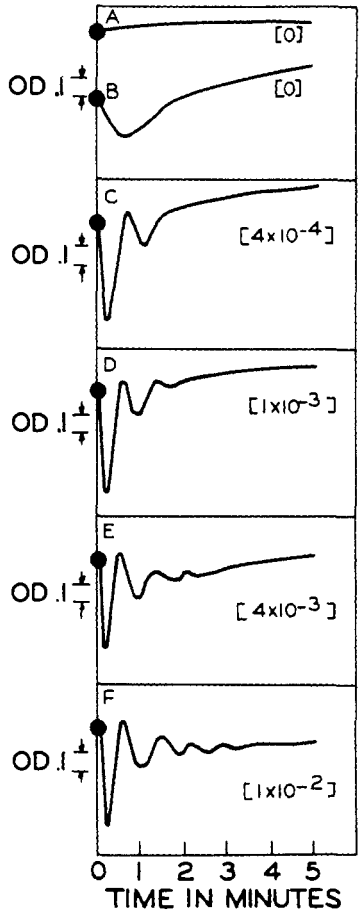

FIGURE 6: The effect of ATP on the oscillations induced by dinactin in presence of Na^+. The medium contained: NaCl 0.075 M; acetate 0.008 M; TEA 0.01 M; sucrose 0.09 M; dinactin 2×10^{-6} M. The molarity of added ATP is shown in brackets. Line A has no added ATP using mitochondria incubated at 30° for 10 min with 1×10^{-5} M 2,4-dinitrophenol and washed three times with cold sucrose. Line B has no added ATP; it and the subsequent lines are with fresh mitochondria. The volume, pH, temperature, and mitochondrial concentration are as in Figure 1.

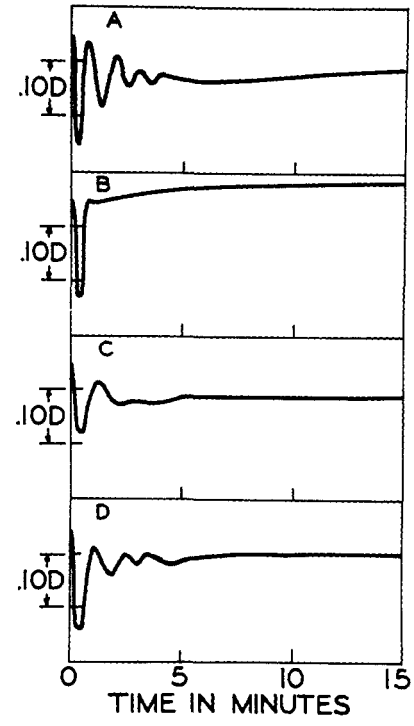

FIGURE 7: The effect of oligomycin, aurovertin, and 2,4-dinitrophenol on the oscillations induced by trinactin in the presence of Na^+. The basic medium is: NaCl 0.06 M; acetate 0.008 M; ATP 0.003 M; TEA (Cl) 0.01 M; sucrose 0.09 M; trinactin 4×10^{-7} M. The volume was 6 ml at 25°. Mitochondria equivalent to *ca.* 0.6 mg of N were used. The optical density was recorded manually using an Evelyn colorimeter with 520 filter. The A line is the control pattern, B is with oligomycin, 1 μg/ml, C is with aurovertin, 1 μg/ml, and D is with 5×10^{-5} M 2,4-dinitrophenol.

Anions. Changes in the optical density of mitochondria induced by nonactin homologs required the presence of a permeant anion of the type described by Sallis and DeLuca (1964) and Rasmussen *et al.* (1964) for ATPase and swelling induced by parathyroid hormone. No swelling was observed with chloride as the only added anion. The effects of different concentrations of phosphate, acetate, and formate on the swelling and oscillations induced by dinactin in the presence of Na^+ are presented in Figure 8. Little swelling and few oscillations were observed with formate as the anion. The greatest number of oscillations occurred at phosphate concentrations between 0.0017 and 0.008 M. Fewer oscillations were observed at an acetate concentration of 0.0017 M than at 0.008 M. At 0.017 M acetate the period of the oscillation was lengthened and the amplitude increased while the oscillations were dampened at a phosphate concentration of 0.017 M.

Substrate-Supported Swelling. In the absence of added ATP, the oxidation of substrates can support the oscillations of light scattering induced by the nonactin homologs (Figures 9–11) but the conditions required for oscillation are much more restricted than with ATP. Nonactin, in the presence of K^+ and acetate, induced oscillations supported by either ATP or substrate oxidation (Figure 9). The substrate-supported oscillations have a longer period but similar amplitude to those supported by ATP. The effect of anaerobiosis on swelling induced by dinactin is presented in Figure 10. When the medium became anaerobic (B at 10 min) the mitochondria contracted but rapidly swelled again on addition of oxygen, a process that could be repeated until the substrate was exhausted. The oscillations supported by ATP were not altered by the presence of substrate (C in Figure 10); however, at the completion of the oscillations the mitochondria remained in a partially swollen condition rather than contracted (Figure 10, *cf.* A and C). Mitochondria in the presence

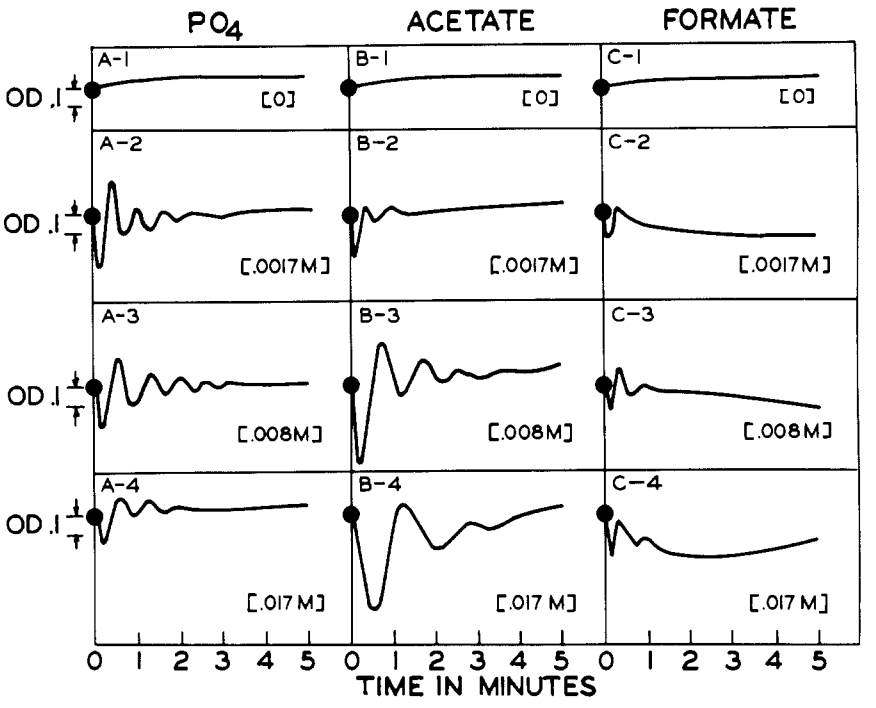

FIGURE 8: The effect of anion concentration on the oscillations induced by dinactin. The basic medium was: NaCl 0.073 M; TEA (Cl) 0.01 M; ATP 0.006 M; sucrose 0.08 M; dinactin 2×10^{-6} M. The concentration of the PO_4, acetate, and formate are as shown in the figure. The volume, temperature, pH, and mitochondrial concentration are as in Figure 1.

of both ATP and substrate contracted when the medium became anaerobic, as they did with substrate alone.

The addition of ADP did not alter significantly the swelling induced by trinactin and supported by β-hydroxybutyrate (Figure 11-I). No swelling was observed with β-hydroxybutyrate alone or in the presence of rotenone and nonactin. Swelling, and in some cases oscillations, were induced by nonactin in the presence of β-hydroxybutyrate or succinate. The oscillation observed with BOHB + nonactin (Figure 11, curve II-D) was essentially the same as that observed with succinate and nonactin. Rotenone inhibited the swelling supported by β-hydroxybutyrate but did not inhibit the swelling supported by succinate (Figure 11-II-E). Antimycin A and 2,4-dinitrophenol inhibited the β-hydroxybutyrate-supported swelling induced by trinactin while oligomycin and aurovertin failed to inhibit the substrate-supported swelling (Figure 11-III and IV). The ATP-supported swelling induced by nonactin was only slightly inhibited by ADP (Figure 11-V). Succinate-supported swelling induced by trinactin was reversed by the addition of malonate. The subsequent addition of ATP caused the mitochondria to swell again (Figure 11-VI).

Discussion

The nonactin homologs have been shown to have profound effects on oxidative phosphorylation and ATPase induction (Graven *et al.*, 1966). The data presented above demonstrate that the nonactin homologs also induce changes in the optical density of mitochondria at concentrations as low as 10^{-10} M. Trinactin, dinactin, and monactin appear to be active in lower concentrations than nonactin, but by a factor of 10^{-1} or 10^{-2} at most. By adjusting the cation environment and concentration of the antibiotic it is possible to produce similar effects with any of the four homologs. In many experiments the antibiotics were interchangeable with only minor modifications of the media. The nonactin homologs uncouple oxidative phosphorylation but only at very high concentrations do they inhibit respiration (Graven *et al.*, 1966). They induce ATPase, and ATP-supported swelling is blocked by oligomycin (Figure 7 and Graven *et al.*, 1966), indicating the participation of ATP hydrolysis in the swelling phenomenon. Substrate-supported swelling induced by nonactin homologs or phosphate (Connelly and Lardy, 1964) is not blocked by oligomycin. Swelling induced by the nonactin homologs and supported by ATP is not inhibited by 2,4-dinitrophenol (DNP) at 5×10^{-5} M while substrate-supported swelling is blocked by that concentration of DNP (Figures 7 and 11). Thus, to induce swelling in mitochondria the nonactin homologs require a source of energy, either substrate oxidation or ATP hydrolysis. If the source of energy is blocked, the mitochondria recontract. The nonactin homologs can induce swelling supported by substrate oxidation

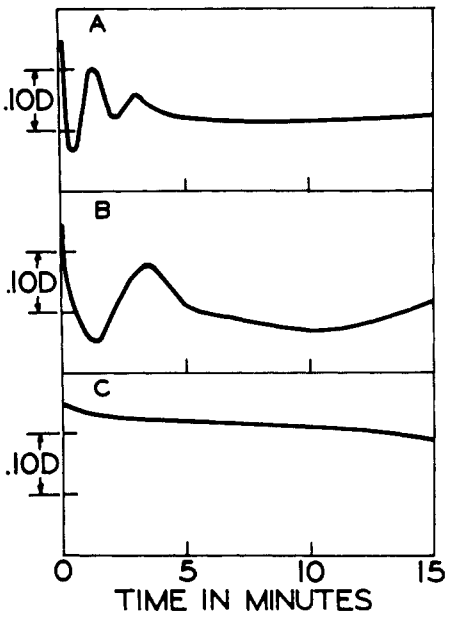

FIGURE 9: The induction of oscillations by nonactin supported by ATP and β-hydroxybutyrate. The basic medium was KCl 0.06 M; acetate 0.008 M; TEA (Cl) 0.01 M; sucrose 0.08 M. The medium for curve A had 0.003 M ATP and 4×10^{-7} M nonactin. The medium for curve B contained 0.013 M β-hydroxybutyrate and 4×10^{-7} M nonactin. The medium for curve C contained 0.013 M β-hydroxybutyrate or 0.003 M ATP but no antibiotic. The volume, pH, and method were the same as for Figure 7.

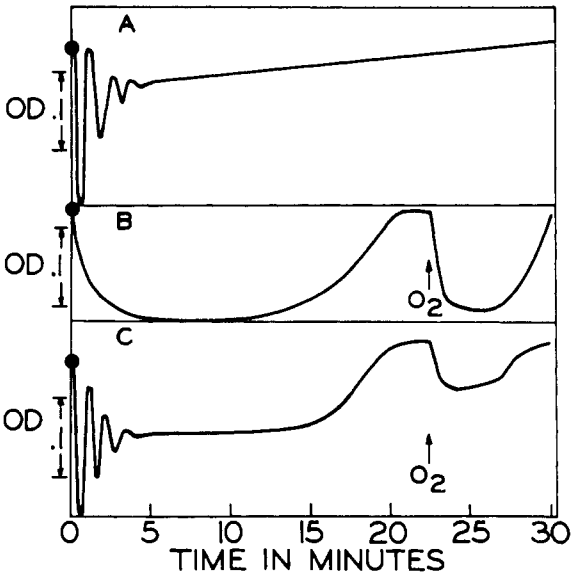

FIGURE 10: The effect of ATP and substrate on the oscillations induced by trinactin. The basic media was: NaCl 0.06 M; acetate 0.008 M; TEA (Cl) 0.01 M; sucrose 0.08 M; trinactin 4×10^{-7} M. Curve A has 0.003 M ATP; curve B 0.015 M glutamate and C has both 0.003 M ATP and 0.015 M glutamate. The O_2 was introduced by shaking the tubes. The volume, pH, and method were the same as for Figure 7.

in the presence of acetate and in the absence of phosphate. This effect is not blocked by oligomycin. This would indicate that there is a high energy sequence which leads to the site of action of the nonactin homologs without involving a phosphorylated intermediate or phosphate as an anion. The nonactin homologs at concentrations of 1×10^{-7} and 3×10^{-8} M have been shown to decrease but not completely inhibit the net uptake of phosphate by ADP. These same concentrations of antibiotics are capable of inducing nearly complete hydrolysis of the ATP added to the reaction mixture (Graven *et al.*, 1966). It would appear that the high energy intermediate involved in the action of the antibiotics is derived from the high energy intermediates involved in the synthesis of ATP but perhaps not on the same pathway (*cf.* Lardy *et al.*, 1964).

The oscillations in apparent optical density of the mitochondria induced by all four of the antibiotics were observed with any of the alkali metal cations except Li^+ and with either acetate or phosphate as the anion. ATP supported oscillations induced by dinactin and trinactin in a Na^+ and acetate containing medium were previously reported (Lardy and Graven, 1965). The ATP-supported oscillations were abolished by oligomycin and the substrate-supported oscillations were abolished by inhibitors of respiration. The amplitude and period of the oscillations were influenced by the concentration of the monovalent cation, the concentration and type of anion present, the availability of energy (ATP concentration or rate of substrate oxidation), the temperature of the reaction mixture, and the age or condition of the mitochondria. The oscillations appear to be the result of conditions which produce cyclic interruptions in the availability of energy to sustain the mitochondria in the swollen state.

The induction of cyclic oscillations in the optical density of mitochondria have also been observed with valinomycin (Pressman, 1965a) and with gramicidin D (S. N. Graven and H. A. Lardy, unpublished data). The substrate-supported oscillations induced by valinomycin were associated with oscillations in the K^+ concentration of the suspending medium. It appeared that the changes in K^+ uptake and the changes in mitochondrial size were both related to interruptions in flow or coupling of the energy required to support the cation movement and mitochondrial swelling. Cyclic oscillations in the apparent optical density of mitochondria induced by the nonactin homologs in a medium containing Na^+ were not associated with cyclic changes in Na^+ concentration (S. N. Graven and H. A. Lardy, unpublished data). The oscillations in optical density would appear to be associated sometimes with cyclic cation movements but cation movements would not appear to be essential to the occurrence of the oscillations.

The evidence that the nonactin homologs require a monovalent cation to induce the hydrolysis of ATP or swelling of the mitochondria suggests that they may act at the mitochondrial membrane by altering the K^+–Na^+ transport system. This could be the result of a stimulation of cation uptake or induction of increased cation exchange between the mitochondria and the surrounding medium. The hydrolysis of ATP induced by the nonactin homologs requires the presence of certain alkali metal cations while the induction of mitochondrial swelling requires both a cation and a permeant anion. Thus the mitochondrial swelling induced by the nonactin homologs is associated with ATP hydrolysis (or substrate oxidation) but the ATP hydrolysis or substrate oxidation in the presence of the nonactin homologs does not necessarily cause the mitochondria to swell. The dependence of the rate of ATP hydrolysis upon the cation concentration would suggest a causal relationship between cation transport and the ATPase activity induced by the nonactin homologs. However, the nature of the relationship between cation transport, ATP hydrolysis, and swelling of mitochondria is not evident from the data presented.

Valinomycin has been observed to have many activities which closely resemble those of the nonactin homologs. Valinomycin causes a greater stimulation of respiration in the presence of P_i or arsenate than in their absence. Substrate-supported, valinomycin-induced swelling of mitochondria is associated with K^+ uptake and H^+ ejection from the mitochondria if P_i or arsenate is present (Moore and Pressman, 1964; Pressman, 1965b). The substrate-supported swelling was inhibited by anaerobiosis, antimycin A, and dinitrophenol. ATP-supported swelling induced by valinomycin was inhibited by oligomycin and required an anion as well as a cation. Valinomycin can also induce substrate-supported swelling of mitochondria, in the presence of K^+ and an anion, in which no net uptake of K^+ occurred (B. C. Pressman, personal communication).

The gramicidins A–D are another group of antibiotics which induce energy-dependent swelling of mitochondria in the presence of a monovalent cation and P_i or arsenate (Neubert and Lehninger, 1962; Chappel and Crofts, 1965; Pressman, 1965; S. N. Graven and H. A. Lardy, unpublished data). They differ from valinomycin in that they induce ATPase activity and mitochondrial swelling in the presence of Na^+ or Li^+ as well as K^+ (Chappell and Crofts, 1965; S. N. Graven and H. A. Lardy, unpublished data). The activity of the gramicidins in Li^+ differentiates them from the nonactin homologs. The gramicidins have been shown to induce an energy-dependent uptake of Na^+ in mitochondria (Pressman, 1965b).

Triamcinolone has been reported to induce swelling of mitochondria in a K^+ medium, an effect which is inhibited by Na^+ and independent of an energy source (Gomez-Puyou *et al.*, 1965). In the study reported, triamcinolone did not require an anion to induce the swelling of mitochondria, an observation which further differentiates its activity from that of the nonactin homologs. The mitochondrial swelling induced by L-thyroxine did not require energy and can be reversed with ATP or substrate oxidation in the presence of Li^+, Na^+, K^+, Rb^+, or NH_4^+ but not in the presence of Cs^+ (Lehninger, 1961). The energy-dependent reversal of thyroxine-induced swelling was not inhibited by most anions. Thyroxine and triamcinolone, like selenite and selenate, appear to induce ATPase and mitochondrial swelling by a mechanism which is distinctly different from that of the nonactin homologs, valino-

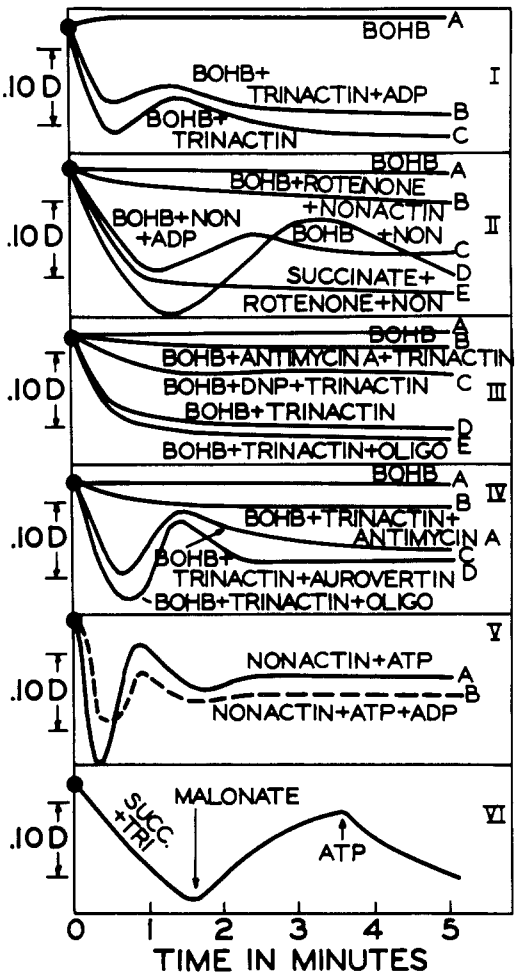

FIGURE 11: The effect of inhibitors on the swelling and oscillations induced by nonactin and trinactin. The basic medium was: NaCl 0.06 M (I and V) or KCl 0.06 M (II, III, IV, and VI); acetate 0.008 M (I, V, VI), or PO_4 0.005 M (II–IV); TEA (Cl) 0.01 M, sucrose 0.10 M. When present, the media contained β-hydroxybutyrate 0.013 M, succinate 0.013 M, ATP 0.003 M, ADP 0.003 M, trinactin 4×10^{-7} M, nonactin 4×10^{-7} M, antimycin A 1 μg/ml, oligomycin D 1.78 μg/ml, aurovertin 1 μg/ml, and malonate 0.015 M. Additions after the initiation of the reactions are indicated by the arrows. The volume, pH, and method were as indicated in Figure 7.

mycin, the gramicidins A–D, and tyrocidine (Lardy *et al.*, 1964). The wide variety of agents that affect mitochondrial swelling and ion movements should be of value in testing the hypothesis of Mitchell (1961) and Mitchell and Moyle (1965) that proton gradients may supply energy for synthesis of ATP.

Acknowledgment

We are grateful to Professor V. Prelog for supplying the principal antibiotics used in this work and especially for preparing a quantity of pure nonactin when none was available in his laboratory.

References

Chappell, J. B., and Crofts, A. R. (1965), *Biochem. J. 95*, 393.

Connelly, J. L., and Lardy, H. A. (1964), *Biochemistry 3*, 19.

Gomez-Puyou, A., Tuena, M., Campillo-Serrano, C., and Diaz-Maqueo, J. (1965), *Arch. Biochem. Biophys. 109*, 221.

Graven, S. N., Lardy, H. A., Johnson, D., and Rutter, A. (1966), *Biochemistry 5*, 1729 (preceding paper).

Lardy, H. A., Connelly, J. L., and Johnson, D. (1964), *Biochemistry 3*, 1961.

Lardy, H. A., and Graven, S. N. (1965), *Federation Proc. 24*, 424.

Lardy, H. A., and Wellman, H. (1952), *J. Biol. Chem. 195*, 215.

Lehninger, A. L. (1961), *Biochim. Biophys. Acta 48*, 324.

Mitchell, P. (1961), *Nature 191*, 144.

Mitchell, P., and Moyle, J. (1965), *Nature 208*, 147, 1205.

Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.

Neubert, D., and Lehninger, A. L. (1962), *Biochim. Biophys. Acta 62*, 556.

Pressman, B. C. (1965a), *Federation Proc. 24*, 425.

Pressman, B. C. (1965b), *Proc. Natl. Acad. Sci. U. S. 53*, 1076.

Rasmussen, H., Fischer, J., and Arnaud, C. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1198.

Sallis, J. D., and DeLuca, H. (1964), *J. Biol. Chem. 239*, 4303.

The Action on Soluble Blood Group A Substances of an α-*N*-Acetylgalactosaminidase from *Helix pomatia**

Hans Tuppy and Walter L. Staudenbauer

ABSTRACT: An α-*N*-acetylgalactosaminidase obtained from the hepatopancreas of *Helix pomatia* and purified 300-fold was allowed to act on soluble blood group A substances from human ovarian cyst fluid and hog gastric mucosa. This treatment resulted in an almost complete loss of serological A specificity and an 8- to 16-fold enhancement of blood group H activity as measured by hemagglutination inhibition tests. *N*-Acetylgalactosamine was found to be released. This indicates that the enzyme destroys the serological A specificity by removing from the antigenic sites terminal *N*-acetylgalactosamine residues thereby exposing structures similar to those present in H substances. Quantitative determinations suggest an average number of 120 antigenic sites per molecule of human blood group A substance, each of them being composed of two *N*-acetylgalactosamine, one *N*-acetylglucosamine, two galactose, and two fucose residues.

There is ample evidence suggesting that the soluble blood group substances obtained from human and other sources consist of a protein backbone to which numerous oligosaccharide units are attached (Kabat, 1956; Morgan, 1959). The latter are considered to carry the group specificity. Enzymes are known which destroy the serological activity of blood group substances by degrading the carbohydrate portions of the mucopolysaccharide molecule with the liberation of reducing sugars.

Enzyme preparations from the hepatopancreas of the snail *Helix pomatia* were found by Freudenberg and Eichel (1935) to inactivate blood group A substance and to release from it *N*-acetylhexosamine and galactose. The presence of A-decomposing enzymes was also demonstrated in liver extracts from the snail *Buscyon* (Howe and Kabat, 1953), and in various microorganisms (Iseki and Masaki, 1953; György *et al.*, 1954; Watkins, 1959; Yamamoto *et al.*, 1962). All these enzyme preparations, however, contained mixtures of

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