Biochemical Pharmacology, Vol. 20, pp. 533–545. Pergamon Press, 1971. Printed in Great Britain

EFFECTS OF GLYCEROL TRINITRATE, MANNITOL HEXANITRATE AND ERYTHRITOL TETRANITRATE ON ELECTRON TRANSPORT AND PHOSPHORYLATION IN LIVER MITOCHONDRIA*

IRVING BOIME and F. EDMUND HUNTER, JR.

The Edward Mallinckrodt Department of Pharmacology, Washington University School of Medicine, Saint Louis, Mo. 63110, U.S.A.

(*Received* 22 *January* 1970; *accepted* 12 *June* 1970)

Abstract—The organic nitrate esters, glycerol trinitrate (GTN), mannitol hexanitrate (MHN) and erythritol tetranitrate (ETN) were tested for effects on the oxygen consumption of rat liver mitochondria. Both MHN and GTN are capable of reducing the respiratory control ratio to 1·0 [rate of O_2 consumption with ADP (state 3)/rate without ADP (state 4)]. However, this requires 1 mM GTN as compared to 0·1 mM MHN, and the actions are effected through different mechanisms. With the NAD-linked substrate, 3-hydroxybutyrate, GTN inhibits the state 3 rate, whereas MHN markedly increases the state 4 rate. GTN, in concentrations that inhibit 3-hydroxybutyrate oxidation, has little or no effect on respiration with succinate as substrate. MHN, on the other hand, accelerates the state 4 rate with succinate just as it does with 3-hydroxybutyrate. Therefore, the action of GTN appears to be selective for the NADH dehydrogenase region of the electron transport chain. In contrast to the generalized uncoupling activity of MHN, the predominant effect of GTN appears to be inhibition of electron transport. Since DNP relieves this inhibition to a large degree, a considerable part of it must be indirect, by way of an inhibition in energy transfer reactions, as with oligomycin or atractyloside. There also appears to be a small direct inhibition of electron transport by GTN and MHN at low concentrations. This inhibition increases with higher concentrations. Erythritol tetranitrate appears to be intermediate between MHN and GTN in the effects produced. It is suggested that lipid solubility of the organic nitrate esters, location of sulfhydryl groups critical for electron transfer and energy conservation, and reactivity of the organic nitrate esters with sulfhydryl groups determine the selective effects observed.

IT WAS first shown by Hunter *et al.*[1, 2] in 1953 that glycerol trinitrate inhibits oxidative phosphorylation in isolated liver mitochondria. Furthermore, these investigators demonstrated that another organic nitrate ester, mannitol hexanitrate (MHN),† is a more potent uncoupler than glycerol trinitrate (GTN). Stam and Honig[3] reported uncoupling by GTN with heart mitochondria. Later Needleman and Hunter,[4] working with a series of organic nitrate derivatives, demonstrated that in liver and heart mitochondria MHN possessed the highest potency of all nitrates tested. It was apparent,

* This work was supported by United States Public Health Service, National Institute of General Medical Sciences Pharmacology Training Grant 5-T01-00096, National Heart Institute Research Grant HE-10107-03, and National Institute for Metabolic Diseases and Arthritis Research Grant AM-11673-02.

† Abbreviations used: GTN, glycerol trinitrate; MHN, mannitol hexanitrate; ETN, erythritol tetranitrate; PETRIN, pentaerythritol trinitrate; DNP, 2,4-dinitrophenol; bovine serum albumin, BSA; ethylenediamine tetraacetate, EDTA; GSH, reduced glutathione.

especially with heart mitochondria, that there was some inhibition of electron transport, either directly or indirectly, as well as uncoupling. In this paper we report further on the localization of the site of action of GTN on electron transport and on oxidative phosphorylation, as well as on selective differences between GTN, MHN and ETN in modes of action on these parameters. Observations which are similar in part have been reported by Blum *et al.*[5, 6] in preliminary communications.

METHODS AND MATERIALS

Isolation of liver mitochondria

Rat liver mitochondria were isolated by the previously described procedure,[7] using 0·33 M sucrose, 0·1 mM EDTA, and 1 mg/ml bovine serum albumin (BSA). The final washing and resuspension were made in this medium also.

Addition of alcoholic solutions of organic nitrates

In some experiments the organic nitrate esters were added to the oxygen electrode cuvette and the organic solvent was removed with a stream of air before the medium was added.[4] However, because of limited solubility and rate of solution of the organic nitrates in aqueous medium, in many other experiments the organic nitrates were added directly to the cuvettes filled with the incubation medium, in most cases before and in some cases after the mitochondria. In such experiments, the solvent for the organic nitrate was absolute ethanol. An equal volume of absolute ethanol was added to the control cuvette. Many controls with 20–100 μl ethanol indicated no significant effect due to the alcohol. In general, the preferable procedure is to add the alcoholic solution after the mitochondria, slowly and with the magnetic stirrer operating. The experiment is then started by the addition of substrate. When the solution of organic nitrate was evaporated to dryness first in the cuvette, somewhat larger amounts of organic nitrate were required, presumably because it had not all dissolved under the conditions used.

Use of bovine serum albumin

BSA at 1 mg/ml was used in the standard medium because it is well known to bind free fatty acids and preserve respiratory control. BSA also can bind uncoupling agents, especially pentachlorophenol.[8] The degree to which this concentration of BSA would influence results with organic nitrate esters and with DNP was checked by making several comparisons with 0, 1, 5 and 7 mg BSA/ml. One mg BSA, as might be anticipated, appears to bind some organic nitrate and some DNP, but the experimental results are not greatly altered. Seven mg BSA/ml binds enough of the drugs to alter greatly the result seen with a given concentration of drug.

Measurement of O_2 consumption

Oxygen consumption was followed by means of a Beckman model 160 physiological gas analyzer, a Beckman macro oxygen electrode, and a Varian dual channel recorder. All experiments were performed in 4-ml cuvettes with a magnetic stirrer in a constant temperature bath at 25°. Controls with normal mitochondria were run simultaneously with the tests on mitochondria treated with organic nitrate esters.

The oxygen consumption test medium consisted of 20 mM potassium phosphate

(pH 7·5), 10 mM $MgCl_2$, 0·1 mM EDTA, 10 mM NAF, 200 mM sucrose, and 1 mg/ml of BSA. The final mitochondrial protein concentration was about 1 mg/ml. The tracings presented are representative experiments which have been repeated several times at three different points in a 2-yr period.

The concentration of the ADP used was standardized enzymatically by measuring the disappearance of NADH spectrophotometrically at 340 mμ in a system which couples the reactions catalyzed by pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27).[9]

Materials

Biochemical reagents were obtained from Sigma Chemical Company, St. Louis, Mo. Organic nitrate esters were obtained from the following sources: GTN, Atlas Chemical Industries, Inc., Wilmington, Del. and Eli Lilly, Indianapolis, Ind.; MHN, William S. Merrill Company, Cincinnati, Ohio; ETN, Burroughs Wellcome, Tuckahoe, N.Y.

RESULTS

Figure 1 shows the polarographic tracings for control mitochondria, mitochondria treated with GTN, and mitochondria treated with MHN when the substrate used was 3-hydroxybutyrate. The concentrations of organic nitrate esters used are the minimal concentrations necessary to give essentially complete loss of respiratory control [rate of O_2 consumption with ADP present (state 3): rate with very low ADP and high ATP (state 4) = 1·0; see Chance and Williams[10]]. Experiment A illustrates the normal response to ADP. From experiment B it can be seen that the primary effect of GTN is to block the response to ADP and lower the state 3 rate of oxygen consumption, with a slight increase in the rate with substrate alone. This effect is the

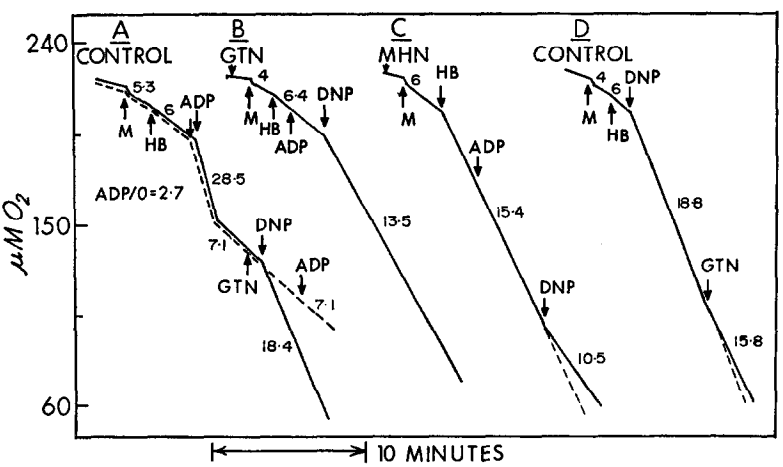

FIG. 1. Effects of GTN and MHN on 3-hydroxybutyrate oxidation by rat liver mitochondria. The medium used in the O_2 electrode cuvette is given under Methods. Additions and final concentrations are indicated as follows: M, mitochondria, approximately 1 mg protein/ml; HB, 5 mM 3-hydroxybutyrate; ADP, 190 μM; DNP, 30 μM. In expt. B, 1 mM GTN was added to the medium before the mitochondria; in expt. C, 90 μM MHN. Other sequences for GTN addition are indicated on the figure. Rate values along the tracings are in nmoles/ml/min.

same whether GTN is present initially (expt. B) or added after an ADP response (expt. A). In contrast to GTN, however, MHN results in a marked increase in the rate with substrate alone (expt. C), although this accelerated rate falls short of the maximal rate with ADP in the control. ADP addition does not increase this rate. Higher concentrations of MHN were inhibitory, decreasing the rate rather than bringing it up to the ADP-stimulated rate in the control.

Also shown in Fig. 1 is the effect of DNP on organic nitrate ester-treated mitochondria with 3-hydroxybutyrate as substrate. DNP at 35 μM increased the respiratory rate of GTN-inhibited mitochondria to a rate approaching that seen with DNP in controls. With mitochondria treated with MHN, no enhancement in the already rapid rate of O_2 consumption was observed. In fact, a significant inhibition was almost always observed. The degree of inhibition increased as the concentration of either MHN or DNP was increased. Experiment D in Fig. 1 illustrates the fact that GTN added after DNP inhibits the uncoupled rate to some extent.

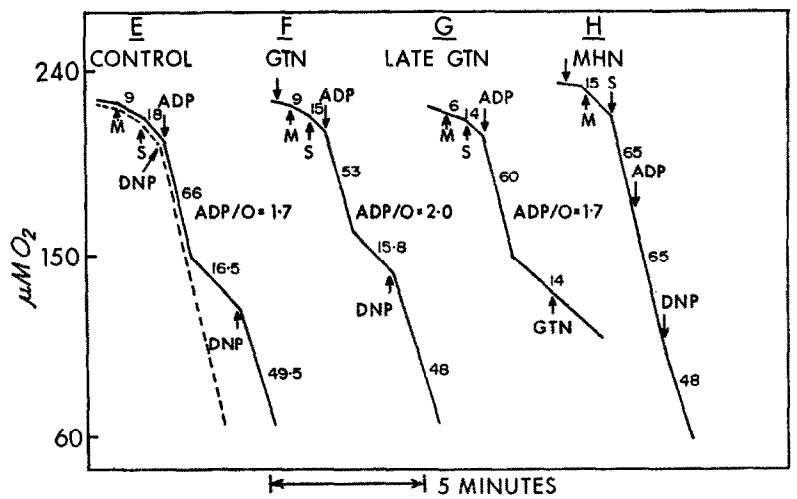

FIG. 2. Effects of GTN and MHN on succinate oxidation by rat liver mitochondria. The medium used in the O_2 electrode cuvette is given under Methods. Additions and final concentrations are indicated as follows: M, mitochondria, approximately 1 mg protein/ml; S, 5 mM succinate; ADP, 190 μM; DNP, 30 μM; in expt. F, 2 mM GTN was added to the medium before mitochondria; in expt. H, 100 μM MHN; in expt. G, 2 mM GTN was added as indicated. Rate values along the tracings are in nmoles/ml/min.

Mitochondria were also examined using succinate as substrate instead of NAD-linked substrate. Figure 2 shows the polarographic tracings for the O_2 consumption with succinate as the substrate when mitochondria were incubated with GTN and with MHN. Experiment E illustrates normal responses with ADP and uncoupling by DNP. With succinate as substrate, the uncoupled rate with DNP was very similar to the rate with ADP. Experiments F and G illustrate that the presence of GTN, in a concentration which inhibits the state 3 rate of respiration and results in a low respiratory control ratio when 3-hydroxybutyrate is substrate, had slight effect on succinate oxidation rates (states 3 or 4) or the ADP:O ratio. Experiment G in Fig. 2 emphasizes the fact that GTN, added after an ADP response with succinate,

was also without a significant effect. Even with higher concentrations of GTN, there was only a slight suggestion of uncoupling with succinate.

When MHN-treated mitochondria were tested with succinate as substrate, there was a marked acceleration of the rate of O_2 consumption with substrate alone and complete elimination of respiratory control (Fig. 2, expt. H). However, in most experiments the maximal rate was not quite so fast as the maximal rate seen with ADP in the control. DNP did not increase the respiratory rate of the MHN-treated mitochondria with succinate as substrate, with or without the presence of ADP. DNP sometimes showed slight inhibitory effects on succinate oxidation in MHN-treated mitochondria, but this was never so marked as that already described with 3-hydroxybutyrate.

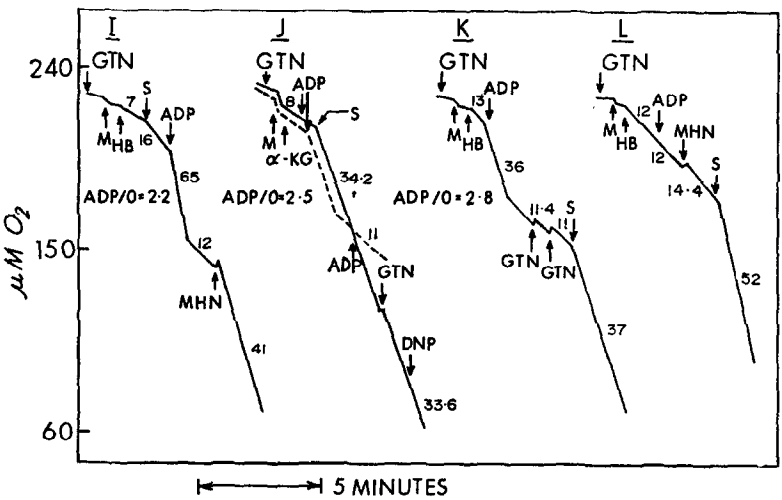

FIG. 3. Effects of GTN and MHN on 3-hydroxybutyrate, α-ketoglutarate and succinate oxidations by rat liver mitochondria. The medium used in the O_2 electrode cuvette is given under Methods. Additions and final concentrations are indicated as follows: M, mitochondria, approximately 1 mg protein/ml; HB, 5 mM 3-hydroxybutyrate; αKG, 4 mM α-ketoglutarate; ADP, 190 μM; DNP, 30 μM. The additions of organic nitrates (expressed as final concentration) were as follows: expt. I, 1·3 mM GTN, 125 μM MHN; expt. J, 2 mM GTN and 1·4 mM GTN; expt. K, 0·7 mM, 0·7 mM GTN, 1·4 mM GTN; expt. L, 1·7 mM GTN and 110 μM MHN. The dashed line in expt. J is a control without GTN. Rate values along the tracings are in nmoles/ml/min.

The experiments presented in Fig. 3 emphasize the fact that relatively normal respiratory control and ADP:O ratios for succinate can be seen when succinate is added to mitochondria in which 3-hydroxybutyrate or α-ketoglutarate oxidation has been inhibited by GTN (expts. I and J). It is clear that GTN inhibits the response to ADP only with NAD-dependent substrates. Figure 3 also illustrates the fact that MHN, added after an ADP response with succinate, produces a stimulation (expt. I) very much like that seen with DNP. Multiple late additions of GTN did not produce inhibition of succinate oxidation (expt. K). The rate was somewhat faster than with succinate alone in the corresponding control, but not equal to the rate with succinate plus ADP. Succinate was also oxidized at a rapid rate when both GTN and MHN were present (expt. L).

Since MHN exhibited the selective property of releasing normal controlled respiration with succinate and with 3-hydroxybutyrate, and since DNP produced a substantial release from GTN inhibition of 3-hydroxybutyrate respiration, experiments were conducted to see if MHN was capable of releasing respiration inhibited by GTN. Experiments such as experiment L in Fig. 3 and experiment M in Fig. 4 indicated that release of GTN-inhibited respiration with 3-hydroxybutyrate was either absent or very limited. Experiment N in Fig. 4 reemphasizes the fact that significant

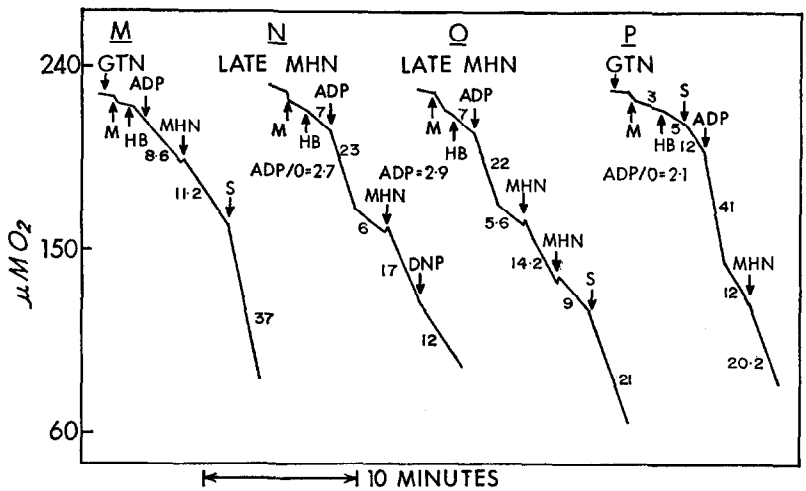

FIG. 4. Effects of GTN and MHN on 3-hydroxybutyrate and succinate oxidations by rat liver mitochondria. The medium used in the O_2 electrode cuvette is given under Methods. Additions and final concentrations are as follows: M, mitochondria, approximately 1 mg protein/ml; HB, 5 mM 3-hydroxybutyrate; S, 5 mM succinate; ADP, 190 μM; DNP, 30 μM. In expts. M and P, 1·7 mM GTN was added to the medium before the mitochondria. The MHN concentrations added in expts. M, N and P were 100 μM. In expt. O, two 130 μM additions of MHN were made after the response to ADP. Rate values along the tracings are in nmoles/ml/min.

release of normal controlled respiration (state 4) with 3-hydroxybutyrate as substrate can be produced by MHN. However, as the concentration of MHN was increased (expt. O), there was increasing inhibition of the released or uncoupled respiration. This indicates that direct inhibition of electron transport is produced by higher concentrations of MHN. Just as with GTN, this inhibition was in the NAD-dependent pathway and did not affect succinate oxidation. The failure to observe a greater release of GTN-inhibited respiration by MHN was probably due to additive inhibition of electron transport. Whether any release will be seen is determined by the exact combination of concentration employed. MHN released controlled respiration with succinate regardless of whether GTN was present or not (Fig. 3, expt. I; Fig. 4, expt. P). With succinate, neither compound inhibited respiration enough to obscure the effect.

ETN also can release controlled respiration with 3-hydroxybutyrate (Fig. 5, expt. Q), but it is less effective than MHN. The occurrence of simultaneous partial inhibition of electron transport was indicated by the absence of stimulation by ADP and DNP even though the rate was far short of the ADP rate in the control. Mitochondria

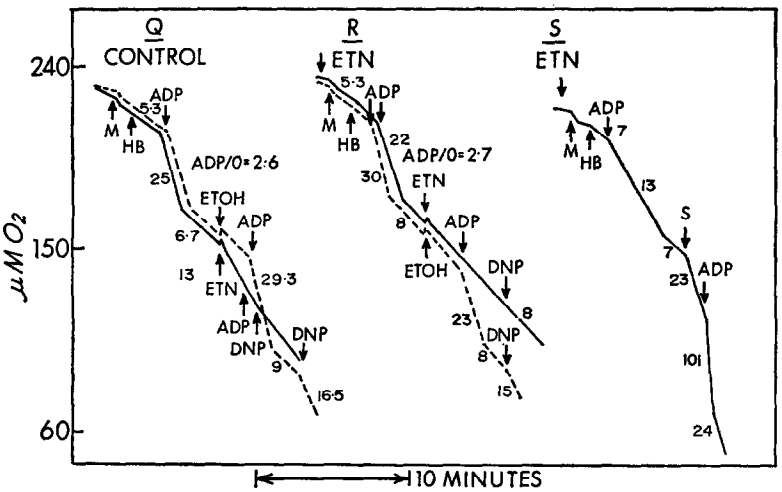

FIG. 5. Effects of ETN on 3-hydroxybutyrate and succinate oxidations by rat liver mitochondria. The medium used in the O_2 electrode cuvette is given under Methods. Additions and final concentrations are indicated as follows: M, mitochondria, approximately 1 mg protein/ml; HB, 5 mM 3-hydroxybutyrate; S, 5 mM succinate; ADP, 190 μM; ETOH, ethanol (2·5% in expt. Q and 0·8% in expt. R); DNP, 30 μM. In expt. Q, the ETN was 0·65 mM. In expt. R, the first aliquot of ETN (0·2 mM) was evaporated to dryness in the cuvette prior to the addition of medium; another addition of 0·3 mM ETN in ethanol was made after the response to ADP. In expt. S, an addition of 0·5 mM ETN in 100 μl ethanol was made to the medium before the mitochondria were added. The dashed line in expt. R is a control without ETN. Rate values along the tracings are in nmoles/ml/min.

can be titrated with ETN until there is complete inhibition of the stimulation by ETN or ADP (Fig. 5, expt. R). With succinate as substrate, ETN did not inhibit electron transport, and response to ADP was relatively normal (Fig. 5, expt. S). Thus limited uncoupling and the inhibition of electron transport appear to involve the NAD-dependent pathway.

DISCUSSION

The most revealing finding in these experiments is selective action of organic nitrate esters at different sites in electron transport and oxidative phosphorylation. The greatest selectivity is seen in a comparison of GTN and MHN. ETN appears to be intermediate in its properties. With all three compounds, the degree of selectivity observed was somewhat dependent on the concentrations used. The concentration of GTN required to produce essentially complete block of the ADP stimulation of O_2 consumption varied the most from one mitochondrial preparation to another. It seemed to be critically dependent on concentration changes between 0·7 and 1·2 mM, and was determined in part by the protein concentration present. The first 0·5 mM GTN may react in some nonspecific noncritical way.

At this point it is appropriate to emphasize the different mechanisms by which GTN and MHN each causes the elimination of respiratory control. This is possible because of the definition of the term "respiratory control", which is the ratio between two rates, either of which may change. In the case of GTN, one observes an inhibition

of the state 3 rate of respiration. In the case of MHN, on the other hand, an accelerated state 4 rate of respiration is observed. In either case, the respiratory control ratio may decrease to 1·0. The rate of respiration is low in the first case and high in the second. When inhibition of respiration in state 3 is indirect, it would be more accurate to say that respiration is still controlled but not increased or released by ADP.

GTN appears to be very weak in the classical uncoupling action, since the increase in state 4 rate of respiration was small or absent and normal respiratory control ratios and ADP:O ratios were obtained with succinate in the presence of GTN until 3 or 4 mM concentrations of GTN were used. With succinate as substrate, GTN inhibited the oxygen consumption rate only 5–10 per cent with ADP present. On the other hand, it abolished ADP stimulation with 3-hydroxybutyrate as substrate. Thus, the presence of GTN resulted in inhibited electron transport in the NADH dehydrogenase area. This could result from a direct inhibition of 3-hydroxybutyrate dehydrogenase or of the electron transport chain in the NADH dehydrogenase region, or it could be the indirect result of an effect on the energy coupling and phosphorylation mechanism in this region. The partial release from this inhibition by DNP indicates that much of the inhibition is in the coupling mechanism rather than in electron transport *per se*. These conclusions are in general agreement with some of the findings of Blum *et al.*[5, 6] with GTN and heart mitochondria. This action of GTN is reminiscent of the action of oligomycin[11] or atractyloside,[12] in that DNP relieves to a large extent the inhibitory effects on O_2 consumption. GTN differs from oligomycin or atractyloside in showing apparent selectivity for just one of the three energy conservation sites (site I, NAD-dependent chain). This means that it must inhibit at a different point than does oligomycin or atractyloside, which can inhibit respiration by blocking ADP phosphorylation at any of the three energy conservation sites. When state 3 respiration is blocked by oligomycin (1 μg/ml), the addition of GTN produces no change, but DNP can still stimulate respiration. MHN releases respiration inhibited by oligomycin.

It is interesting that an oligomycin type of effect might be the explanation for the report of improved phosphorylation with GTN in aged mitochondrial preparations.[5] Lee and Ernster[13] have reported that low concentrations of oligomycin can improve low P:O ratios in specific types of submitochondrial particles, presumably through changes which reduce hydrolysis of intermediates of phosphorylation.

MHN, in contrast to GTN, markedly accelerates the state 4 rate of respiration both with an NAD-linked substrate and with succinate. The most sensitive and selective effect of MHN seems to be generalized uncoupling in a manner analogous with DNP, although it need not be at the same point as for DNP. Like DNP, MHN to a large degree releases the respiration from oligomycin inhibition. However, the fact that the MHN-accelerated rate is not identical with the normal maximal ADP rate (state 3) indicates that MHN may produce some direct inhibition of electron transport as well. The failure of ADP or DNP to increase the rate seen with MHN is consistent with this suggestion. This partial inhibition of uncoupled electron transport is seen primarily with 3-hydroxybutyrate, although there may be a small effect with succinate. This means that inhibition of electron transport by MHN, as with GTN, occurs in the NADH dehydrogenase region.

ETN is essentially without ability to inhibit selectively energy transfer mechanisms as does GTN. Whenever respiration is inhibited significantly, there is no release by

DNP. ETN does show evidence of uncoupling action in lower concentrations (increase in states 2 and 4), before extensive inhibition of electron transport occurs. Since the two do occur simultaneously at slightly higher concentrations, raising the concentration of ETN inhibits electron transfer from 3-hydroxybutyrate and makes it impossible to observe the uncoupling by any increase in rate. As with GTN and MHN, there is little or no inhibition of electron transfer from succinate. We have not studied further pentaerythritol trinitrate (PETRIN), an organic nitrate, which Blum *et al.*[6] have reported to inhibit respiration with succinate in heart mitochondria. In earlier work with heart mitochondria in our laboratory, all organic nitrates tested significantly inhibited maximal respiration with ADP when glutamate was used as the substrate.[4]

Therefore, the organic nitrate esters, in different degrees with different compounds, show evidence of: (1) uncoupling of energy conservation mechanisms; (2) inhibition of energy transfer mechanisms; and (3) inhibition of electron transfer mechanism in the NADH dehydrogenase flavoprotein area.

It has been pointed out previously[4] that the potency of many common biologically active organic nitrate esters parallels their oil–water partition coefficient. Our data confirm the greater activity of the more lipid-soluble MHN with respect to actions on oxidative phosphorylation. However, there is the added dimension of marked qualitative as well as quantitative differences between MHN and GTN. Reactivity with sulfhydryl groups may contribute to these differences. Organic nitrate esters are known to react nonenzymatically with several sulfhydryl compounds and not with others.[14] They react nonenzymatically with reduced glutathione (GSH) at different rates, and there is at least one enzyme, glutathione organic nitrate ester reductase, which catalyzes the reaction between several pharmacologically active organic nitrates and GSH.[14–16] It is interesting that the enzymatic studies indicate that the rate of attack by this enzyme parallels the oil–water partition coefficient for MHN, ETN, GTN and other pharmacologically active nitrate esters.[4, 15, 16] GTN, being more polar than MHN, would be somewhat amphipathic. Although GTN might act through a nonspecific mechanism which alters membrane structure, it seems more likely that it would act through a specific interaction, possibly with a sulfhydryl group critical for energy conservation[17] or for phosphate transfer to ADP[18] and located in a hydrophilic area. MHN, being less water soluble and more lipid soluble, may penetrate more readily to sites involved in the primary energy conservation process possibly sulfhydryl groups in more hydrophobic areas, while being less reactive with sulfhydryl groups in hydrophilic areas. Thus, selectivity in site of action could be determined by: (1) the lipid solubility of the organic nitrate ester; (2) the hydrophobic or hydrophilic location of the sulfhydryl groups; and (3) reactivity of the organic nitrate ester with sulfhydryl groups. These suggestions are consistent with previous demonstrations of the participation of several sulfhydryl groups[17] of different properties[19] in the overall process of electron transport and energy conservation by phosphorylation.

Additional possibilities which must be considered in relation to effects of organic nitrate esters on overall rates of respiration are interaction with substrate anion carriers,[20, 21] ADP–ATP exchange carrier,[12] and the phosphate carrier in the mitochondrial membrane.[22, 23] The latter has already been shown to have an essential sulfhydryl group. Harris[24] has demonstrated distinct differences between the ability of 3-hydroxybutyrate and succinate to penetrate into the mitochondria in the presence

of K^+ and valinomycin. Further work is necessary to determine whether inhibition of penetration of 3-hydroxybutyrate, partially relieved by a permeability increase due to DNP, is responsible for GTN producing an apparent oligomycin-like action limited to site I energy conservation. The greater inhibition of respiration by DNP in the presence of organic nitrate esters with 3-hydroxybutyrate than with succinate may be related to differences in competition for the carrier and penetration mechanisms for the two substrates.[25]

The oligomycin or atractyloside type of effect reported here was not observed in earlier work.[4] The reasons for the difference are not known. Variations in mitochondrial preparation, experimental procedure, and different sources and samples of GTN have been examined. None of the GTN samples used in earlier work is available for testing, but contamination with an uncoupling substance in a sample used for part of the work would explain the earlier finding.

In the previous study,[4] the action of organic nitrates in initiating or inhibiting mitochondrial swelling was studied. Although the compounds tested seemed to fall into two groups, interpretation was restricted because the findings did not seem consistent with all that was known about the actions of uncouplers and reagents that react with sulfhydryl groups. A prime discrepancy was in the failure of GTN to inhibit energy-dependent swelling, as all uncouplers do. It is worthy of note that the failure of GTN to inhibit this swelling is consistent with the oligomycin type of effect with little uncoupling that was found in the present study.

The concentrations of organic nitrate esters needed to produce effects on isolated liver mitochondria are much higher than those likely to occur at any point in the body and to be responsible for relaxation of smooth muscle, unless there is an unusual accumulation at some lipoidal site. However, it appears that these drugs may be very fuluse tools for further studies on the mechanism of oxidative phosphorylation.

Addendum—While this paper was being modified according to editorial suggestions, a paper by J. V. Levy appeared in *Br. J. Pharmac. Chemother.* **38,** 743 (1970). Using rabbit atrial tissue as his test object, he reached conclusions completely consistent with our studies on the oxygen consumption of isolated liver mitochondria. MHN and GTN behave quite differently, and ETN is intermediate.

REFERENCES

1. F. E. HUNTER, JR. and L. FORD, *J. Pharmac. exp. Ther.* **113,** 186 (1955).
2. F. E. HUNTER, JR., S. KAHANA and L. FORD, *Fedn Proc.* **12,** 221 (1953).
3. A. STAM and C. R. HONIG, unpublished results cited by C. R. HONIG, S. M. TENNY and S. M. GABEL, *Am. J. Med.* **29,** 910 (1960).
4. P. NEEDLEMAN and F. E. HUNTER, JR., *Molec. Pharmac.* **2,** 134 (1966).
5. S. W. BLUM, B. DUBNICK and G. E. PHILLIPS, *Fedn Proc.* **26,** 740 (1967).
6. S. W. BLUM, J. B. QUINN and B. DUBNICK, *Fedn Proc.* **28,** 479 (1969).
7. I. BOIME, E. E. SMITH and F. E. HUNTER, JR., *Archs Biochem. Biophys.* **128,** 704 (1968).
8. E. C. WEINBACH and J. GARBUS, *J. biol. Chem.* **241,** 169 (1966).
9. O. H. LOWRY, J. V. PASSONNEAU, F. X. HASSELBERGER and D. M. SCHULZ, *J. biol. Chem.* **239,** 18 (1964).
10. B. CHANCE and G. R. WILLIAMS, *J. biol. Chem.* **217,** 383 (1955).
11. H. A. LARDY, D. JOHNSON and W. C. MCMURRAY, *Archs Biochem. Biophys.* **78,** 587 (1958).
12. H. MEISNER and M. KLINGENBERG, *J. biol. Chem.* **243,** 3631 (1968).
13. C. P. LEE and L. ERNSTER, *Biochem. biophys. Res. Commun.* **18,** 523 (1965).
14. L. A. HEPPEL and R. J. HILMOE, *J. biol. Chem.* **183,** 129 (1950).
15. P. NEEDLEMAN and F. E. HUNTER, JR., *Molec. Pharmac.* **1,** 77 (1965).
16. P. NEEDLEMAN, D. J. BLEHM and K. S. ROTSKOFF, *J. Pharmac. exp. Ther.* **165,** 286 (1969).
17. E. K. R. KURUP and D. R. SANADI, *Biochemistry, N.Y.* **7,** 4483 (1968).

18. H. S. PENEFSKY and R. C. WARREN, *J. biol. Chem.* **240**, 4694 (1965).
19. R. W. ESTABROOK, D. D. TYLER, J. GONZE and J. A. PETERSON, in *Flavins and Flavoproteins* (Ed. K. YAGI), p. 268. University of Tokyo Press, Tokyo (1968).
20. J. B. CHAPPELL, *Br. med. Bull.* **24**, 150 (1968).
21. E. C. SLATER, E. QUAGLIARIELLO, S. PAPA and J. M. TAGER, in *The Energy Level and Metabolic Control in Mitochondria* (Eds. S. PAPA, J. M. TAGER, E. QUAGLIARIELLO and E. C. SLATER), p. 1. Adriatica Editrice, Bari (1969).
22. A. FONYO, *Biochem. biophys. Res. Commun.* **32**, 624 (1968).
23. D. D. TYLER, *Biochem. J.* **107**, 121 (1968).
24. E. J. HARRIS, in *The Energy Level and Metabolic Control in Mitochondria* (Eds. S. PAPA, J. M. TAGER, E. QUAGLIARIELLO and E. C. SLATER), pp. 31 and 135. Adriatica Editrice, Bari (1969).
25. K. VAN DAM and R. KRAAIJENHOF, in *The Energy Level and Metabolic Control in Mitochondria* (Eds. S. PAPA, J. M. TAGER, E. QUAGLIARIELLO and E. C. SLATER), p. 299, Adriatica Editrice, Bari (1969).