Regulation of Mitochondrial Metabolism by Specific Cellular Substances. II. The Nature of Stimulation of Mitochondrial Glutamate Metabolism by a Cytoplasmic Component*

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ABSTRACT: Oxidative metabolism of glutamate by liver mitochondria is not stimulated by dinitrophenol when the mitochondria are preincubated with the uncoupler for 15 min without added glutamate unless adenosine diphosphate or adenosine triphosphate is also present during preincubation. In the presence of adenosine diphosphate or adenosine triphosphate, the metabolic response to increasing concentrations of dinitrophenol is biphasic. A cytoplasmic component, which contains the readily extractable cytoplasmic Mg^{2+} of the tissue it was isolated from, raises the activating effect of dinitrophenol. The apparent increase in metabolism by a cytoplasmic component occurs at a concentration of the uncoupler, which, without a cytoplasmic component, does not augment the metabolism of mitochondria. The activity of a cytoplasmic component is related to its Mg^{2+} content; $MgCl_2$ is far less effective than an equimolar amount of Mg^{2+} added as a cytoplasmic component. Mitochondrial Mg^{2+} is selectively labilized by preincubation with adenosine diphosphate + dinitrophenol and the loss of mitochondrial Mg^{2+} coincides with a loss of metabolic response to dinitrophenol. A cytoplasmic component prevents the loss of mitochondrial Mg^{2+} and sustains the responsiveness of mitochondria to stimulation of metabolism by dinitrophenol. Analyses of experimental conditions indicate that the cytoplasmic component may function as a Mg^{2+} carrier, or as an activator of a Mg^{2+}-dependent mitochondrial system which is essential for the metabolic stimulatory effect of dinitrophenol.

In the course of preceding studies (Loh *et al.*, 1968), it was found that relatively short (15 min) preincubation of intact mitochondria with 2.3 mM ADP or ATP and varying concentrations of DNP determined the magnitude of metabolic rates measured after subsequent addition of glutamate. The rate of O_2 consumption exhibited a characteristic biphasic dependence upon the concentration of DNP. At a critical concentration of DNP, metabolic rates reached a maximum; a further increase in DNP levels lowered rates approaching those of the controls containing no DNP, and at even higher concentrations of DNP, inhibition of metabolism was observed. The shape of curves obtained by plotting metabolic rates against concentrations of DNP (defined as "DNP profiles") was markedly altered when small quantities of a cytoplasmic cellular component (CMF)[1] were also present during preincubation. In the previously described assay system, the characteristic effect of CMF consisted of an apparent increase in the rate of metabolism, induced by amounts of DNP which, in the absence of CMF, did not stimulate O_2 uptake and were often high enough to be inhibitory (*cf.* Loh *et al.*, 1968). It was also found (*cf.* Loh *et al.*, 1968) that the effect of CMF could not be simulated by agents which are well known to preserve mitochondrial integrity (*e.g.*, sucrose and serum albumin); therefore the specific nature of the metabolic effect of CMF was further investigated.

Elucidation of the nature of CMF depended upon a better understanding of its complex metabolic effect. This in turn required more detailed information concerning the rate-limiting role of each component in the metabolic assay system for CMF. As the result of studies concerned with this question it became apparent that the (DNP + ADP)-dependent stimulation of mitochondrial metabolism by CMF could be related to the operation of a normally intramitochondrial Mg^{2+}-requiring system. The present report deals with experiments which lead to this conclusion.

Experimental Procedures

Mitochondrial Preparations and Standard Assay Conditions. Although the preparative methods and handling of rat liver mitochondria were mentioned in previous papers (Kun *et al.*, 1964; Loh *et al.*, 1968), certain details require further emphasis. In all experiments Sprague–Dawley male rats weighing 150–200 g were used. The rats were kept on normal rat diet and fed *ad libitum*. For preparation of mitochondria, the rats were killed by cervical dislocation and exsanguination, an operation which should not take longer than 2–3 min/rat.

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[1] Abbreviation used is: CMF, cytoplasmic metabolic factor. This abbreviation was used previously (*cf.* Loh *et al.*, 1968) to mean "cytoplasmic macromolecular fraction." Recent studies, in part reported in this paper, indicate that Mg^{2+} and an apparently macromolecular constituent both contribute to the effect of CMF. Until its chemical structure is conclusively established, the term "metabolic" is to replace "macromolecular."

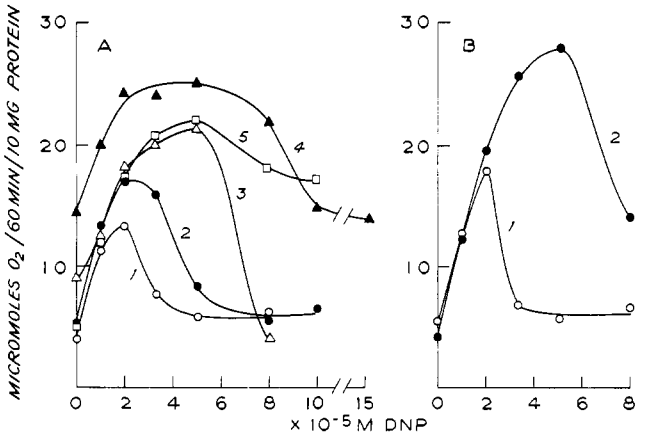

FIGURE 1: The effects of ADP and ATP (curves 1A, 1B with ADP and 2A with ATP, both 2.3 mM); HPO_4^{2-} (30 mM) and ADP (curve 3A); HPO_4^{2-}, $MgCl_2$ (1 mM), and ATP (curve 4A); Mg^{2+} and ADP (curve 5A); and ADP and CMF (3.4 μequiv of reducing substance, curve 2B) on the rates of glutamate metabolism of liver mitochondria at varying concentrations of DNP. Experimental conditions were described in Methods. The amount of Mg^{2+} added as CMF gives a final concentration of 0.1 mM (curve 2 in part B).

The livers were immediately removed, placed into a chilled sucrose solution (0.25 M sucrose, 1.3 mM EDTA, and 10 mM Tris, pH 7.7, at 0°), cut into small pieces of approximately 4–8 mm diameter, and washed free from blood by repeated decantation (at 0°) of the sucrose–EDTA–Tris medium until the supernatant solution was colorless. Homogenization was carried out in an ice bath in about 10-fold excess of sucrose–EDTA–Tris (w/v), by means of a motor-driven losse-fitting Teflon-glass homogenizer, with not more than one to two slow and gentle upward movements of the plunger. Differential centrifugation was performed in a Sorvall refrigerated centrifuge at 0–4°; nuclei plus unbroken cells were sedimented at 800*g* for 10 min and the mitochondria at 12,000*g* for 10 min. The mitochondria were washed once with the original volume of sucrose–EDTA–Tris solution and, after rehomogenization by hand, once with the same volume of 0.15 M KCl containing 50 mM Tris (pH 7.6 at 5°) and resuspended in KCl–Tris solution to yield a suspension of about 20 mg of protein/ml. The extreme care in preparation and subsequent handling of mitochondria is essential for reproducibility of the effect of DNP as well as metabolic stimulation by CMF. Experiments should be performed preferably with freshly prepared mitochondria. In some instances stability of mitochondria prepared as described is maintained up to 4 hr if kept at 4°. Aged or injured mitochondria are readily recognized by a diminished and broadened DNP profile and a loss of sensitivity toward CMF. Other criteria of mitochondrial integrity (*e.g.*, acceptor control ratio and P:O ratio with glutamate, determined polarographically) generally predict metabolic sensitivity of mitochondria toward both DNP and CMF. For manometric experiments the Gilson respirometer was used. The reaction vessels were kept in an ice bath while components of the test system were added. All reaction components except glutamate were pipetted into the main compartment of the reaction vessels. The standard manometric system contained 150 mM KCl, 30 mM Tris-HCl (pH 7.7 at 23°), 2.3 mM adenine nucleotides, 10 mM glutamate (tipped in from the side arm after 15-min preincubation), 4.5–6.0 mg of mitochondrial protein/3-ml reaction mixture, and DNP at varying concentrations (0–8 × 10^{-5} M). After 5-min temperature equilibration (30°), rates of O_2 uptake were followed for 10 min prior to the addition of glutamate. Metabolic CO_2 was absorbed by 15% KOH in the center well. The gas phase was air. Modifications of this standard system will be described under Results.

Analytical Procedures and Reagents. Mitochondrial protein was determined colorimetrically (Beisenherz *et al.*, 1953) after solubilization of mitochondria with 1% (v/v) Triton X-100. Proteins were precipitated by 2.3% perchloric acid at 0° when analyses for adenine nucleotides were performed. The perchloric acid extracts were brought to pH 7.5–8.0 with 4 M K_2CO_3 to remove the perchlorate. ATP, ADP, and AMP were assayed in deproteinized extracts by enzymatic methods (Lamprecht and Trautschold, 1963; Adam, 1963; Markland and Wadkins, 1966), and orthophosphate according to Lowry and Lopez (1946).

In the experiments reported here CMF was prepared from a homogenate of rabbit liver by the method described previously (Loh *et al.*, 1968), except water was substituted for 0.15 M KCl. The active fraction was separated by chromatography on Sephadex G-25, developed with distilled water. This fraction was treated batchwise with Dowex 1-X8 ion-exchange resin (bicarbonate cycle) to remove contaminating material which absorbs at 260 mμ, and the filtrate was concentrated to 0.1 of the original by freeze drying. As shown previously, CMF as detected by the manometric assay appeared to be related to the amount of reducing component present in the active chromatographic fraction. Consequently, the amount of CMF was arbitrarily expressed in terms of glucose equivalents (*cf.* Loh *et al.*, 1968). Since recent experiments revealed that CMF contained Mg^{2+}, the metabolic effect of this factor could be related to its Mg^{2+} content (see Results). Analyses for Mg^{2+} were performed with the Perkin-Elmer 303 atomic absorption spectrophotometer. An emission spectrum of rabbit liver CMF was taken in the Spectrographic Laboratory of the Department of Pharmaceutical Chemistry (School of Pharmacy) by Mr. M. Hrenoff and evaluated by Dr. Louis H. Strait.

Results

The Influence of Components of the Test System on the DNP Profile. Mitochondria preincubated with DNP for 15 min in the absence of added ADP or ATP oxidized glutamate at relatively low rates which were independent of DNP concentration; *i.e.*, DNP did not stimulate O_2 uptake in the absence of added nucleotides. Similarly, no stimulation of O_2 uptake occurred by CMF, 1 mM $MgCl_2$, or 30 mM HPO_4^{2-} when preincubation of mitochondria with these agents and varying levels of DNP was performed in the absence of added adenine nucleotides.

Significantly different results were obtained when glutamate metabolism was studied after preincubation of mitochondria with 2.3 mM ADP or ATP and increasing concentrations of DNP. While the ascending parts of the DNP profiles (*i.e.*, the DNP concentration-dependent stimulation of metabolism) were similar (up to about 2 × 10^{-5} M DNP), the location of the peak and the descending portion was

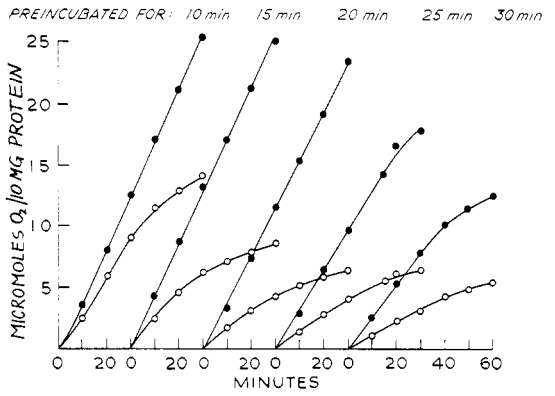

FIGURE 2: The effect of the time of preincubation of mitochondria on the rates of glutamate metabolism in the presence of 2.3 mM ADP and 5×10^{-5} M DNP, with (solid circles) and without (open circles) CMF (equivalent to a final concentration of 0.1 mM Mg^{2+}). The time of preincubation (*i.e.*, prior to addition of glutamate) is indicated on the top of the figure. Ordinate = μmoles of O_2 per 10 mg of protein, measured at time intervals indicated in minutes on the abscissa. All other conditions were described in the legend of Figure 1.

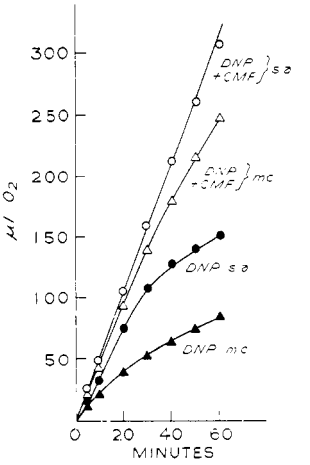

FIGURE 3: The influence of the order of additions of DNP and CMF on the rates of glutamate metabolism expressed as microliters of O_2 (ordinate). Standard assay conditions (see Methods) and a preincubation period of 15 min were employed. Each vessel contained mitochondria (6.1 mg of protein) suspended in the KCl–Tris medium and 2.3 mM ADP. When present, DNP concentration was 5×10^{-5} M. Side arms (abbreviated SA) of vessels contained glutamate and, where indicated, DNP or DNP + CMF. Upper curve (=0) shows the reaction rate when CMF + DNP was tipped in together with glutamate from the side arm. When DNP + CMF were present in the main compartment (abbreviated mc) and glutamate alone was added after 15-min preincubation, results shown in the second curve (Δ) were obtained. In the experiments shown by the remaining curves, DNP (solid circle) was in the side arm and CMF in the main compartment. In the curve indicated by solid triangles, CMF was in the side arm and DPN in the main compartment (abbreviated mc).

highly sensitive to the presence of individual components of the system. With ADP alone, a sharp maximum was observed close to 2×10^{-5} M DNP, followed by a decrease in O_2 uptake to the level of the corresponding controls without DNP, as shown in curve 1 of Figure 1A,B. The DNP profile with ATP as the single added nucleotide was similar (Figure 1A, curve 2). Neither AMP, GTP, nor ITP replaced ADP or ATP. Orthophosphate (30 mM) and ADP (curve 3), or orthophosphate together with 1 mM $MgCl_2$ and ATP (curve 4) markedly broadened the DNP profile. A similar effect of 1 mM $MgCl_2$ in the presence of ADP is shown in curve 5 of Figure 1. The most important aspect of the experiments summarized in Figure 1 is illustrated in curve 2 of Figure 1B. This system contains only CMF (besides ADP + DNP and glutamate) and the amount of Mg^{2+} added as CMF yields a final concentration of 0.1 mM Mg^{2+}, yet the increase of DNP-dependent metabolic rates exceeds the effect of 30 mM HPO_4^{2-} or 1 mM $MgCl_2$ (see III).

In earlier experiments (Kun *et al.*, 1967; Loh *et al.*, 1968), the test system for CMF was composed of 30 mM phosphate, 1 mM $MgCl_2$, 2.3 mM ATP or ADP, and varying amounts of DNP. As seen in Figure 1A (curve 4), this reaction mixture gave rise to a broad (and variable) DNP profile which sometimes partially masked the activating effect of CMF. If the simpler system (DNP–Tris–KCl–ADP, with $MgCl_2$ and phosphate deleted) was used, a sharp and much more reproducible profile was seen (Figure 1A,B, curve 1), and the activating effect of CMF exceeded the maximal stimulation by DNP obtained in the presence of added ADP alone. In contrast to results obtained with the high HPO_4^{2-}- and $MgCl_2^-$- containing assay (Loh *et al.*, 1968), the range of DNP concentration in the Tris–KCl–ADP system necessary for maximal stimulation, did not depress O_2 uptake in the absence of CMF below the basal metabolism of mitochondria. Basal metabolism is defined as the metabolic rate of controls containing no DNP. It is clear that CMF in the KCl–Tris–ADP system does not merely reverse the inhibitory effect of DNP, as seemed to be the case in the originally developed assay (Loh *et al.*, 1968), but markedly enhances the stimulatory effect of DNP.

Because of its high degree of reproducibility, the DNP–Tris–KCl–ADP system was employed for further studies concerned with the nature of metabolic activation by CMF.

The Influence of Time of Preincubation and the Order of Addition of Components on the Stimulation of Glutamate Metabolism. This question was reinvestigated with the presently developed ADP–KCl–Tris system. Mitochondria were preincubated with 2.3 mM ADP and 5×10^{-5} M DNP in the absence and presence of a constant amount of CMF, and glutamate was added at time intervals varying from 10 to 30 min (Figure 2). While the rate of O_2 uptake remained linear in the presence of CMF, preincubation with DNP and ADP alone resulted in a progressive decline in metabolism. Beyond the preincubation period of about 25 min, the rate of respiration diminished in all systems, although the relative stimulatory effect of CMF was still noticeable. It is evident that quantitative assays for CMF are complicated by the fact that the rates without CMF deviate from linearity beyond a reaction time of 20 min. The determination of CMF content by the metabolic assay is subject to errors unless initial linear portions of rates without CMF are compared with linear rates obtained in the presence of various quantities of CMF.

In further experiments, the period of temperature equilibration was reduced to 3–4 min. The oxidation of glutamate was initiated by the addition of mitochondria to the preequilibrated assay system, containing glutamate in all cases and

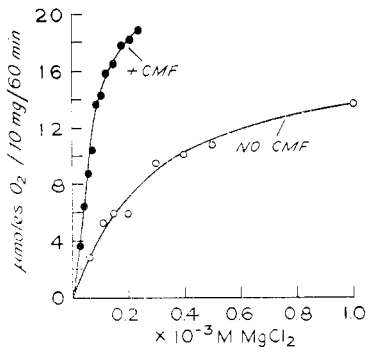

FIGURE 4: Relationship between rates of glutamate metabolism, concentration of $MgCl_2$, and the concentration of Mg^{2+} present in CMF. Experimental conditions were the same as described for Figure 1B. Reaction rates (ordinate) are expressed as μmoles of O_2/10 mg of mitochondrial protein per 60 min. Abscissa = concentration of added Mg^{2+}. The lower curve shows the $MgCl_2$ concentration–reaction rate relationship, while the upper curve indicates the effect of Mg^{2+} present in increasing amounts of CMF. The stock liver CMF contained 9.7 mM Mg^{2+}. The reaction system contained 5.9 mg of mitochondrial protein and components of the standard assay (2.3 mM ADP and 5×10^{-5} M DNP). Rates of O_2 uptake were corrected for controls containing only DNP but no $MgCl_2$ or CMF.

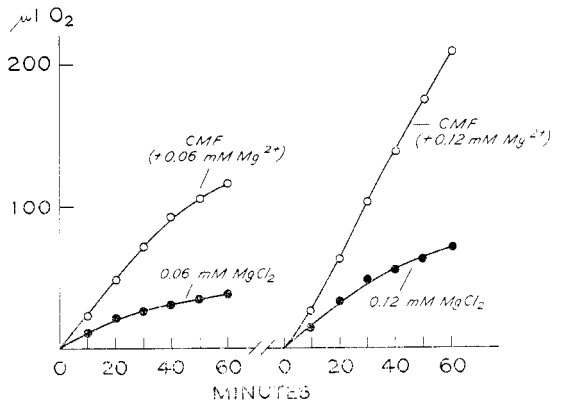

FIGURE 5: The effect of $MgCl_2$ and equivalent amounts of CMF (in terms of Mg^{2+}) on glutamate metabolism as a function of time. Experimental conditions were the same as described in the legend of Figure 4, except two fixed concentrations of Mg^{2+} (as $MgCl_2$ or Mg^{2+} present in CMF) were employed.

adenine nucleotides, and DNP and CMF in various combinations. Added ADP or a mixture of ADP, ATP, AMP, and HPO_4^{2-} in proportions formed during 15-min preincubation of mitochondria, with ADP + DNP supported metabolic stimulation in the presence of DNP + CMF in a similar manner. The increase in glutamate metabolism by CMF became apparent only after a reaction period of 15–20 min.

The significance of the order of addition of DNP and CMF was also studied. In the experiments illustrated in Figure 3, mitochondria were preincubated for 15 min in the ADP–KCl–Tris medium with CMF alone, with DNP alone, with both CMF and DNP, and with neither DNP nor CMF present. In each case, the missing component was placed together with glutamate into the side arm of reaction vessels and tipped into the main compartment after 15-min preincubation. The characteristic stimulation of O_2 uptake by CMF occurred only when DNP + CMF were added simultaneously to mitochondria from the side arm or were preincubated together with mitochondria in the main compartment. In these experiments ADP was present with the mitochondria during preincubations.

The Role of Mg^{2+} and HPO_4^{2-} in the Metabolic Effect of CMF. Since in the DNP–KCl–Tris–ADP system the rate of glutamate metabolism was stimulated by both 1 mM Mg^{2+} and 30 mM HPO_4^{2-}, although generally not to the same extent as by CMF, a relationship between these ion effects and the effect of CMF was sought. It was found that CMF isolated as described contained almost all the Mg^{2+} present in aqueous liver extracts, which were the source of CMF. Spectrographic analysis of rabbit liver CMF revealed Mg^{2+} and some Ca^{2+}, but no significant amounts of other metals except Na^+. According to atomic absorption analyses, the preparation of rabbit liver CMF used in the experiments shown in Figures 4 and 5 contained 9.7 mM Mg^{2+} and 0.15 mM Ca^{2+}. The concentration of Mg^{2+} which was introduced by CMF (40 μl/3 ml) into the metabolic assay system was close to 0.1 mM in all experiments. When the rate of O_2 uptake per 60 min was correlated with the amount of Mg^{2+} added either as $MgCl_2$ (lower curve of Figure 4) or in the form of CMF (upper curve of Figure 4), a marked difference between the activating efficiencies of the two sources of Mg^{2+} was consistently observed. The difference between $MgCl_2$ and CMF became even more apparent when the time course of metabolism was followed. As shown in Figure 5, the rate of O_2 uptake was maintained at a much higher level when Mg^{2+} was introduced as CMF than in the presence of equivalent amounts of $MgCl_2$. On the other hand, Ca^{2+} content of CMF could not be readily related to metabolic stimulation. The effect of $MgCl_2$ was uninfluenced by added amounts of $CaCl_2$, which were equal to the concentration of Ca^{2+} found in CMF. Higher concentrations of $CaCl_2$ (*e.g.*, 5×10^{-5} M) completely inhibited the metabolic effect of CMF.

Preliminary experiments indicate that the activity of CMF preparations from rabbit liver is lost upon heating at 100° at pH 1.0 or below for 5–10 min without the appearance of appreciable amounts of inhibitors. More drastic treatment, such as boiling CMF in 0.2 N HCl on an open flame for 2 min, not only destroys the activating effect but produces inhibitors of mitochondrial metabolism, most likely due to acid-degradation products of the organic constituents of CMF. Wet ashing with concentrated HNO_3 destroyed the activity of CMF, but contaminating heavy metals (introduced by HNO_3) produced some inhibitory side effects. Direct analyses of CMF for ATP, ADP, UDP, IDP, CDP, and pyridine nucleotides yielded negative results; therefore these substances as ligands for Mg^{2+} could be readily excluded. The essential role of Mg^{2+} in the mitochondrial effect of CMF was further illustrated by the fact that removal of Mg^{2+} from CMF by a chelating resin (Chelex-100) drastically reduced its metabolic effect and abolished the protective influence of CMF on the loss of mitochondrial Mg^{2+} (see Figure 6 and Table I). Both of these effects were completely restored when Mg^{2+} (as $MgCl_2$) was added back to Chelex-100-treated CMF in amounts present in the original preparation.

Because of the apparent significance of Mg^{2+} in the metabolic effect of CMF, the Mg^{2+} content of mitochondria was

TABLE I: The Effect of Experimental Conditions on Mg^{2+} Release from Rat Liver Mitochondria during Incubation for 15 min at 30°.[a]

No.	Experimental Conditions	O_2 Uptake (μmoles)[b]	% Mitochondrial Mg^{2+} Released[c]
1	Control (in KCl-Tris)		27
2	Control + ADP (2.3 mM)		28
3	Control + DNP (0.05 mM)		25
4	Control + DNP + ADP	5.2	67
5	Control + DNP + ATP		43
6	Control + DNP + ATP + oligomycin		40
7	Control + DNP + AMP		21
8	Control + DNP + ADP + CMF (0.13 mM Mg^{2+})	21.0	28
9	Control + DNP + ADP + $MgCl_2$ (0.13 mM Mg^{2+})	10.5	58

[a] Liver mitochondria (5.6 mg of protein) were incubated without added substrate in 3 ml of KCl–Tris suspending medium (see Methods) for 15 min at 30°. The suspending medium was then separated from mitochondria by centrifugation at 4° and Mg^{2+} analyses were performed on the supernatant fluid and at zero time on mitochondria. The release of Mg^{2+} from mitochondria is expressed as per cent of mitochondrial Mg^{2+} found in the supernatant fluid after 15-min incubation (*b*). When the rate of glutamate metabolism was correlated with Mg^{2+} loss, glutamate was tipped in from the side arm after 15-min preincubation and O_2 consumption (*c*) followed for 60 min (as described in Methods). All adenine nucleotides were added at a concentration of 2.3 mM and DNP at 0.05 mM. The amount of oligomycin was 10 μg. The mitochondrial Mg^{2+} content at zero time was 22 mμmoles of Mg^{2+}/mg of mitochondrial protein.

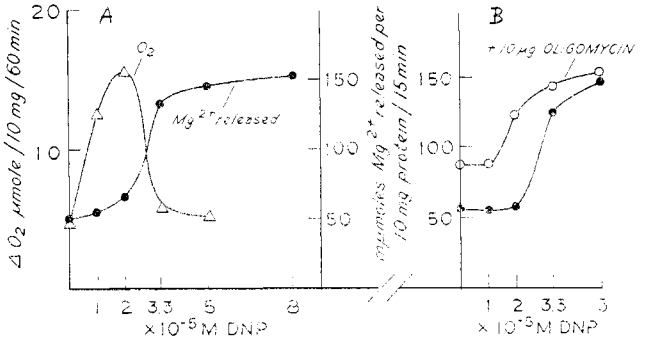

FIGURE 6: Correlation between DNP profile and the release of mitochondrial Mg^{2+} into the suspending medium. In part A the rate of O_2 consumption (upper curves = Δ) was determined under conditions described in Figure 1B (curve 1) and is shown on the left ordinate. The release of mitochondrial Mg^{2+} was determined by incubating mitochondria without glutamate in 3 ml of Tris–KCl medium (see Methods) in the presence of 2.3 mM ADP, and varying concentrations of DNP (shown on the abscissa) for 15 min at 30°. Analyses for Mg^{2+} were performed at zero time (on mitochondria) and after 15-min incubation on the supernatant fluid. This is shown on the right ordinate of A and the left ordinate of part B. Mitochondrial Mg^{2+} content was 190 mμmoles of Mg^{2+}/10 mg of protein in the experiment illustrated in A, while this value was 228 mμmoles of Mg^{2+}/10 mg of protein in expt B. The effect of 10 μg of oligomycin on the DNP concentration-dependent Mg^{2+} release (in the presence of 2.3 mM ADP) is shown in the upper curve of B (O), whereas the lower curve of B indicates Mg^{2+} release under the same conditions without oligomycin (compare with lower curve of A). Mitochondrial protein in A = 6.0 mg and in B = 5.6 mg.

determined under various experimental conditions. As shown in Table I (expt 1), preincubation of mitochondria for 15 min at 30° in 150 mM KCl–30 mM Tris (pH 7.4 at 30°) resulted in a 27% loss of mitochondrial Mg^{2+} content. Addition of 2.3 mM ADP or 0.05 mM DNP alone did not influence Mg^{2+} loss from mitochondria. The simultaneous presence of both ADP and DNP, however, greatly increased extrusion of Mg^{2+} from mitochondria (expt 4), and during 15 min, 67% of mitochondrial Mg^{2+} appeared in the suspending medium. The loss of Mg^{2+} from various batches of mitochondria in the presence of ADP + DNP ranged between 55 and 70% of the originally present mitochondrial Mg^{2+}. All mitochondrial Mg^{2+} was lost when incubation with ADP + DNP was prolonged for 60 min, while Mg^{2+} content of the same mitochondria was preserved up to half of the original level for 60 min when ADP or DNP was present separately. Addition of glutamate after preincubation with ADP + DNP did not restore mitochondrial Mg^{2+} content. Attempts to replace ADP by AMP (expt 7) or ATP (expt 5 and 6) were unsuccessful. There is some increase in Mg^{2+} release over control values when 2.3 mM ATP + 0.05 mM DNP are preincubated with mitochondria, but this small effect was traced to ADP contamination of ATP solutions, which was 0.07 mM ADP. Moreover, the small increase in Mg^{2+} release in the presence of ATP was independent of DNP concentration, while with ADP as the initially added nucleotide, a characteristic DNP concentration-dependent sigmoidal relationship was found (see Figure 6). It is of further interest that oligomycin, which is known to inhibit DNP-activated ATPase (Lardy *et al.*, 1964; Kun *et al.*, 1966), has no influence on Mg^{2+} release when ATP is present, but increases Mg^{2+} loss of mitochondria when ADP is the initially added nucleotide (Figure 6B). Present results suggest that in the absence of metabolizable substrate, ADP specificity of the (DNP + ADP)-induced loss of mitochondrial Mg^{2+} is determined during initial exposure of mitochondria to ADP, and events which are responsible for nucleotide specificity are unrelated to the rate of enzymatic hydrolysis of ATP.

The loss of mitochondrial Mg^{2+} induced by ADP + DNP is inhibited by CMF. The same amount of CMF prevents loss of mitochondrial Mg^{2+} as is required to support maximal metabolism of glutamate (expt 8). A concentration of $MgCl_2$ equivalent to the Mg^{2+} content of CMF has very little effect on the loss of mitochondrial Mg^{2+} and only raises metabolism

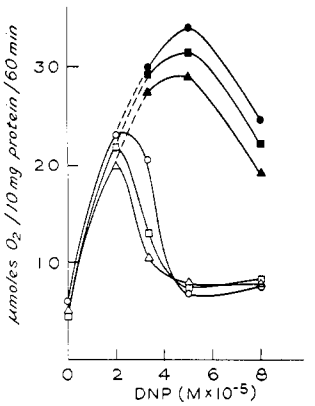

FIGURE 7: The influence of added HPO_4^{2-} (0.5 and 1.66 mM) on the DNP profile (see text), determined in the presence of 2.3 mM ADP alone (lower curves) and in the presence of ADP + CMF (upper curves). Experimental conditions were the same as described in the legend of Figure 1. (Δ) DNP, (▲) DNP + CMF, (□) DNP + 0.5 mM HPO_4^{2-}, (■) DNP + 0.5 mM HPO_4^{2-} + CMF, (○) DNP + 1.66 mM HPO_4^{2-}, and (●) DNP + 1.66 mM HPO_4^{2-} + CMF.

to about half-maximal levels (expt 9). Further experiments which indicate a close correlation of mitochondrial Mg^{2+} content and the biphasic metabolic response of mitochondria to varying levels of DNP are illustrated in Figure 7A. The disappearance of metabolic stimulation by DNP coincides with the loss of Mg^{2+} from mitochondria.

The specificity of the loss of mitochondrial Mg^{2+} during preincubation with ADP + DNP was tested by simultaneous analyses of oxidized and reduced pyridine nucleotides in the suspending medium of mitochondria. Under conditions when about 80% of mitochondrial Mg^{2+} was recovered in the suspending medium, no reduced or oxidized pyridine nucleotides could be detected by specific enzymatic tests (Estabrook and Maitre, 1962). The sensitivity of detection of pyridine nucleotides is close to 10^{-8} M.

The second ionic constituent which at a level of 30 mM stimulated O_2 uptake in the DNP–KCl–Tris–ADP system was HPO_4^{2-}. It was of importance to test the possibility that HPO_4^{2-} formed from ADP in the presence of DNP might contribute to the increase in O_2 uptake in the system containing CMF. The steady-state level of HPO_4^{2-} in the presently developed CMF assay system was found to be close to 1.6 mM. As shown in Figure 7, addition of 0.5 or 1.66 mM HPO_4^{2-} did not influence either the DNP profile or the stimulatory effect of CMF. This amount of HPO_4^{2-} had no effect on mitochondrial Mg^{2+} content. The elucidation of the mechanism of the activating effect of 30 mM HPO_4^{2-} requires further studies. It is certain, however, that the effect of high, clearly unphysiological levels of HPO_4^{2-} is readily separable from the lack of effect of a low concentration of HPO_4^{2-}, which is found in our assay systems.

The essential role of added adenine nucleotides in the metabolic stimulation by $MgCl_2$, as well as by CMF in the presence of DNP, could not be related to the activity of adenylate kinase as determined by kinetics of ATP, AMP, and HPO_4^{2-} formation from ADP. The activity of isolated adenylate kinase (Boehringer) or of adenylate kinase activity of isolated mitochondria was influenced in an identical manner by $MgCl_2$ and by an amount of CMF containing an equivalent concentration of Mg^{2+}. The lack of discrimination by adenylate kinase between $MgCl_2$ and Mg^{2+} present in CMF indicates that adenine nucleotide substrates of the adenylate kinase system successfully compete for Mg^{2+} present in CMF.

Discussion

It is readily seen from an analysis of experimental conditions that events which occur during preincubation of mitochondria with ADP + DNP determine the magnitude of increase in metabolism induced by CMF or to a much lesser extent (Figure 4 and 5) by $MgCl_2$. The unique reaction which could be correlated with the modifications of the mitochondrial metabolism of glutamate was an (ADP + DNP)-dependent ejection of mitochondrial-bound Mg^{2+} into the suspending medium (see Figure 6). The process of Mg^{2+} ejection is prevented under specific conditions by CMF and, to a much lesser extent, by $MgCl_2$, in amounts equivalent to the Mg^{2+} present in CMF (see Table I). The augmenting effect of the organic (ligand) constituent of CMF on the effectivity of Mg^{2+} is compatible with at least two reaction mechanisms. It is possible that CMF serves as a Mg^{2+} carrier; or a constituent of CMF may activate a mitochondrial site for Mg^{2+} which plays a rate-limiting role in mitochondrial metabolism. Distinction between these two mechanisms, or establishment of coexistence of both types of actions of CMF requires further studies. It is, however, certain that results reported in this paper provided significant clues, helpful in continued experimental work, aimed at the identification of the active component of CMF. Metabolic activity of CMF can now be related to a Mg^{2+} binding constituent of CMF, and its effect on mitochondria narrowed to a Mg^{2+}-sensitive mitochondrial site.

The obviously unphysiological requirement for DNP for the experimental demonstration of the metabolic effect of CMF suggests that a defect has first to be created in the function of a mitochondrial system before the effect of CMF is demonstrable. This defect is then specifically corrected or replaced by CMF. Clearly, the understanding of the molecular mechanism of action of uncouplers is a prerequisite for the formulation of the mode of action of CMF. Although the mechanism of "uncoupling" is still unknown (*cf.* Lardy and Ferguson, 1969), our results permit certain predictions related to the mitochondrial site of uncouplers. Ever since the pioneering studies of Lardy and Wellman (1953), the site of action of uncouplers has been assumed to be the energy transducing system of mitochondria. This site has been located in the inner membrane of mitochondria (*cf.* Sottocasa *et al.*, 1967), but its chemical nature has remained unknown. According to our results, the mitochondrial system which responds to DNP with an increase in metabolism requires a certain level of bound mitochondrial Mg^{2+} and the presence of one specific substrate of oxidative phosphorylation, namely, ADP. These experimental criteria tend to indicate that the site of action of DNP may be a Mg^{2+}-requiring and CMF-sensitive constituent of the oxidative phosphorylating system.

The role of DNP in our assay system may become more understandable if one considers this substance as a specific reagent capable of donating protons to a hydrophobic region in mitochondria, as proposed by Skulachev *et al.* (1967).

The results of this effect of DNP may be a dissociation of some component, which is otherwise tightly bound to a system, located in a hydrophobic membrane site. Release of bound mitochondrial Mg^{2+} by DNP in the presence of ADP is likely to be the consequence of the specific dissociating effect of the uncoupler. This phenomenon may be analogous to the dissociation of a coenzyme from its apoenzyme, except on a more complex scale. This working hypothesis serves as a guide in further experimental studies.

Although the specificity of ADP for the induction of Mg^{2+} loss of mitochondria is apparent, this requirement is difficult to assess under conditions when mitochondria perform active metabolism of glutamate. It is possible that the nearly equivalent role of ADP and ATP under *metabolic* conditions (Figure 1) may be explained by the hydrolysis of ATP and active transfer of ADP to mitochondrial sites which are inaccessible to ADP formed from ATP in the absence of oxidizable substrates.

The validity of the proposed connection between a Mg^{2+}-requiring mitochondrial system, the site of action of uncouplers, and the reactivation of this site by CMF depends upon the specificity of Mg^{2+} release from mitochondria. It may be argued that even brief preincubation of mitochondria without oxidizable substrates may cause disintegration of mitochondrial structures, and the loss of Mg^{2+} is merely one of the signs of mitochondrial decay. While preincubation of mitochondria with DNP without added nucleotides results in a progressive loss of metabolic functions, DNP itself does *not* increase the rate of loss of mitochondrial Mg^{2+} (see Table I). Moreover, ADP, as shown by Connelly and Hallstrom (1967), is an allosteric stabilizer of mitochondria; therefore incubation with 2.3 mM ADP for 15 min is not likely to cause a nonspecific decay of mitochondrial integrity. Since no detectable leakage of mitochondrial pyridine nucleotides occurs under conditions when almost all mitochondrial Mg^{2+} is lost (in presence of DNP + ADP), the latter process may be considered reasonably specific.

Van Dam and Tsou (1968) reported that the inhibitory effect of DNP on mitochondrial oxidation of succinate and some other carboxylic acid bears a competitive relationship to substrate concentration. It should be noted that these workers employed 0.5 mM DNP, which is about 10–15 times more than was used in our experiments. The biphasic DNP profile, which requires added adenine nucleotides, and was observed at low levels of DNP, could not have been detected under conditions described by Van Dam and Tsou (1968). It was shown previously (*cf.* Loh *et al.*, 1968) and confirmed recently, that the oxidation of succinate is not influenced by CMF. It is therefore unlikely that the phenomenon of inhibition of respiration by high levels of DNP and its reversal by succinate and other substrates is directly related to our experiments.

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References

Adam, H. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic, p 573.

Beisenherz, G., Boltze, H. S., Bücher, T., Czok, K. H., Garbade, K. H., Meyer-Arendt, E., and Pleiderer, G. (1953), *Z. Naturforsch. 83*, 555.

Connelly, J. L., and Hallstrom, C. H. (1967), *Biochemistry 6*, 1567.

Estabrook, D. W., and Maitre, P. K. (1962), *Anal. Biochem. 3*, 369.

Kun, E., Ayling, J. E., and Baltimore, B. G. (1964), *J. Biol. Chem. 239*, 2896.

Kun, E., Loh, H. H., and El-Fiky, S. B. I. (1966), *Mol. Pharmacol. 2*, 481.

Kun, E., Loh, H. H., and Volfin, P. (1967), *Proc. 7th Intern. Congress Biochem., Tokyo 5*, 868.

Lamprecht, W., and Trautschold, I. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic, p 543.

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

Lardy, H. A., and Ferguson, S. M. (1969), *Ann. Rev. Biochem. 38*, 713.

Lardy, H. A., and Wellman, H. (1953), *J. Biol. Chem. 201*, 357.

Loh, H. H., Volfin, P., and Kun, E. (1968), *Biochemistry 7*, 726.

Lowry, O. H., and Lopez, J. A. (1946), *J. Biol. Chem. 162*, 421.

Markland, F. S., and Wadkins, C. L. (1966), *J. Biol. Chem. 241*, 4124, 4136.

Skulachev, V. P., Sharaf, A. A., and Liberman, E. A. (1967), *Nature 216*, 718.

Sottocasa, G. L., Kulenstierna, B., Ernster, L., and Bergstrand, A. (1967), *J. Cell Biol. 32*, 415.

Van Dam, K., and Tsou, C. S. (1968), *Biochim. Biophys. Acta 162*, 301.