reaction if *d*-TC was added before (see Fig. 2) are in agreement with the suppositions made above The back reaction caused by the fraction W_3, A, B and C were not antagonized by *d*-TC.

Fraction D was further fractionated by gel filtration on Sephadex G-75 with 0·05 M sodium phosphate buffer (pH 6·6). The size of the column was 2·0 cm × 38·2 cm and the flow rate was about 15 ml/hr. Finally, fraction D was separated into fraction I (1·6 per cent of total), fraction II (0·7 per cent) and fraction III (97·7 per cent).

These three fractions were subjected to agar gel electrophoresis (13·5 V/cm at 5° for 15 min. and on a 1·5-mm thick layer of 1·5 per cent agar gel in Tris-EDTA borate buffer). This fraction D contained about 98 per cent of a pure single substance.

It was observed that fraction D (in 0·05 M sodium phosphate buffer, pH 6·6) had absorption maxima at 280, 409, 500 and 630 mμ, with the strongest absorption at 409 mμ. This pattern corresponds to haem, suggesting that fraction D might, mainly, consist of myoglobin.[6] To provide further support for this suggestion, fraction D was fractionated by IEC on DEAE-cellulose with Tris-HCl-buffer (pH 8·4) with a step-wise change in the ionic strength.[7] It was observed that ferrimyoglobin was eluted first, and this was followed by oxymyoglobin.

In view of the above results it is concluded that the ACh-binding fraction isolated from ox diaphragm muscle mainly consisted of myoglobin.

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Study on the stability of lysosome membranes—IV. Protection of liver lysosomes from the labilizing effect of chlorpromazine with succinate and glutamate *in vivo*

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SMALL doses of chlorpromazine (CPZ) stabilize and large ones labilize lysosomes [1]ATP and riboflavine protect these particles from the injuring action of the drug [2, 3] It is possible that the stabilizing effect of ATP is due to the protection of succindehydrogenase (SD) This interpretation is in agreement with the hypothesis according to which *in vivo* CPZ labilizes lysosome membranes indirectly, by causing primary disturbances in a bioenergetic pathway [2–4] ATP may protect SD from the inhibitory

action of oxaloacetate,[5–8] but it is not clear, whether *in vivo* large doses of CPZ inhibit SD only by competing with the flavine component of the enzyme,[9] or whether oxaloacetate plays some role in this process It is known that in the presence of other depressants of oxidative phosphorylation (some ancouplers), inhibition of SD by oxaloacetate occurs.[5, 8] Also since the oxidation of succinate is less sensitive in respect to the inhibitory action of CPZ than the oxidation of NAD.H,[10] it can be concluded that the effect of CPZ on lysosomes *in vivo*, if it is indirect it may be correlated with the functioning of succinate oxidizing pathway, in respect to the SD activity.

Experiments were carried out on male and female albino rats of the Wistar breed with body weight 110–150 g. The animals were fed on a diet containing: casein 15 parts; corn starch 28; sucrose 40; olive oil 6; dried baker's yeast 7; mineral mixture 4[11] and adequate quantities of vitamins A, D, E and K.

TABLE 1. SUBCUTANEOUS TREATMENT OF EXPERIMENTAL RATS WITH VARIOUS SUBSTANCES

Serial No.	Substances	Treatment: At zero hr	Treatment: 1·5 hr after first treatment	Treatment: 3 hr after first treatment
1.	—	—	—	—
2.	CPZ	1 mg/100 g.b.w.	—	—
3.	CPZ	1 mg/100 g.b.w.	—	—
	Succinate*	0·2 ml/100 g.b.w.	0·2 ml/100 g.b.w.	0·2 ml/100 g.b.w.
4.	CPZ	1 mg/100 g.b.w.	—	—
	Succinate	0·2 ml/100 g.b.w.	0·2 ml/100 g.b.w.	0·2 ml/100 g.b.w.
	Malonate†	50 mg/100 g.b.w.	50 mg/100 g.b.w.	50 mg/100 g.b.w.
5.	CPZ	1 mg/100 g.b.w.	—	—
	Glutamate‡	15 mg/100 g.b.w.	15 mg/100 g.b.w.	15 mg/100 g.b.w.
6.	Succinate	0·2 ml/100 g.b.w.	0·2 ml/100 g.b.w.	0·2 ml/100 g.b.w.
7.	Glutamate	15 mg/100 g.b.w.	15 mg/100 g.b.w.	15 mg/100 g.b.w.
8.	Malonate	50 mg/100 g.b.w.	50 mg/100 g.b.w.	50 mg/100 g.b.w.

* 0·4 M solution; † Solution of sodium malonate injected intraperitoneally; ‡ Neutral solution of glutamic acid.

After 12 hr of starvation the experimental rats were treated as indicated in Table 1. The control and treated animals were kept under a temperature of 19–21° and were killed by decapitation, 4·5–5 hr after the first treatment. Livers were rapidly removed and put in an ice-cold 0·25 M solution of sucrose. Preparation of homogenates and their fractionation was done in the same way as described before,[2] with the only difference being that the nuclei and debris were sedimented by centrifugation at 1500 *g* for 10 min.

An assessment of lysosome membrane stability has been made according to the rate of release of the acid phosphatase and proteins from isolated large-granule fractions (mitochondrial–lysosomal fractions isolated at 20,000 *g* for 20 min, which will be referred to below as ML fractions). The experiments with ML fractions were made by the method of De Duve[12] and by the method of Dingle[13] with the modification of Weissmann.[14] The activity of the acid phosphatase (EC 3.1.3.2) was determined by the method of Gianetto and De Duve,[15] the total activity in the presence of Triton X-100 (final concentration 0·1% v/v) and the free activity in its absence. Proteins were determined by the method of Lowry *et al.*[16] with bovine serum albumin as a standard. The following chemicals were used in the experiments: Sodium β-glycerophosphate and glutamic acid (Merck, Darmstadt); Chlorpromazine, Plegomazine (Egyt, Hungary); Triton X-100 (Nymco, Milano); Dinatriumsuccinate (VEB Laborchemie, Apolda).

The results from the experiments are shown in Figs. 1 and 2. It is seen that when ML fractions isolated from liver homogenats of rats treated with CPZ are preincubated in media containing 0·25 M of sucrose, at 37° and pH 5 (0·1 M acetate buffer), rapid release of proteins and lysosome-bound acid phosphatase, takes place. This is an indication for labilized lysosomes *in vivo*. Simultaneous

treatment of rats with CPZ and succinate or glutamate, delays the CPZ accelerated release of acid phosphatase and proteins from ML fractions. Succinate and glutamate would appear to have preserving effect against the CPZ induced fragility of the lysosomes *in vivo*. The succinate is more effective as a stabilizer than glutamate, but when its oxidizing pathway is blocked with malonate (rats treated with CPZ, succinate and malonate), the succinate does not act as a stabilizer but increase the labilizing effect of CPZ (Fig. 1).

The application of succinate, glutamate and malonate separately on experimental rats, has no

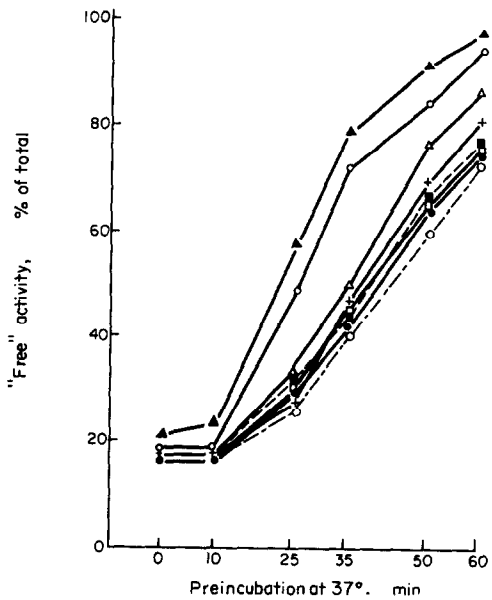

FIG. 1. Release of acid phosphatase from the ML fractions isolated from liver homogenates of control and treated rats.

Amounts of the fractions used for determining the enzyme activity were preincubated for 0, 10, 25, 35, 50 and 60 min in a medium containing 0·25 M sucrose at 37° and pH 5 (0·1 M acetate buffer). Substrate (Na β-Glycerophosphate) ensuring a concentration of 0·05 M was added at the end of the period of time indicated for preincubation, after which the incubation continued for 10 min.

The "free" activity is expressed in percentages to the total one determined in the presence of Triton X-100 (final concentration 0·1% v/v).

●——● controls(5), ○——○ treated with CPZ(8), ×——× treated with CPZ + succinate (9), △——△ treated with CPZ + glutamate(5), ▲——▲ treated with CPZ + succinate + malonate(3), ○-·-○ treated with succinate(3), □——□ treated with glutamate(3), ■ - - - ■ treated with malonate(3).

Number of the experiments are given in brackets.

effect on the stability of the lysosomes (Fig. 1). Consequently the stabilizing action of succinate and glutamate is manifested only when each of them is simultaneously applied with CPZ. Succinate and glutamate in physiological concentrations by *in vitro* experiments (experimental conditions as indicated in the text for Fig. 1) do not have any stabilizing effect on lysosomes, which is an indication that their action is not direct. The results obtained in the present study are not enough to elucidate the manner by which the succinate and glutamate protect the lysosomes from the labilizing action of CPZ *in vivo*, and, thus, further experiments in this direction are neccessary. Most probably the

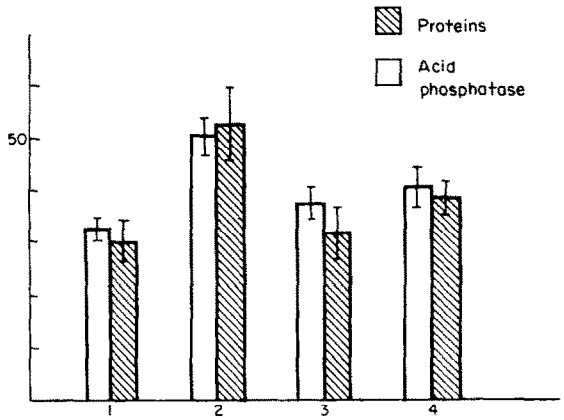

FIG. 2. Release of acid phosphatase and proteins from the ML fractions isolated from liver homogenates of control and treated rats.

Two samples (one containing Triton X-100 0·1% v/v, a concentration enough to rupture all lysosomes) from each ML fraction were preincubated in a medium containing 0·25 M sucrose at 37° and pH 5 (0·1 M acetate buffer) for 35 min. The samples were cooled and centrifuged at 20,000 *g* for 20 min. Unsedimentable (free) acid phosphatase activity and released proteins determined in the supernatants obtained from the samples without treatment with Triton X-100 are expressed in percentages to the total activity of the enzyme and total protein amounts released in the presence of Triton X-100.

1. ML fractions isolated from liver homogenates of untreated rats.
2. ML fractions isolated from liver homogenates of rats treated with CPZ.
3. ML fractions isolated from liver homogenates of rats treated with CPZ + succinate.
4. ML fractions isolated from liver homogenates of rats treated with CPZ + glutamate.

Average value from five experiments S.E.M. are given.

succinate and glutamate play the role of protectors of SD against inhibition due to CPZ. This presumption is in agreement with the hypothesis according to which *in vivo* CPZ labilizes lysosomes indirectly, by causing primary disturbances in a bioenergetic pathway.[2–4]

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