Regulation of Mitochondrial Metabolism by Specific Cellular Substances. I. Isolation of a Cytoplasmic Fraction and Its Effect on Mitochondrial Oxidations*

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ABSTRACT: Increasing concentrations of 2,4-dinitrophenol exert a biphasic effect on the O_2 consumption of mitochondria oxidizing certain carboxylic acid substrates in 0.15 M KCl in the presence of adenosine triphosphate (ATP) or adenosine diphosphate (ADP) and Mg^{2+}. This effect of 2,4-dinitrophenol is modified by a soluble cytoplasmic cellular fraction. The modification consists of a large increase of O_2 consumption within a limiting range of concentrations of 2,4-dinitrophenol.

The cytoplasmic component responsible for this effect was obtained from rat liver, kidney, and heart by gel filtration on Sephadex G-25 and G-50 as a macromolecular complex of apparent molecular weight of 5000–6000. The metabolically active cytoplasmic fraction inhibits the activating effect of 2,4-dinitrophenol on mitochondrial adenosine triphosphatase (ATPase) only in the presence of carboxylic acid substrates. Prerequisities for *in vitro* metabolic action of the cytoplasmic fraction are preservation of mitochondrial membrane system and presence of ADP or ATP (adenosine monophosphate is ineffective), isotonic KCl (present both during preparation of the fraction and during metabolic experiments), and a specific concentration of 2,4-dinitrophenol, which is characteristic for certain substrates and varies with the tissue source of mitochondria. A possible interpretation of these results is proposed in terms of extramitochondrial regulation of mitochondrial permease systems.

During experiments concerned with the mechanism of action of synthetic fluorocarboxylic acids in tissue homogenates (Kun *et al.*, 1964; Loh and Kun, 1966; Kun *et al.*, 1966a; Kun, 1967), 2,4-dinitrophenol (DNP) was employed in order to simplify metabolic control in this system by eliminating the effects of oxidative phosphorylation. It was assumed that in multienzyme systems kinetic consequences of competitive (*i.e.*, substrate type) inhibitors would be easier to evaluate in the absence of oxidative phosphorylation. In the course of preliminary studies, which were intended to test this assumption, it was found that the soluble fraction of tissue homogenates (cytosol)[1] markedly influenced the metabolic effect of DNP on mitochondria. More detailed analyses of this phenomenon led to the isolation of a cytoplasmic macromolecular fraction (CMF)[2] from the cytosol of liver, kidney, and heart which could replace cytosol with respect to an apparent stimulation of the oxidation of certain carboxylic acids by mitochondria. Because of the unusual effects of CMF on mitochondrial metabolism, studies were initiated in order to clarify its mechanism of action, composition, and possible biological significance. The present report deals with a method of recognition and isolation of CMF, providing the foundation for further work. A preliminary paper describing the stimulation of glutamate metabolism by CMF appeared in the form of an abstract (Kun *et al.*, 1967).

Experimental Procedures

Mitochondria from rat liver were prepared by differential centrifugation of 15% tissue homogenates in 0.25 M sucrose containing 1.34×10^{-3} M EDTA adjusted to pH 7.4 with 0.01 M Tris buffer, as described previously (Kun *et al.*, 1964). Sedimented mitochondria were washed once with 0.25 M sucrose–EDTA, then with 0.15 M KCl. Mitochondria were then resuspended

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[1] The term *cytosol*, introduced by H. A. Lardy, denotes the particle-free supernatant of tissue homogenates (in our case prepared in 0.15 M KCl) obtained after ultracentrifugation at 105,000*g* for 60 min.

[2] Abbreviations used: CMF, cytoplasmic macromolecular fraction; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates; DPN, and DPNH, oxidized and reduced diphosphopyridine nucleotides; FAD, flavin–adenine dinucleotide.

in 0.15 M KCl and the volume of the suspension was adjusted with 0.15 M KCl to give a mitochondrial protein concentration (analyzed according to Beisenherz *et al.*, 1953) of 10 mg/test system.

Manometric assays were carried out in Gilson respirometers at 30°, with air as gas phase. The main compartment contained 10 mg of mitochondrial protein, 2.3×10^{-3} M ADP (or ATP), 10^{-3} M $MgCl_2$, 3×10^{-2} M phosphate buffer (pH 7.4), and CMF or cytosol prepared in 0.15 M KCl. Substrates, 30 mmoles in 0.3 ml, were placed into the side arm. The volume of the reaction mixture was 3 ml, adjusted with 0.15 M KCl; metabolic CO_2 was absorbed by 10% KOH, placed together with a filter paper into the center well. During pipetting of reaction components, the respirometer vessels (of about 20-ml capacity) were chilled in crushed ice. After temperature equilibration (30°), endogenous respiration (*i.e.*, with substrate in side arm) was measured for 15 min; then the substrate was tipped into the main compartment and O_2 uptake was followed for 30 min. The rate of endogenous respiration was less than 5% of the rate measured after addition of substrates.

The reaction was terminated by 0.1 ml of 70% $HClO_4$. Analyses for glutamate (Holzer *et al.*, 1963), aspartate (Pfleiderer, 1963), adenine nucleotides, and NH_4^+ (Kun *et al.*, 1966b) were performed on deproteinized centrifugal extracts of incubation mixtures after removal of perchlorate by KOH (pH was adjusted to 8.4 at 0°). Orthophosphate was assayed according to Lowry and López (1946) and total bound phosphate as orthophosphate after digestion with concentrated H_2SO_4. Ninhydrin-positive components of CMF were determined by the procedure of Alberti and Bartley (1963). Reducing equivalents of CMF were measured by the colorimetric procedure of Somogyi (1945). Nucleotides, glutamic and malic dehydrogenase, and glutamate–aspartate transaminase were purchased from Sigma Chemical Co. and Boehringer Co. 2,4-Dinitrophenol was recrystallized from ethanol–water. Oligomycin was a gift from Dr. H. A. Lardy, University of Madison, Wis. Spectrophotometric assays were carried out on a Zeiss PMQ II spectrophotometer equipped with chart recorder and scale expansion. Sephadex G-25 and G-50 were commercial preparations obtained from Pharmacia Chemicals, Uppsala, Sweden.

Results

A. Assay Conditions for CMF. When liver mitochondria, prepared as described, oxidize glutamate (tipped in after 15-min preincubation) in the presence of 2.3×10^{-3} M ADP or ATP in KCl–phosphate–$MgCl_2$ medium, increasing concentrations of DNP (between 0 and 2×10^{-4} M) exert a biphasic influence on the rate of O_2 consumption. It should be noted that during 30 min the rate of O_2 uptake in liver mitochondria oxidizing glutamate is the same in the presence of either 2.3×10^{-3} M added ATP or ADP. In the absence of DNP the steady-state concentration of ATP varied between 1.2 and 1.3×10^{-3} M and that

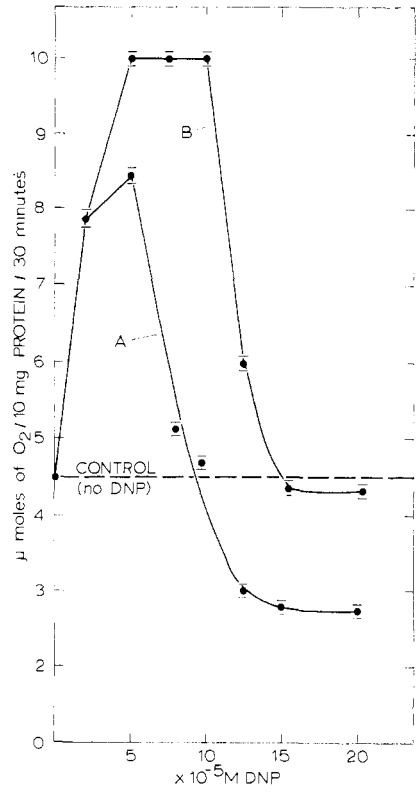

FIGURE 1: The effect of CMF or cytosol on the oxidation of glutamate by liver mitochondria in the presence of 2.3×10^{-3} M ATP, 10^{-3} M $MgCl_2$, 3×10^{-2} M phosphate, and various concentrations of DNP. Curve A: no CMF or cytosol present. Curve B: CMF (1.2 μmoles of reducing equivalents) or cytosol (3.5 mg of protein) was present in all reaction vessels, containing 0–20 $\times$ 10^{-5} M DNP. The experimental procedure was the same as described in the text.

of ADP is close to 1×10^{-3} M, regardless of which of the two nucleotides was added at the beginning of the incubation. Dinitrophenol at 5×10^{-5} M slightly lowers the steady-state level of ATP to 0.53–0.65 $\times$ 10^{-3} M, while the concentration of ADP remains between 0.85 and 0.95 $\times$ 10^{-3} M. Only negligible oxidation of glutamate occurs in the absence of either added ATP, ADP, or DNP. It appears that the stimulatory effect of 5×10^{-5} M DNP in the presence of added ATP or ADP exceeds the well-known stimulation of respiration by ADP alone (in the absence of DNP) or the increase of O_2 uptake caused by DNP in the absence of added adenine nucleotides (Lardy and Wellman, 1953). The nature of this effect of DNP is a subject of further experimental studies.

As shown in Figure 1, an increase of O_2 uptake is observed up to a concentration of DNP of about 2×10^{-5} M. The increase of O_2 uptake due to DNP until this level of the uncoupling agent is exactly the same in the presence or absence of a constant amount of CMF (or cytosol). Above 2×10^{-5} M DNP, the effect of CMF becomes noticeable. Without CMF, an

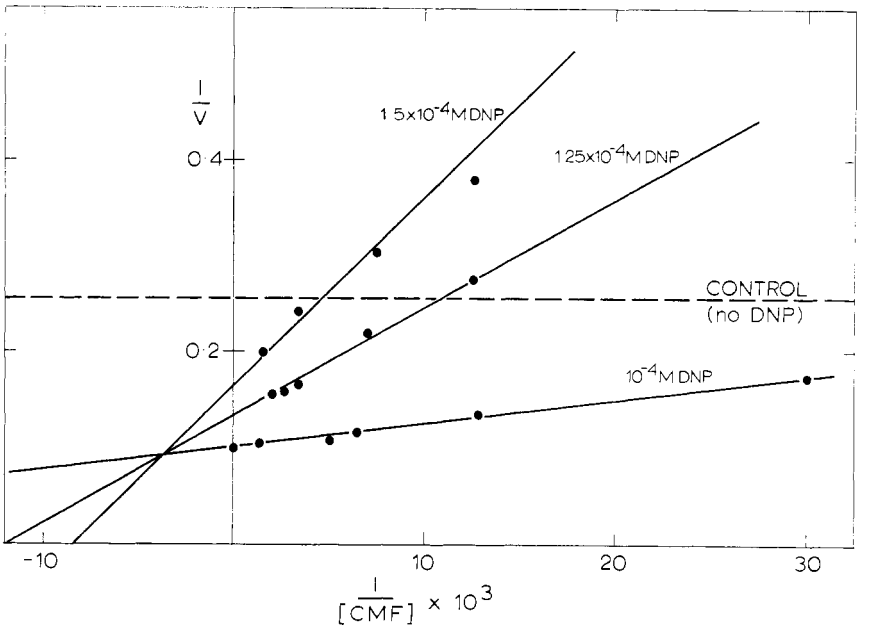

FIGURE 2: Kinetic relationship between DNP and CMF. CMF concentration was varied at three levels of DNP. Ordinate: O_2 uptake (μmoles of O_2/10 mg of protein per 30 min). Abscissa: CMF in μmoles of reducing equivalents. Substrate was glutamate. Experimental conditions were the same as described in the legend to Figure 1.

increase in DNP concentration above 5×10^{-5} M results in a sharp decline of O_2 uptake, and at close to 10^{-4} M DNP the rate of O_2 uptake is the same as in the controls (containing no DNP). Further increase of DNP levels produces a partial inhibition of O_2 uptake. The degree of inhibition between 1.5 and 2×10^{-4} M DNP is almost the same. As illustrated by Figure 1, the shape of the curve obtained by connecting rates of O_2 uptake at different levels of DNP is markedly altered in the presence of a constant amount of isolated CMF or cytosol (equivalent to 3–6 mg of cytoplasmic protein). At 10^{-4} M DNP the rate of oxygen uptake is increased 2.5-fold by CMF. Curves obtained in the presence of either CMF or appropriate amounts of crude cytosol are superimposable. Although with different mitochondrial preparations the concentrations of DNP required to demonstrate this effect of CMF show some variation (*e.g.*, concentration of DNP, which in the absence of CMF no longer increases O_2 uptake over the control values, may vary between 1 and 1.25×10^{-4} M), the phenomenon of increase of O_2 uptake by CMF is entirely reproducible. This was observed with more than 200 different mitochondrial preparations in a total of over 1000 manometric experiments.

Determination of the "DNP profile" of mitochondrial preparations (*i.e.*, the rates of O_2 uptake as a function of DNP concentration in the absence and presence of CMF) is essential for the establishment of conditions suitable for the quantitative assay for CMF activity. The manometric assay consists of quantitative determination of the degree of activation of respiration which occurs as a function of the amount of CMF in the range of DNP concentrations where respiration without CMF approaches the control values, or begins to show partial inhibition. A hyperbolic relationship exists between the degree of activation of O_2 uptake and the amount of CMF. The quantity of CMF which under these conditions raises the O_2 uptake to half-maximal value is the experimentally determined K_{CMF}. This value (defined as 1 catalytic unit), by analogy with K_m, can be determined by a double-reciprocal plot (1/% activation *vs.* 1/[CMF]). The accuracy of the method under these conditions is limited by the accuracy of the manometric technique ($\pm 5\%$).

When the reciprocal of the rate of O_2 uptake is plotted against the reciprocal of the concentration of CMF at various levels of DNP within the range where CMF increases mitochondrial O_2 uptake, a family of curves is obtained which intersect in the left upper quadrant (Figure 2). By analogy with the steady-state kinetics of two substrate systems, this intersection (projected to the abscissa) is a complex expression ($-K_{DNP}/(K_{DNP \times CMF})$) which is hard to evaluate at present. A simpler illustration of the same relationship is obtained by plotting the increase in the rate of O_2 uptake caused by increasing amounts of CMF against the quantity of CMF within a critical range of DNP concentrations (Figure 3).

An increase of substrate concentration above apparent saturation level (10 mM for all substrates tested) has no detectable effect on the degree of activation by CMF. At lower substrate levels, below apparent

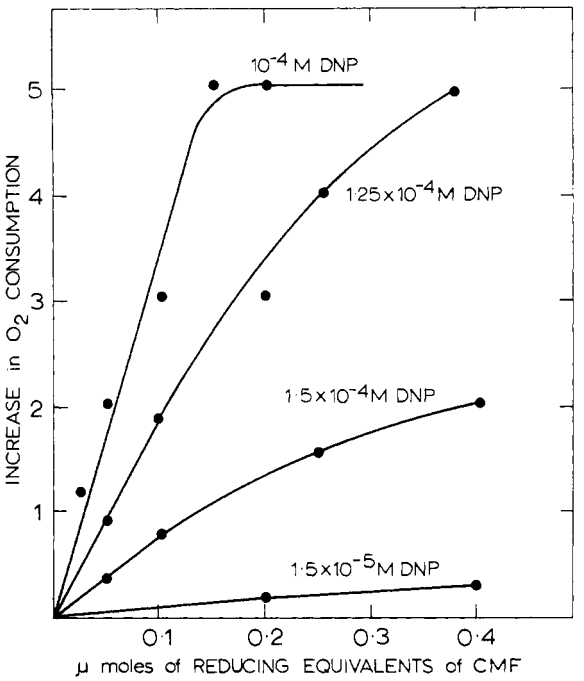

FIGURE 3: Kinetic relationship between DNP and CMF. CMF concentration was varied at three levels of DNP. Ordinate: O_2 uptake (μmoles of O_2/10 mg of protein per 30 min). Abscissa: CMF in μmoles of reducing equivalents. Substrate was glutamate. Experimental conditions were the same as described in the legend to Figure 1.

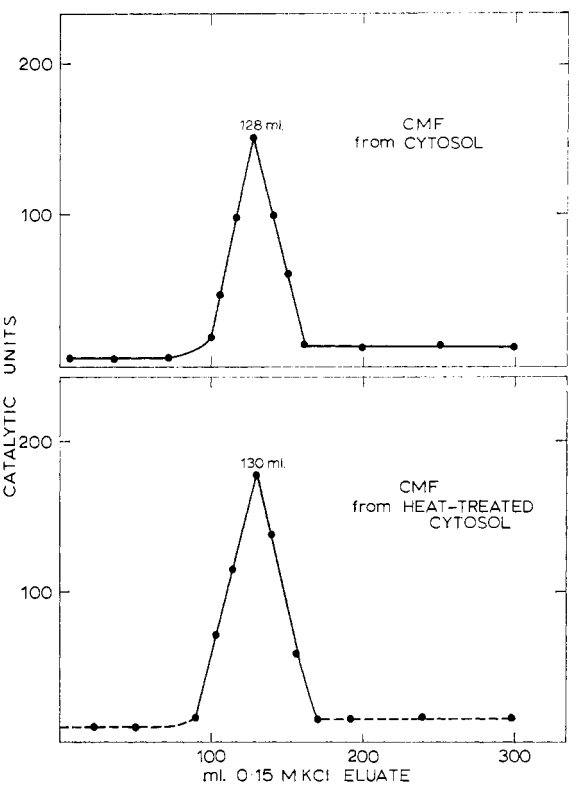

FIGURE 4: Elution pattern of CMF from liver cytosol from a Sephadex G-25 column. Catalytic units (K_{CMF}) were determined in the manometric system. Colorimetric analysis of reducing equivalents gives an elution pattern which is indistinguishable from that obtained by the manometric assay. The upper elution pattern is obtained from cytosol without prior deproteinization by heat treatment, the lower one after heat treatment. V = 395 ml; V_0 = 104 ml.

saturation of the mitochondrial system, CMF has proportionally smaller activating effect on the rate of O_2 uptake.

Preliminary studies suggest that DNP can be replaced by other uncoupling agents which activate latent mitochondrial ATPase. It is also essential that mitochondria used in the assay have preserved a degree of functional integrity which permits them to respond to DNP with induction of ATPase and that this effect be inhibited by oligomycin in a manner described earlier (Kun *et al.*, 1966b). ADP and orthophosphate or ATP (without added orthophosphate) are essential and interchangeable for the metabolic effect of CMF; AMP does not replace ADP or ATP. The more rapid (and for certain experimental purposes, often preferred) polarographic kinetic assay of initial rates of O_2 consumption of mitochondrial suspensions (Kun *et al.*, 1964) did not yield reliable quantitative results suitable for the bioassay of CMF.

B. Method of Isolation of CMF. Once conditions for detection of the activating effect of CMF on glutamate oxidation were established, isolation of CMF from cytosol could be readily accomplished with the aid of quantitative analyses by the manometric method. The activating effect of CMF was stable for at least 1 month when kept at −15° (without thawing and refreezing); consequently manometric assays could be performed on fractions obtained from cytosol with the same batch of mitochondria for which a DNP profile had been determined just prior to assays. It was found, furthermore, that CMF isolated from cytosol of liver, kidney, or heart tissues reduces the Nelson–Somogyi reagent (Somogyi, 1945) directly. This colorimetric assay was used routinely for the quantitative chemical estimation of the presence of CMF, since reducing capacity and stimulatory effect of CMF on mitochondrial O_2 uptake in the presence of glutamate run parallel. Since the nature of reducing groups in CMF was not known, the amount of CMF was expressed as micromoles of reducing equivalents. Glucose was used as primary standard. Simultaneous catalytic and colorimetric assays revealed that 1 catalytic unit (*i.e.*, K_{CMF}) of liver CMF corresponded to 0.6–0.9 μmole of reducing equivalent. In control experiments Sephadex G-25 and G-50 columns were eluted with 0.15 M KCl in the absence of added cytosol. The Nelson–Somogyi reagent was not reduced by fractions collected over a volume span of 0.15 M KCl corresponding to elution patterns of CMF.

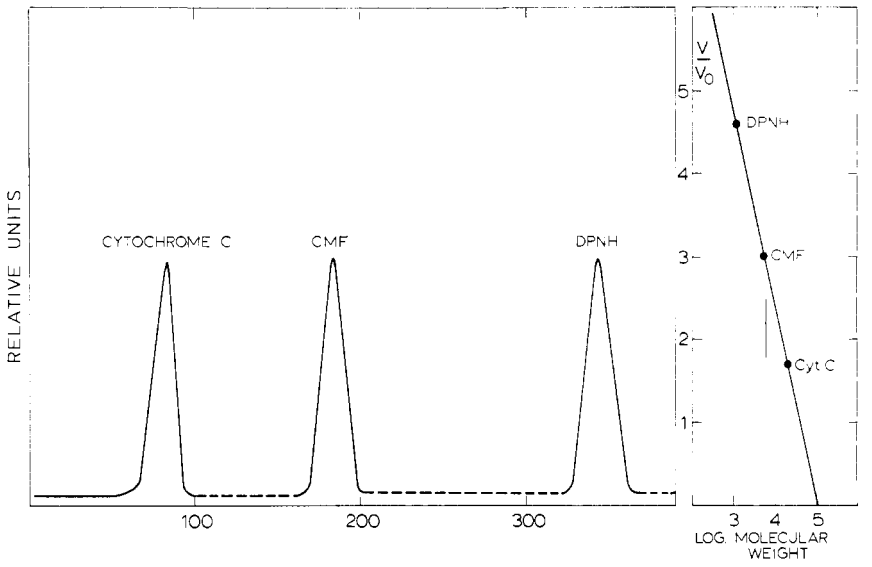

FIGURE 5: Elution pattern of CMF, cytochrome *c*, and DPNH from Sephadex G-50 column. Ordinate: relative units, either catalytic units for CMF or absorbance values for cytochrome *c* or DPNH, normalized to identical dimensions. Abscissa: milliliters of 0.15 M KCl. The values for V/V_0 *vs.* log molecular weight (right end of Figure 5) were derived from the elution pattern. V = 380 ml; V_0 = 96.5 ml.

Contamination of CMF by fortuitous degradation products of Sephadex was therefore excluded.

A routinely used procedure for isolation of CMF is as follows. All operations were carried out at 0–4° (ice bath). Rat livers (from ten rats) or 67 g (fresh weight) were homogenized in an approximately equal weight of 0.15 M KCl. The homogenate was centrifuged at 105,000*g* for 1 hr in a preparative ultracentrifuge. Cytosol (64 ml), containing 70 mg of protein/ml, were obtained. One unit of CMF activity was found to correspond to 0.05 ml of cytosol (containing 3.5 mg of protein). In terms of total liver weight, one unit of CMF (equal to K_{CMF}) is present in 58 mg of fresh liver. Based on the manometric test, the total number of CMF units present in cytosol was 1280. About 93% of total protein was removed by submerging the cytosol (64 ml in 250-ml erlenmeyer flasks with continued agitation) for 2.5 min into a boiling water bath, followed by centrifugation at 0°. The supernatant (containing 5 mg/ml of residual protein) quantitatively retained CMF activity. Isolation of CMF from the centrifugal supernatant fluid of heat-treated cytosol was carried out by the gel filtration technique on Sephadex G-25, which was washed, equilibrated, and eluted with 0.15 M KCl. For separation of CMF, the flow rate of the column was adjusted to 2 ml/min. Residual protein appeared in the first three 5-ml fractions immediately following the void volume of the column. CMF was eluted as a symmetrical peak, first appearing in the eleventh 2-ml fraction following the last tube containing residual protein. In this particular experiment, 1040 units of CMF (corresponding to 72 μmoles of reducing equivalents) was obtained in a total volume of 150 ml of eluate (82% recovery). In all instances, CMF was monitored by both colorimetric and manometric assays of 5-ml eluate fractions.

Instability of the activating effect of CMF prohibited the use of commonly employed methods of deproteinization (organic solvents, trichloroacetic acid, and $(NH_4)_2SO_4$). It was determined in separate experiments that gel filtration alone on Sephadex G-25 is sufficient to separate CMF. This is shown in Figure 4, which also illustrates that heat treatment does not alter the elution pattern of CMF. Gel filtration without previous deproteinization is not suitable for a general preparative procedure since excessive amounts of protein tend to overload the column. The particle size of CMF was determined by repeated gel filtration on G-25, followed by rechromatography on G-50 Sephadex columns. Cytochrome *c* and DPNH were used as markers. Elution patterns are shown in Figure 5. An approximate molecular weight of 5000–6000 was calculated from the straight line obtained by plotting v/v_0 against the logarithm of molecular weights of added markers (Figure 5) (Whitaker, 1963). On the basis of numerous large-scale preparations, it was estimated that rat liver contains about 15–20 units of CMF/g of liver. Attempts to isolate CMF from liver mitochondria were unsuccessful. No fraction with CMF activity was found in brain tissue, but highly active preparations could be obtained from cytosol of kidney and heart muscle. These active CMF fractions from kidney and heart tissue were not characterized beyond elution patterns on Sephadex G-25 and total carbohydrate content.

Numerous substances were tested for CMF activity. Taurine, hypotaurine, cysteine, cystine, oxidized and reduced glutathione, and cysteamine in amounts

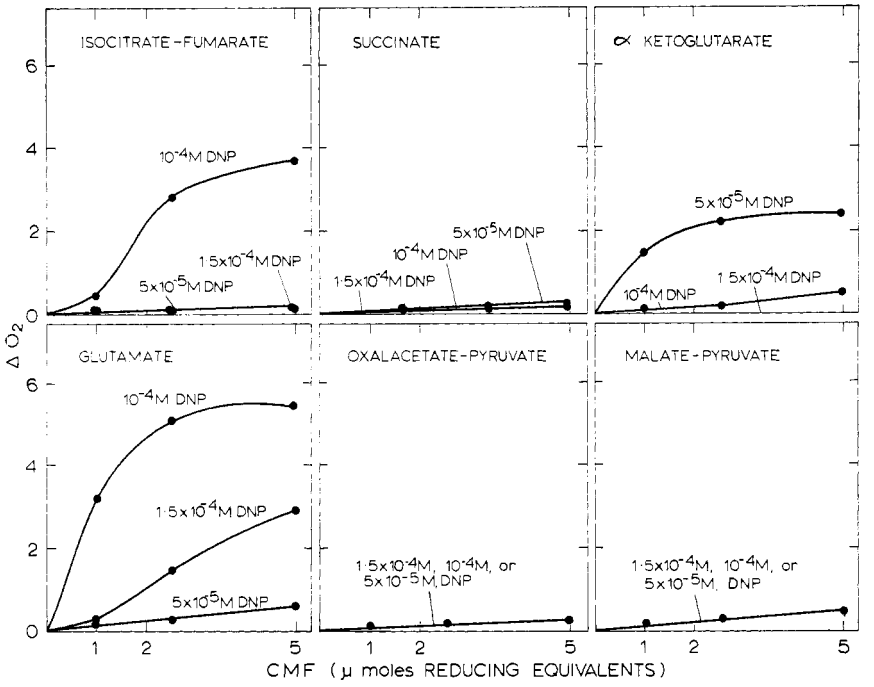

FIGURE 6: The effect of increasing concentrations of CMF (prepared from liver cytosol) on the oxidation of carboxylic acid substrates by liver mitochondria. The manometric system is described in Experimental Procedures. Various substrates and substrate pairs were added to mitochondria preincubated for 15 min with DNP (5×10^{-5}–1.5×10^{-4} M) and CMF (1–5 μmoles of reducing equivalents) in the presence of 2.3×10^{-3} M ATP (and components described under methods). Ordinate: μmoles of O_2/30 min per 10 mg of mitochondrial protein. Abscissa: amounts of CMF; concentrations of DNP are indicated in the figure.

ranging from 10 to 500 μg were found to be without effect in the CMF test system. Thyroxine, epinephrine, insulin (1–30 μg), DPN, DPNH, and FAD (1–100 μg) were also ineffective. Carnitine in a range of 100–200 μg/flask increased O_2 uptake 15–25%, but this effect could hardly be compared with the two- to threefold augmentation of O_2 uptake caused by CMF.

C. Properties of CMF Isolated from Rat Liver. Metabolic activity of CMF prepared in 0.15 M KCl is preserved at −15° for at least 1 month, but is unstable to repeated thawing and freezing, storage at 0° or higher temperatures, and changes of pH below 3.0 or above 8.0 at room temperature. At 0° metabolic activity can be retained between pH 3 and 9, provided readjustment of pH to neutrality is carried out within 5 to 10 min after lowering or raising the pH. Loss of metabolic activity runs parallel with the appearance of oxidizable substances, readily detectable by the manometric assay during preincubation. Absence of oxidizable material in CMF is a sensitive test of its intactness, which on the other hand is a prerequisite for the detection of its metabolic effect on mitochondria. A cytoplasmic fraction corresponding to CMF can be isolated also from liver homogenates prepared in distilled water. The elution pattern of CMF prepared from water homogenates appears to be indistinguishable from CMF isolated in the presence of 0.15 M KCl. However, the metabolic effect is associated only with CMF prepared in isotonic KCl. It is presently unknown whether or not the loss of metabolic effect caused by low ionic strength is due to a structural change of macromolecular constituents of CMF or is the consequence of dissociation and loss of an active component from this fraction which occurs only at low salt concentrations. While CMF isolated on Sephadex (in 0.15 M KCl) behaves like a macromolecular fraction containing no orthophosphate or ammonia, treatment with Dowex 50 (in the H^+ form) readily releases about 40% of organically bound phosphate as orthophosphate with simultaneous appearance of NH_4^+ (240 μmoles of NH_4^+ from an amount of CMF containing 62 μmoles of reducing equivalents). The Dowex column retains the bulk of ninhydrin-positive constituents of CMF as free amino acids and small peptides (equivalent to about 44% of the weight of CMF), while the strongly acidic effluent contains the carbohydrate moiety of CMF (about 55% by weight) and a constituent of CMF which has a sharp absorption maximum at 263 mμ. It is not known whether degradation of CMF by Dowex 50 is solely due to its acidic nature or to some additional catalytic effect of this resin.

After freeze drying and dehydration with a tenfold excess of isopropyl alcohol, CMF prepared from water homogenates may be obtained as a dry powder (after removal of isopropyl alcohol *in vacuo* at 60°).

TABLE I: Substrate Specificity of Liver CMF Tested with Liver Mitochondria.[a]

No.	Variable Components of the System			O_2 Consumption[b]	K_{CMF} (μmoles of reducing equiv)
	Substrate	DNP (M)	CMF		
1	Glutamate		−	4.2	
	Glutamate	10^{-4}	−	4.2	
	Glutamate	10^{-4}	+	8.3	0.8
2	α-Ketoglutarate		−	4.2	
	α-Ketoglutarate	5×10^{-5}	−	1.0	
	α-Ketoglutarate	5×10^{-5}	+	3.2	0.9
3	Succinate		−	9.0	
	Succinate	5×10^{-5}	−	2.7	
	Succinate	5×10^{-5}	+	3.0	
	Succinate	1×10^{-4}	−	1.3	
	Succinate	1×10^{-4}	+	1.8	
4	Fumarate–isocitrate		−	4.7	
	Fumarate–isocitrate	10^{-4}	−	1.4	
	Fumarate–isocitrate	10^{-4}	+	5.1	1.6
5	Malate–pyruvate		−	4.2	
	Malate–pyruvate	5×10^{-5}	−	4.3	
	Malate–pyruvate	5×10^{-5}	+	4.4	
	Malate–pyruvate	10^{-4}	−	1.7	
	Malate–pyruvate	10^{-4}	+	1.9	
6	Oxalacetate–pyruvate		−	3.5	
	Oxalacetate–pyruvate	5×10^{-5}	−	2.7	
	Oxalacetate–pyruvate	5×10^{-5}	+	2.7	
	Oxalacetate–pyruvate	10^{-4}	−	1.5	
	Oxalacetate–pyruvate	10^{-4}	+	1.4	

[a] All manometric assays were carried out as described under methods. ADP (2.3×10^{-3} M) was the added nucleotide; substrate concentration was 10^{-2} M for each carboxylic acid. K_{CMF} values were determined by double-reciprocal plots. [b] O_2 (μmole)/10 mg of mitochondrial protein per 30 min.

A sample of this preparation (dissolved in 10^{-2} M HCl) was subjected to amino acid analyses in the Beckman 120C amino acid analyzer with both short and long columns. An aliquot of 30 μg (analyzed without prior hydrolysis) contained approximately 103 mμmoles of amino acid residues (not counting NH_4^+), in fair agreement with results of direct analyses for ninhydrin-reactive material in the metabolically active CMF. No aromatic amino acids were present, but predominance of serine, alanine, and NH_4^+ (23 mμmoles each), aspartic (3.2) and glutamic (9.1) acids, glycine plus cystine (8.0), valine (3.4), isoleucine (2.6), leucine (6.5), lysine (3.5), histidine (2.2), methionine (1.0), and about 20 mμmoles of small peptides of unknown composition was ascertained.

CMF prepared from water homogenates contains 0.7% chloroform–methanol-soluble material, which was shown by thin-layer chromatography not to be fatty acid. The nature of this component is presently unknown.

At present, the only criteria of apparent molecular homogeneity of CMF are its behavior on Sephadex and its reproducible composition in terms of carbohydrate and amino acid constituents. These parameters may suggest but do not prove that CMF is a real molecular entity. Application of other experimental tests (*e.g.*, electrophoresis) have met thus far with technical difficulties since the most important criterion of detection, the metabolic effect on mitochondria, is usually lost during various operations. This problem is the subject of further studies.

D. Substrate and Tissue Specificity of the Metabolic Effect of CMF. Increased rate of oxidation of certain but not all dicarboxylic acids occurred in the presence of CMF, an effect which was critically dependent on the concentration of DNP. In other words, an apparent selection of the increased oxidation of a specific substrate in the presence of CMF is brought about by a specific concentration of the uncoupler. The effect of CMF prepared from liver on the oxidation of various sub-

TABLE II: Effect of Heart CMF on Heart Sarcosomes in the Presence of 1.5×10^{-4} M DNP.[a]

Variable Components			
Substrate	CMF (μmoles of reducing equiv)	μmoles of O_2 Consumed[b]	% Activation of O_2 Uptake by CMF
Glutamate		5.4	
Glutamate	0.6	13.0	140
Malate		2.6	
Malate	0.6	2.0	
Malate + pyruvate		3.0	
Malate + pyruvate	0.6	3.8	37
Succinate		3.0	
Succinate	0.6	3.6	20
α-Ketoglutarate		3.0	
α-Ketoglutarate	0.6	11.2	365
Succinate + glutamate		9.9	
Succinate + glutamate	0.6	17.0	70
Pyruvate		1.0	
Pyruvate	0.6	3.8	280

[a] Experimental conditions were identical with those described in the legend to Figure 1, except heart mitochondria and CMF from heart cytosol were employed. [b] Per 10 mg of mitochondrial protein per 30 min.

strates by liver mitochondria is shown in Figure 6. A constant amount of CMF (1 μmole of reducing equivalent) in the presence of 10^{-4} M DNP increases the oxidation of glutamate more than six times above the level reached by the isocitrate–fumarate substrate couple. No increase in the oxidation of added α-ketoglutarate occurs under the same conditions unless the concentration of DNP is diminished to 5×10^{-5} M. On the other hand, increased oxidation of isocitrate–fumarate couple in the presence of CMF does not occur at all at 5×10^{-5} M DNP. At this concentration of DNP, oxidation of glutamate is only slightly increased by CMF. Oxidation of succinate was not influenced significantly by CMF. As shown in Table I, the nature of added mitochondrial substrate influences also the value of K_{CMF} determined at critical DNP levels where CMF is effective (Table I).

CMF isolated from heart cytosol was similar to that obtained from liver with respect to both K_{CMF} and its activating effect on glutamate oxidation by liver mitochondria. However, certain differences in apparent substrate specificity of heart CMF were observed when its effects on heart sarcosomes were determined (Table II). For example, pyruvate oxidation by heart sarcosomes was greatly stimulated by heart CMF. On the other hand, in liver mitochondria, oxidation of pyruvate is unaffected by liver CMF. It is interesting that in the heart system O_2 uptake in the presence of glutamate–succinate substrate pair still responds to CMF with an increase in rate (Table II), even though succinate oxidation alone is unaffected by CMF. This experiment indicates that selective stimulation of the oxidation of certain substrates takes place by CMF even under conditions when simultaneous oxidation of several substrates appears to be possible. This experiment illustrates substrate selection by mitochondria in the presence of CMF. Oxidation of α-ketoglutarate by heart sarcosomes is activated by heart CMF to the largest extent at 1.5×10^{-4} M DNP. As shown in Figure 6, oxidation of α-ketoglutarate by liver mitochondria is unaffected by liver CMF at 1.5×10^{-4} M DNP, clearly demonstrating a hitherto unknown form of tissue-specific metabolic response which escapes detection by ordinary metabolic measurements.

Mitochondria isolated from kidney are completely inert to apparent metabolic effects of CMF isolated from kidney, liver, or heart cytosol. However, CMF obtained from kidney cytosol is ten times more effective than CMF prepared from liver cytosol, tested on the oxidation of glutamate by liver mitochondria, when activity is based on the amount of reducing equivalents present in CMF. Metabolic responses of various types of mitochondria to all possible combinations of substrates in the presence of CMF prepared from various tissues have not yet been explored, but it appears that an unexpectedly specific metabolic pattern may emerge, projecting experimental models for the study of mechanisms of tissue-specific metabolic control.

E. The Effect of CMF on DNP-Activated ATPase. Activation of latent mitochondrial ATPase is the most conspicuous enzymatic effect of DNP (Lardy and Wellman, 1953; Kun *et al.*, 1966a). The effect of CMF on this reaction was investigated because of the DNP requirement of the metabolic effect of CMF. It was found that CMF by itself had no effect on latent mitochondrial ATPase, nor did CMF influence the activat-

TABLE III: Apparent Inhibition of DNP-Induced ATPase of Liver Mitochondria by Liver CMF in the Presence of Substrates.[a]

Variable Components of the System					
No.	Substrate	DNP (M)	CMF (μmoles of reducing equiv)	O_2 Uptake[b]	Orthophosphate Released[c]
1	Glutamate			1.5	0.7
	Glutamate	10^{-4}		1.5	2.7
	Glutamate	10^{-4}	2.2	4.1	0.2
2	α-Ketoglutarate			2.1	0.7
	α-Ketoglutarate	5×10^{-5}		1.5	2.1
	α-Ketoglutarate	5×10^{-5}	2.2	2.6	1.0
3	Malate–pyruvate			1.9	0.7
	Malate–pyruvate	2.5×10^{-5}		3.7	2.7
	Malate–pyruvate	2.5×10^{-5}	2.2	4.2	0.7

[a] Manometric assays were performed as described under methods, except phosphate buffer was replaced by equimola Tris-hydrochloride (pH 7.4). Orthophosphate was determined at zero time and at the end of the manometric experiments The orthophosphate values, therefore, express the net increase in concentration which occurred during incubation. [b] Micromoles per 10 mg of mitochondrial protein per 30 min. [c] Micromoles of orthophosphate present in the system at the end of incubation.

ing effect of DNP on the rate of ATP hydrolysis by mitochondria. However, large inhibition of DNP-activated ATPase by CMF occurs when dicarboxylic acid substrates are oxidized by mitochondria in the pres ence of DNP plus CMF (Table III). Inhibition of DNP-induced ATPase occurs in the presence of all carboxylic acid substrates tested. In the case of substrates which respond with increased O_2 uptake to the presence of DNP plus CMF, inhibition of orthophosphate accumulation by CMF coincides with an increase of O_2 uptake. Inhibition of DNP stimulation of mitochondrial ATPase is the only hitherto observed enzymatic effect of CMF. Mitochondrial metabolism and oxidative phosphorylation appear to be unaffected by CMF in the absence of uncouplers.

Discussion

A relatively uninteresting mechanism of action of CMF may be proposed, suggesting that CMF stimulates metabolic activity by removing inhibitory DNP through binding. This mechanism does not explain either the substrate-specific metabolic action of CMF or the substrate-dependent inhibition of DNP-activated ATPase. Furthermore, if CMF binds DNP, similarly to serum albumin (Weinbach and Garbus, 1966), low concentrations of DNP should not have any metabolic effect on mitochondrial O_2 uptake (which is not the case; see Figure 1), since a constant amount of CMF would be expected to remove most effectively low concentrations of DNP. For comparison, the effect of serum albumin was also tested. Serum albumin had no effect on the CMF-detecting manometric system so long as concentrations of serum albumin were not sufficiently high to remove DNP from the system. This is predictable from the known stoichiometric binding of DNP to serum albumin (Weinbach and Garbus, 1966).

Additional experimental results as well as arguments derived from work carried out in other laboratories indicate that the mitochondrial membrane system plays an important role in the mechanism of action of CMF. When liver mitochondria are suspended in distilled water for 5 to 10 min at 0° and isotonicity is quickly reestablished by the addition of solid KCl, acceptor control ratios and P:O ratios of water-treated mitochondria in the presence of carboxylate substrates remain the same as in untreated ones. This indicates that the oxidative phosphorylative system is not greatly altered by H_2O treatment. However, CMF is completely ineffective on water-treated mitochondria.[3] Since osmotic effects are known to alter primarily the mitochondrial membrane system (Sottacosa *et al.*, 1967), it is evident that intactness of this system is necessary for the action of CMF. The most probable metabolic role of mitochondrial membranes is their involvement in the transfer of substrates from extra- to intramitochondrial systems. It is proposed that CMF may act on a carboxylic acid substrate transfer (permease) system of mitochondria which becomes detectable only under specific experimental conditions. It appears that this system has an ATP requirement. As a part of this permease system, DNP-activated ATPase of mitochondria may play a role similar to that of the K^+,Na^+-activated ATPase in the active transport system of cell membranes (Skou, 1957). The proposed hypothesis permits interpretation of

[3] E. Kun and H. H. Loh, unpublished experiments.

important published observations of Lardy and Wellman (1953), who originally found that activation of mitochondrial ATPase by DNP is modified by dicarboxylic acid substrates. It is suggested that the modifying effect of substrates on ATPase reflects the function of the same component of the system which is more fully recognized in terms of a metabolic effect when CMF acts on mitochondria in the presence of DNP and substrates. In 1964, Lardy *et al.* discovered that oligomycin counteracts DNP activation of ATPase. This antagonistic effect of oligomycin to DNP exhibits no carboxylate substrate requirement. The seemingly analogous action of oligomycin and CMF on ATPase does not necessarily indicate the same site of action of the two agents, but identifies ATPase merely as a common component of the target systems involved. It was indeed found that oligomycin does not inhibit the metabolic effect of CMF but appears to stabilize the increase in O_2 uptake caused by CMF when the manometric assay is carried out over longer (1–2 hr) periods.

It is of further importance that the action of CMF is abolished by 0.25 M sucrose. The inhibitory effect of sucrose on various mitochondrial systems is well known (Johnson and Lardy, 1958; Lehninger, 1961; Carafoli *et al.*, 1966). Consequently mitochondria studied in sucrose-containing suspending media are likely to possess a partially modified substrate transfer system due to sucrose itself. This conclusion can be drawn also from the results of Chappell and Haarhoff (1967). It is somewhat surprising that in view of this information metabolic studies carried out with sucrose suspensions of mitochondria are still considered to have predictive value for the understanding of cellular physiology. As a consequence of this uncertainty, the role of mitochondrial suspending media (*e.g.*, ionic specificity) in the action of CMF is being further studied.

Although the more decisive documentation of the involvement of the operation of mitochondrial substrate permeases in the action of CMF will depend on simultaneous quantitative analyses of extra- and intramitochondrial distribution of numerous metabolites, preliminary experiments with glutamate as substrate already indicate the participation of these systems. It was found in preliminary studies[3] that O_2 consumption in the presence of a limiting DNP level is raised two- to threefold by CMF with glutamate as substrate; yet aspartate accumulation is augmented six- to sevenfold. Since aspartate in the mitochondrial systems employed is the end product of metabolism, a greater rate of its formation must necessarily occur as a consequence of an increased rate of penetration of glutamate into mitochondria where oxalacetate derived from oxidative metabolism of glutamate serves as a second substrate to intramitochondrial glutamate–aspartate transaminase (*cf.* Kun *et al.*, 1964).

Further support of the proposed working hypothesis may be found in the results of Papa *et al.* (1967), who recently observed that uncouplers drastically change the large intramitochondrial accumulation of α-ketoglutarate formed from glutamate in the presence of arsenite. Their results as well as those of Harris *et al.* (1967) may explain the DNP requirement for the demonstration of the metabolic effect of CMF. These authors discovered that mitochondrial permeability toward various carboxylic acids is drastically altered by DNP; consequently a highly complex change in rates of entry as well as exit of substrates must take place. It is predicted that CMF may change the pattern of substrate permeation in and out of mitochondria and produce selective metabolic effects as a consequence of selective antagonism to the inhibitory effect of DNP. This working hypothesis is the basis for presently pursued experimental work.

It is premature to ascribe a physiological significance to CMF, particularly in view of the apparently artificial conditions necessary for its detection. On the other hand, it would be expected that in tightly coupled mitochondria the control strength of oxidative phosphorylation outweighs the rate-limiting role of any substrate-selecting mechanism (such as permeases); for this reason, loose coupling appears to be a logical prerequisite for the rate-controlling function of substrate transfer systems. It follows also that in a cellular environment, where active substrate flux is known to exist, mitochondria may be kept in a metabolic state resembling that of loose coupling by specific cellular substances. Under these circumstances, CMF could participate in cellular metabolic control.

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The Extracellular Thiaminase I of *Bacillus Thiaminolyticus*. I. Purification and Physicochemical Properties*

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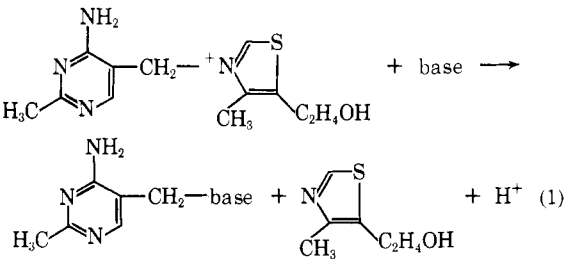
ABSTRACT: Thiaminase I catalyzes the decomposition of vitamin B_1 by means of a base-exchange reaction. The enzyme was purified 200-fold from culture filtrates of *Bacillus thiaminolyticus* using a combination of ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. Homogeneity of the enzyme preparations was determined by ultracentrifugation, polyacrylamide gel electrophoresis, and immunodiffusion in agar gel. The absorption spectrum of thiaminase I is that of a simple protein with a maximum at 277 mμ and a minimum at 252 mμ. The enzyme has an $s_{20,w}$ = 3.1 S and a $D_{20,w}$ = 6.6 × 10^{-7} cm^2/sec which suggest a molecular weight of 44,000. Thiaminase I migrates as an anion at pH 9.5 when subjected to disc electrophoresis and displays maximum enzymatic activity over a broad pH range of 5.8–6.8. The K_m values of thiaminase I at pH 5.8 and 25° are 8.7 × 10^{-6} M for thiamine and 2.9 × 10^{-3} M for aniline. The temperature optimum of the enzyme activity is 37° and Q_{10}(10–20°) = 1.93 and Q_{10}(20–30°) = 1.33. Energies of activation of 9800 cal/mole and 2700 cal/mole were determined for the thiaminase I reaction. The change in the value of the activation energy of the reaction is thought to be due to a reversible inactivation of the enzyme. Thiaminase I has a temperature inactivation coefficient (T_i) of 63.5°.

Various thiamine-decomposing enzymes have been found in fish, mollusks, arthropods, ferns, and bacteria (see reviews by Fujita, 1954; Metzler, 1960; Kimura, 1965; Murata, 1965). Two mechanisms of vitamin B_1 degradation by thiaminases from several sources have been studied (Fujita, 1954; Metzler, 1960). One reaction was shown to require a basic substance as a cosubstrate and was catalyzed by an enzyme subsequently named thiaminase I (thiamine:base 2-methyl-4-aminopyrimidine-5-methenyltransferase, EC 2.5.1.2). The reaction is represented in eq 1. Two bacteria, *Bacillus thiaminolyticus* Matsukawa et Misawa and *Clostridium thiaminolyticum* Kimura et Liao, which have been



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