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Cupric Ion Stimulation of Mitochondrial Protein Synthesis and Dependence on the Osmolarity of Incubation Medium (41193)

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Abstract. The effect of cupric ion on amino acid incorporation into protein by isolated rat liver mitochondria was investigated. In confirmation of previous observations, the incorporation was stimulated as much as 80% by the addition of 2 to 10 μM $CuCl_2$ when the mitochondria were incubated in the medium of Buchanan *et al.* The stimulation was maximal at 6 μM Cu^{2+}. By contrast, cupric ion at comparable concentrations showed no significant effect on the incorporation when the mitochondria were incubated in the medium of Haldar and Freeman. Experiments involving omission or addition of the components which made the two media different showed that the stimulatory effect of cupric ion depended upon the presence of 50 mM Tris–HCl. Additional experiments with Tris–HCl and sucrose demonstrated that the extent of stimulation depended on the osmolarity of the incubation medium and that cupric ion stimulated the incorporation by preventing the inhibition caused by the hyperosmolarity of the medium.

The metal ions Cu^{2+}, Ag^{+}, and Au^{3+} have been shown to cause over 100% stimulation in amino acid incorporation into protein by isolated rat liver mitochondria (1). It has been suggested that cupric ion may regulate mitochondrial protein synthesis since the level of the ion used in the incubation medium (2–10 μM) is comparable to that found *in vivo* (2, 3). Many types of incubation media differing in their composition and concentration of individual components, final osmolarity, and pH are used (4–9). Our results presented in this paper show contrasting effect of cupric ion in stimulating amino acid incorporation into protein by isolated mitochondria depending on the final osmolarity of the incubation medium. In hyperosmotic, but not in isoosmotic medium, Cu^{2+} and Ag^{+} stimulated amino acid incorporation into protein by isolated mitochondria. The apparent stimulation was due to a partial prevention of the inhibitory action of hyperosmolarity on the incorporation process.

Materials and Methods. Male Sprague–Dawley rats weighing 100–125 g were obtained from Taconic Farms, Germantown, New York. They were fed *ad libitum* with Purina Laboratory Chow and were killed by decapitation. The rat liver mitochondrial fraction was isolated in 0.3 M sucrose (adjusted to pH 7.2 with KOH) following essentially the method of Roodyn *et al.* (4). The mitochondrial fraction was sedimented and washed three times at 6500*g* for 10 min and finally resuspended in isolation medium (10 mg/ml).

Incorporation of radioactive amino acids into protein by isolated mitochondria was done in two media. One medium (medium 1) described by Buchanan *et al.* (6) contained: 0.1 M sucrose, 50 mM KCl, 10 mM sodium succinate, 10 mM K_3PO_4 (pH 7.4), 10 mM $MgCl_2$, 50 mM Tris(hydroxymethyl)aminomethane–HCl (Tris–HCl), and 19 amino acids at 5 μM each; final pH 7.4. The other medium (medium 2) described by Haldar and Freeman (5) had the following composition: 0.1 M sucrose, 50 mM KCl, 10 mM sodium succinate, 20 mM K_3PO_4 (adjusted to pH 7.6 with HCl), 0.67 mM ethylenediaminetetracetic acid (EDTA), 2 mM adenosine 5′-pyrophosphate (ADP), 5 mM $MgCl_2$, and 50 μg of synthetic amino acid mixture/ml; final pH 7.2. In all cases L-[4,5-^{3}H(N)]leucine was added to the incu-

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bation medium to a final concentration of 0.67 μCi/ml. There was approximately 3 mg/ml mitochondrial protein.

Reaction was started by addition of mitochondrial fraction to ice-cold incubation medium in 18 × 150-mm test tubes and incubating the mixture (0.75 ml) at 37° in a waterbath with a shaking rate of 80 cycles/min. Reaction was stopped by the addition of 0.25 ml of 1.0 N NaOH and 0.1 ml of a saturated solution of L-leucine. The mixture was allowed to stand for at least 1 hr followed by the addition of 2.0 ml of 10% (w/v) trichloroacetic acid. After 1 hr, the precipitate was collected by centrifugation and was washed two times with 5% (w/v) trichloroacetic acid. The washed pellet was dissolved in 0.5 ml of 0.1 N NaOH and mixed with 4.5 ml of Biofluor in minivials. The samples were milky but became clear on standing for 1 hr under refrigeration in a Packard Tri-Carb liquid scintillation spectrometer. The counting efficiency was 34%. Protein was estimated using bovine serum albumin as standard (10).

All chemicals were of reagent grade. Amino acids, ADP, and Tris were obtained from Sigma Chemical Company. L-[4,5-^{3}H(N)]leucine was obtained from New England Nuclear and had a specific activity of 5.0 Ci/mmole. It was diluted with calculated amount of nonradioactive leucine to 1 Ci/mmole.

Results and Discussion. When incubation was performed in the medium 1 of Buchanan *et al.* (6), a substantial enhancement in the rate of amino acid incorporation into protein by isolated mitochondria was observed in the presence of 6 μM $CuCl_2$ or 6 μM $AgNO_3$. Table I, Experiment 1, documents the results of such experiments using $CuCl_2$. In six different experiments with different mitochondrial preparations, the change in amino acid incorporation due to the addition of Cu^{2+} to medium 1 ranged between 30 to 80% higher than that of controls.

However, when the same mitochondrial preparation was used in medium 2 of Haldar and Freeman (5) very little change was observed in the rate of incorporation due to addition of cupric chloride. As EDTA present in medium 2 may be expected to interfere with the effect of Cu^{2+}, experiments were performed in which EDTA was excluded from medium 2. No significant effect of $CuCl_2$ could be detected. Thus, addition of $CuCl_2$ caused contrasting ef-

TABLE I. CONTRASTING EFFECT OF Cu^{2+} AND Ag^+ IN STIMULATING AMINO ACID INCORPORATION INTO PROTEIN BY ISOLATED MITOCHONDRIA DEPENDING ON THE FINAL OSMOLARITY OF THE INCUBATION MEDIUM

Experiment	Medium with any additions or omissions	Incorporation (cpm/mg protein)	Percentage change relative to the same medium without metal ion
I	1	290 ± 4[a]	0
	1 plus 6 μM Cu^{2+}	449 ± 55	55
	2	943 ± 40	0
	2 plus 6 μM Cu^{2+}	937 ± 25	−1
	2 minus 0.67 mM EDTA	953 ± 89	0
	2 minus 0.67 mM EDTA plus 6 μM Cu^{2+}	957 ± 72	0
II	2[b]	731 ± 13	0
	2 plus 50 mM Tris–HCl[c]	170 ± 15	0
	2 plus 50 mM Tris–HCl and 6 μM Cu^{2+}	420 ± 63	147
	2 plus 50 mM tris-HCl and 6 μM Ag^+	305 ± 36	79
	2 plus 150 mM sucrose[d]	161 ± 27	0
	2 plus 150 mM sucrose and 6 μM Cu^{2+}	241 ± 6	50
	2 plus 150 mM sucrose and 6 μM Ag^+	261 ± 22	62

[a] Mean ± SE for samples incubated in triplicate.
[b] This medium has no EDTA and has an osmolarity of 301 mOsm equivalent to the osmolarity of 0.25 M sucrose.
[c] Osmolarity of 385 mOsm equivalent to 0.31 M sucrose (5).
[d] Osmolarity of 495 mOsm equivalent to 0.39 M sucrose (5).

fects on amino acid incorporation into protein by isolated mitochondria incubated in the two media. It should also be noted that the incorporation of [^{3}H]leucine into protein was substantially higher in medium 2 than in medium 1 (Table I, Experiment 1).

Similar experiments have shown that $CuSO_4$ (6 μM) but not NaCl (6 μM) was as effective as 6 μM $CuCl_2$ in stimulating leucine incorporation and confirmed the observation of Primack (1) that the cation (Cu^{2+}) is responsible for the observed effect in medium 1. Furthermore, in agreement with Primack (1), using two different mitochondrial preparations and the addition of 2, 4, 6, 8, and 10 μM $CuCl_2$ to medium 1, we found that 6 μM Cu^{2+} produced an optimal stimulation. The shape of the curve of stimulation at different concentrations of Cu^{2+} was similar to that obtained by plotting previously reported results (1). However, the same levels of Cu^{2+} produced no stimulation when medium 2 was used.

To understand why Cu^{2+} showed contrasting effects in medium 1 and medium 2 on amino acid incorporation into protein by isolated mitochondria, the composition of these two media was examined (cf. Methods). The amino acid incorporation proceeded virtually at the same rate in the two media in the presence of 50 mM Tris–HCl (187 ± 8 cpm/mg protein for medium 1, and 193 ± 20 cpm/mg for medium 2 plus 50 mM Tris–HCl). In the absence of Tris–HCl, however, the incorporation proceeded significantly more in medium 2 (338 ± 12 cpm/mg for medium 1 minus Tris–HCl, and 804 ± 28 cpm/mg for medium 2).

The presence or absence of ADP in medium 1 or 2 had no effect on the Cu^{2+} stimulation (results not shown). The difference in the response to the addition of Cu^{2+} found for the two media (Table I, Experiment 1) was shown to solely depend on the presence and concentration of Tris–HCl. Figure 1 shows that the higher the concentration of Tris–HCl in either medium, the more pronounced was the stimulatory effect of Cu^{2+} on the amino acid incorporation into protein by isolated mitochondria.

Further experiments were performed to determine whether for the Cu^{2+} dependent stimulation of amino acid incorporation, the presence of Tris–HCl is necessary as such or because Tris–HCl increases the osmolarity of the incubation medium (5). It is known that addition of 150 mM sucrose to medium 2 causes inhibition, comparable to that produced by 50 mM Tris–HCl, on the incorporation process by increasing the osmolarity of the mitochondrial incubation medium (5). The results presented in Table I, Experiment II, demonstrate that the presence of either 50 mM Tris–HCl or 150 mM sucrose in medium 2 brought about the stimulatory effect of Cu^{2+} on the incorporation of [^{3}H]leucine into protein by isolated mitochondria. Ag^+, another mitochondrial swelling agent (11) produced similar stimulatory effect on the incorporation process in the presence but not in the absence of 50 mM Tris–HCl or 150 mM sucrose in medium 2 (Table I, Experiment II).

The dependence of the enhancement of amino acid incorporation into protein by cupric ion on the Tris–HCl concentration in media 1 and 2 (Fig. 1) and on the presence of additional 150 mM sucrose in medium 2 (Table I, Experiment II) demonstrate that the effect of the metal ion is mediated by partially overcoming the inhibitory action of hyperosmolarity of the

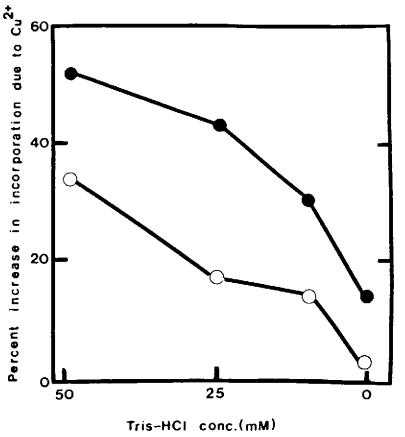

FIG. 1. Effect of cupric ion on the amino acid incorporation by isolated mitochondria incubated in the presence of different concentrations of Tris–HCl. Incubations were carried out as described in Table I with different concentrations of Tris–HCl in the presence or absence of 6 μM $CuCl_2$. The percentage increase in incorporation due to Cu^{2+} in medium 1 (○) and medium 2 without EDTA (●) is given.

incubation medium on the incorporation process. How the metal ions do this is unclear. It is known that the mitochondrial inner membrane is impermeable to Tris (12) and sucrose (13, 14). Cu^{2+} and Ag^{+} may act as swelling agents (11, 15–17) and thus partially reverse the contracted state (18) of the mitochondria resulting from the hyperosmotic effect of excess Tris–HCl or sucrose. It is the contracted state of mitochondria which seems to be responsible for inhibition of amino acid incorporation into protein in hyperosmotic medium (5). However, swelling alone can not explain the entire phenomenon of stimulation, because, while the stimulation is optimal at 6 μM Cu^{2+} or Ag^{+}, the extent of mitochondrial swelling is more at 10 μM (approximately 25%) than at 6 μM (5 to 12%) concentrations of the metal ions (1). Other possibilities include that the metal ions might either increase the amino acid transport or change the permeability of the inner membrane so that some Tris or sucrose can get into the mitochondrial matrix (12) and, thus, partially release the inhibition by hyperosmolarity. One point is clear, however, the apparent stimulation of amino acid incorporation into protein by isolated mitochondria due to cupric ion is mostly, if not entirely, an experimental artifact and, contrary to previous suggestion (1), has little or no physiological relevance.

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