(44:1:22). The radioactive zones were cut out and their radioactivity was measured in a Packard liquid-scintillation spectrometer.

Results and discussion. Zinc-precipitated and lyophilized protein fraction, partially purified from rat kidney cytosol by 20–35% ammonium sulfate saturation[5], retains its activity to dephosphorylate UMP as the soluble enzyme. The insoluble fraction of precipitated proteins is able to phosphorylate in the presence of ATP uridine and pyrimidine nucleoside analogues to corresponding 5′-nucleotides (active uridine kinase[1]) but not to transform uridine to uracil or to catalyze its synthesis from uracil and ribose-1-phosphate. The fraction of precipitated proteins displays the activity of nonspecific phosphomonoesterase and catalyzes also the splitting of uridine 2′(3′)-phosphate and 2,4-dinitrophenyl phosphate.

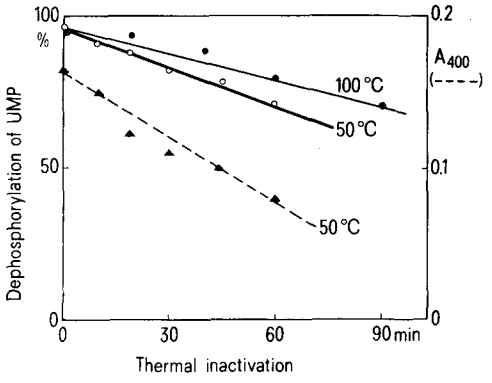

Fig. 2. Thermal stability of zinc-precipitated phosphomonoesterase in solution at 50 °C and in dry state at 100 °C. 0.25 m*M* UMP-2-^{14}C was incubated 5 min at 37 °C in 0.3 ml of 66 m*M* *tris*-HCl buffer (pH 7.4) containing Zn-precipitated enzyme (1 mg) heated for different period of time at 100 °C (●—●) or preincubated in the above buffer at 50 °C (○—○). The degradation of 12 m*M* 2,4-dinitrophenyl phosphate by the enzyme preincubated at 50 °C was measured (A_{400}) at pH 7.4 after 10 min incubation at 37 °C and dilution with 0.5 *M* NaOH (▲—▲).

The time course of UMP dephosphorylation using soluble and zinc-precipitated fractions of the kidney enzyme (Figure 1) indicates that both enzyme preparations split UMP to the same degree. Also kinetic constants of UMP dephosphorylation by the two forms of the enzyme are similar. Since in our system the inhibition by excess of the substrate occurred and a complex character of UMP degradation by the present enzymes was observed, the dephosphorylation was characterized only by the substrate concentration (2.7 m*M*) at which the rate of enzyme reaction was equal to one-half of maximal velocity of the substrate disappearance.

In contrast to the soluble from of phosphomonoesterase, zinc-precipitated enzyme is highly resistant to thermal inactivation. While the soluble enzyme fraction is completely inactivated by a 5 min incubation at 100 °C, the activity of zinc-precipitated and lyophilized proteins after their heating at 100 °C (in unsealed ampules using a sterilizing oven) is almost unchanged (Figure 2). Also the activity of metal complexed enzyme preincubated at 50 °C in a buffered medium is 30–40% higher than that of the soluble one. For comparison, the decomposition of 2,4-dinitrophenyl phosphate by precipitated and freeze-dried enzyme fraction preincubated at 50 °C is presented (Figure 2).

The partially purified extract of rat kidney used for the precipitation contains a number of proteins obviously participating in the final form of the precipitate. Although it is difficult to speculate about the form of active enzymes in coagulated precipitates, the precipitation by zinc ions offers a simple method to prepare a stable protein fraction possessing phosphomonoesterase activity.

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The action of D-penicillamine on cytochrome oxidase in vivo and in vitro

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Summary. The activity of cytochrome oxidase greatly decreases in the organs of rats treated with D-penicillamine for 20 days (30 mg/100 g/day). Although the drug does not affect cytochrome oxidase in vitro, it readily reduces oxidized cyt. c.

D-penicillamine is the well-known chelating agent used in the treatment of Wilson's disease[1] and of several heavy metal intoxications[2,3]. In a previous paper[4] we have reported that D-penicillamine decreases the content of iron, zinc and copper of most organs and tissues of the rat, and that this reduction significantly affects the concentration of blood ceruloplasmin and of the superoxide-dismutase activity of some tissues. With the aim of finding out whether D-penicillamine acts also upon other metalloproteins, we have studied the activity of cytochrome oxidase in rats treated with the drug. It has been reported[5] that cytochrome oxidase is very sensitive to copper deficiency and that the evaluation of its activity is a satisfactory indicator of the clinical copper status in mammals.

The marked effect observed in vivo suggested to us to extend the study to liver mitochondria preparations to investigate whether D-penicillamine affects cytochrome oxidase also in vitro.

Materials and methods. 12 male Wistar rats of about 250 g, fed with pellets of standard composition, were injected parenterally with D-penicillamine (15 mg/100 g body weight every 12 h) for 20 days. 10 rats were used as controls. The organs of each animal were homogenized with 9 vol. of 0.25 M sucrose in 0.1 M phosphate buffer pH 7; the suspended particles were solubilized by adding Tween 80, then centrifuged at 15,000 × g and the clear supernatant was employed for the determination of the

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Table 1. Cytochrome oxidase activity in normal and treated rats. Enzymatic units/g fresh tissue

Tissue	Control	Treated	p	Variation %
Liver	36.73±10.99	20.00± 4.06	<0.05	45.55
Spleen	20.30± 4.21	11.21± 2.65	<0.001	44.78
Kidneys	79.06±10.66	33.79± 8.30	<0.001	57.26
Heart	139.74±20.78	62.08±17.83	<0.001	55.57
Brain	45.85± 9.48	19.71± 5.06	<0.001	57.01
Lungs	17.90± 3.64	10.35± 1.58	<0.001	42.18

Table 2. Copper content in normal and treated rats. μg/g fresh tissue.

Tissue	Control	Treated	p	Variation %
Liver	4.22±0.86	3.23±0.48	<0.02	24.22
Spleen	2.59±1.02	2.11±0.45	n.s.*	18.53
Kidneys	7.13±1.31	3.81±0.68	<0.001	46.56
Heart	5.84±1.07	5.12±0.32	n.s.	12.33
Brain	3.13±0.95	2.72±0.55	n.s.	13.10
Lungs	2.68±0.39	1.62±0.34	<0.001	39.55

* n.s. for p-values >0.05.

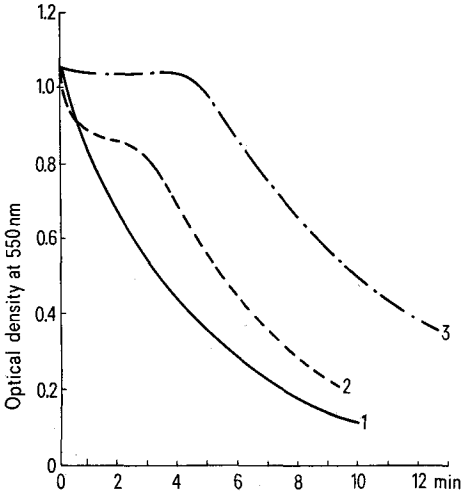

Fig. 1. Oxidation of reduced cyt. c (10^{-4} M), by mitochondrial cytochrome oxidase, in presence of D-Pam.
1 Control. *2* D-Penicillamine 5×10^{-5} M. *3* D-Penicillamine 10^{-4} M.

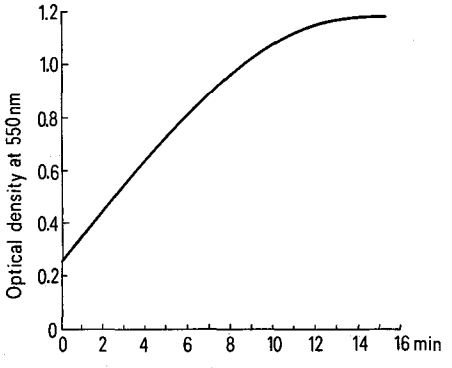

Fig. 2. Reduction of oxidized cyt. c (10^{-4} M), by D-Penicillamine (10^{-4} M).

Table 3. Ratio: Citochrome oxidase activity/copper content in normal and treated rats

Tissue	Control	Treated	p
Liver	7.08±1.71	6.32±1.28	n.s.*
Spleen	8.61±2.61	5.32±1.69	<0.02
Kidneys	12.11±2.74	9.31±2.19	n.s.
Heart	24.47±6.62	12.21±4.51	<0.001
Brain	14.70±4.76	7.61±2.79	<0.02
Lungs	7.23±2.01	6.75±1.61	n.s.

* n.s. for p-values >0.05.

cytochrome oxidase activity. All the operations were carried out at 4 °C. Heart mitochondria were prepared from control rats according to Green and Ziegler[6]. The oxidation of cyt. c previously reduced with ascorbate was followed spectrophotometrically[7] and using the extinction coefficient $\varepsilon = 19.6$[8]. A unit of enzyme was made equal to 1 μM of cyt. c which is oxidized per min. Copper was determined by atomic absorption (Perkin Elmer mod. 300); ferro-cyt. c and D-penicillamine were products from Sigma.

Results and discussion. After treatment with D-penicillamine, the cytochrome oxidase activity significantly decreases in almost all the organs examined (table 1). In the brain, the heart and the kidneys, more than 50% reduction in observed. The copper content of the corresponding organs is not reduced at the same rate (table 2). The ratio enzymatic activity/Cu content is significantly lower in the brain, the heart and the spleen (table 3). Actually these organs show small reduction of the copper content as if the metal was entirely bound; if so, any decrease of the total copper is matched by a strong reduction of the copper enzymes. On the contrary, in the liver, the kidneys and the lungs, where a pool of labile copper is present[9], the ratio remains almost unchanged. It is interesting to point out that, whereas other copper proteins such as ceruloplasmin and superoxide dismutase, are not uniformly reduced[4], in the same experimental conditions there is always 50% decrease of the cytochrome oxidase activity.

The action of D-penicillamine on cytochrome oxidase activity in vitro is shown in figure 1. The chelating agent has a delaying effect on the reoxidation of cyt. c which is proportional to the amount of D-penicillamine. This we interprete as due to the reducing action of the drug on cyt. c (figure 2).

The experiments here reported indicate that:

1. The compartimentation of copper is not equal in all the tissues examined.
2. When injected into rats, D-penicillamine decreases the copper content of several organs; at the same time the cytochrome oxidase activity is reduced to about 50% of the initial values.
3. In vitro D-penicillamine has no effect on the cytochrome oxidase activity. The drug, however, is readily oxidized by cit. c.

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