tary stimulating hormones, seems to be a small polypeptide, with a molecular weight between 1500 and 2000.

The rapidly induced depletion of hypophyseal MSH produced by hypothalamic extracts has been interpreted to mean that a release of stored MSH has occurred and consequently the active agent has been termed the MSH-RF. Final proof that a release of MSH has taken place requires the demonstration of increased plasma levels of MSH after injection of hypothalamic extracts.

Kastin(9) has recently reported in an abstract that crude hypothalamic extracts inhibited the release of MSH by pituitaries incubated *in vitro*. He postulated the existence of an MSH-inhibiting factor (MIF) in the extract. Our results do not negate the possibility that hypothalamic extracts may contain a MIF in addition to the MSH-RF the purification of which is reported here.

Summary. Acetone powders prepared from ovine stalk-median eminence tissue were extracted with glacial acetic acid and the glacial acetic acid extract was lyophilized. An ammonium acetate extract of the resultant powder was filtered through a column of Sephadex G-25. The eluent was assayed for its ability to deplete the pituitary of MSH. Extracts were injected i.v. into normal male rats which were sacrificed 20 minutes later. The MSH activity in pituitaries from rats receiving extracts was compared to that found in pituitaries from diluent-injected controls. MSH activity was estimated in an *in vitro* assay by microscopic grading of the melanocytes. An active zone was eluted from the column. At the peak of this zone 65-70% of the MSH activity had disappeared from the pituitaries of the test rats. The MSH-releasing zone was separated from the zones which contained CRF, GH-RF, LH-RF, PIF and vasopressin, but overlapped the zone which contained FSH-RF. It was concluded that MSH-RF can be added to the family of hypothalamic polypeptides which influence adenohypophyseal secretion.

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Rat Liver Acid Phosphatase: Differences in Lysosomal and Cytoplasmic Forms. (30947)

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Acid phosphatase is primarily a lysosomal enzyme. In liver tissue approximately 70 to 80% of its activity is confined to these particles, the remainder being distributed throughout the cytoplasm(1). Since, however, acid phosphatase is composed of multiple molecular forms(2-4), it is possible that certain organelles may contain specific forms of the enzyme. Indeed, differences have been reported in the acid phosphatases of the lysosomal and supernatant fractions of the liver of the guinea pig(5), but this duality of acid phosphatase has not been clearly established in another species(6). It was felt that further

studies on the nature of liver acid phosphatase in the rat was warranted. This study has shown that there are two distinctly different acid phosphatase fractions in the rat liver.

Materials and methods. Fractions containing lysosomal and supernatant acid phosphatases were prepared from the livers of adult, male Osborne-Mendel rats. One gram of tissue was homogenized in 9 vol of cold 0.25 M sucrose for 30 sec, and a lysosomal-rich pellet was separated from the supernatant fraction by the method of Weissmann and Thomas (7). The isolated pellet was washed, resuspended in 10 ml of 0.25 M sucrose containing 1% Triton X-100 (Rohm and Haas), and rehomogenized for 30 sec to activate the lysosomal enzymes. This procedure resulted in a 9- to 10-fold increase in acid phosphatase activity, and accounted for approximately 70% of the total activity in the homogenate. The remaining 30% was located in the supernatant fraction. The lysed pellet, and the original supernatant material from the homogenate, were both recentrifuged at 23,000 $\times$ *g* for 20 minutes and the resulting supernatants were used in the assay of lysosomal and supernatant acid phosphatases.

Acid phosphatase was assayed using p-nitrophenylphosphate (Sigma) as the substrate. Routine assays contained 5.5 mM p-nitrophenylphosphate; 0.05 M acetate buffer, pH 5.0; 10 mM EDTA and 0.2 ml sample; total volume, 1.2 ml. When studying the effects of inhibitors, EDTA was excluded from the media. Reactions were incubated for 15 minutes at 37°C, after which the color was developed by addition of 4 ml of 0.05 N NaOH. The optical density was read at 400 mμ in a Beckman DU spectrophotometer.

Chromatography of the lysosomal enzyme was carried out on DEAE-cellulose, using a 10 $\times$ 150 mm column equilibrated with either .01 M Tris-maleate(2) or .02 M phosphate buffers, both at pH 6.3. The enzymes were eluted in 2 steps, the first with 30 ml of the starting buffer, the second with 30 ml of 1.0 M NaCl. Fractions of 3 or 5 ml were collected.

Results and discussion. Inhibitors. Table I shows the effect of L-tartrate and fluoride on acid phosphatase activity in the lysosomal and supernatant fractions of rat liver. It is apparent that the two enzymes are dissimilar, thus confirming previous findings(5-6) that a lysosomal and a supernatant acid phosphatase can be distinguished by their sensitivity to tartrate and fluoride when p-nitrophenylphosphate is used as the substrate. Formalin (final concentration 1%) inhibited both enzymes by only 6-7%.

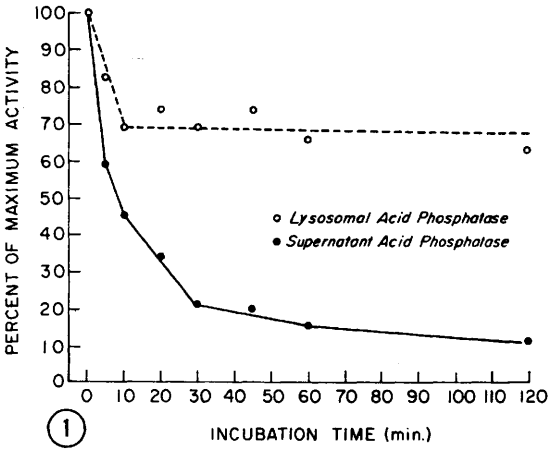
TABLE I. Fluoride and L-Tartrate Inhibition of Rat Liver Lysosomal and Supernatant Acid Phosphatases.

	Percent inhibition	
	Exp 1	Exp 2
A. Lysosomal		
L-tartrate*	98.1	93.5
Fluoride	100.0	98.0
B. Supernatant		
L-tartrate	19.9	27.5
Fluoride	26.6	24.3

* All inhibitors were present in a final concentration of 20 mM.

Heat inactivation. Heating the enzymes at 50°C revealed further differences (Fig. 1). Approximately 25 to 30% of the total activity in the lysosomal fraction was destroyed after 10 minutes of incubation. With continued incubation, however, no further loss was detected. In contrast, incubation of the supernatant fraction resulted in a progressive loss of acid phosphatase activity. Thus, heat inactivation not only distinguishes the lysosomal and supernatant forms, but, because of the initial drop in activity, suggests that more than one acid phosphatase may be present in the lysosomal fraction. Heterogeneity of the lysosomal enzyme is also inferred from the results of chromatography on DEAE-cellulose. Approximately 80 to 85% of the lysosomal enzyme did not bind to the ion exchange cellulose and was eluted with the starting buffer. The remaining 15-20% was eluted with 1.0 M NaCl. Chromatography resulted in a 3-fold increase in the specific activity of the non-binding form.

pH activity curves. Slight differences in pH activity curves were also observed for the 2 enzymes. In 0.2 M acetate (Fig. 2) and 0.1 M citrate buffers, the pH optimum was about 5.0 for the lysosomal, and 5.6 to 6.0 for the supernatant enzyme. With citrate, however,



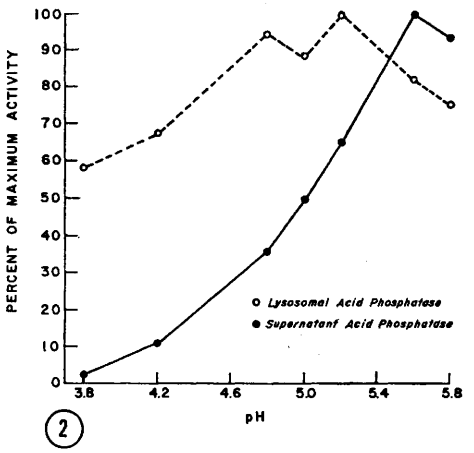


FIG. 1. Thermal instability of lysosomal and supernatant acid phosphatases at 50°C.
FIG. 2. pH activity curves for lysosomal and supernatant acid phosphatases in 0.2 M acetate buffer.

the curve for the supernatant enzyme was broader over the lower pH ranges (4.6 to 5.4). The shape of this curve did not change in the presence of 20 mM fluoride, indicating that increased activity in the lower pH ranges is not due to contamination of the supernatant fraction with lysosomal enzyme.

Since thermal inactivation experiments suggested that a second more heat sensitive enzyme might be present in lysosomes, the pH activity curve was repeated using a lysosomal fraction heated at 50°C for 30 minutes. In spite of a 30% decrease in activity, heating did not change the shape of the curve, or the pH optimum which was 5.0.

Others have obtained pH activity curves similar to those reported here using guinea pig livers(5) and also unfractionated rat livers(3). In the latter study, a fluoride-sensitive enzyme (pH optimum 4.4-4.8) which did not bind to DEAE-cellulose, and a fluoride-insensitive form (pH optimum 5.8-6.0) which bound strongly to DEAE-cellulose, were found. Because of the chromatographic behavior of these enzymes, as well as their pH optima and sensitivity to fluoride, they appear similar to the lysosomal and supernatant forms described here. The inability of earlier workers(2,6) to distinguish differences in the pH optima of the various rat liver acid phosphatases may have been due to the wide range of pH units employed in constructing the curves.

Effects of substrate concentrations. Increasing the concentration of p-nitrophenylphosphate from 0.25 mM to 20 mM had no inhibitory effects on acid phosphatase in either the lysosomal or supernatant fractions. Km values calculated from these data were found to be 1.25 and 0.68 mM for the lysosomal and supernatant enzymes respectively. In view of the relative impurity of the enzyme preparation, these differences may be equivocal. Moore and Angeletti(3), however, obtained a Km of 4.1 mM for the fluoride-sensitive (lysosomal?) enzyme, as opposed to values of 0.41 and 0.15 mM for 2 fluoride-insensitive enzymes.

It is apparent from the present experiments that rat liver contains at least two forms of acid phosphatase, thereby confirming its heterogeneity(2-4). More importantly, however, it was shown that the acid p-nitrophenylphosphatases have, for the most part, specific intracellular locations. The lysosomal fraction contains, predominantly, an acid p-nitrophenylphosphatase which is stable when heated at 50°C for two hours, has a pH optimum of approximately 5.0, is completely inhibited by 20 mM tartrate and fluoride, and does not bind to DEAE-cellulose. In contrast, the supernatant form is unstable when heated for even short periods at 50°C, has a pH optimum of 5.6-6.0, is only partially inhibited by tartrate and fluoride and apparently does bind to DEAE(3,6). Although some data suggest

that two forms of acid phosphatase may be present in the lysosomes, the possibility cannot be excluded that the minor component represents merely supernatant enzyme which was absorbed to the lysosomes prior to their disruption.

Present findings confirm and extend an earlier report that acid p-nitrophenylphosphatases in rat liver lysosomal and supernatant fractions can be distinguished in the presence of inhibitors(6). Association of a specific acid phosphatase with the lysosomal fraction is not, however, species specific for the rat. In guinea pig livers a lysosomal p-nitrophenylphosphatase can be distinguished from a microsomal p-nitrophenylphosphatase (5). The microsomal enzyme is more sensitive to heat and alloxin inhibition, and less sensitive to fluoride and tartrate than the lysosomal enzyme. Since microsomes were not separated from the soluble fraction in the present study, it is possible that the thermolabile acid phosphatase in the supernatant fraction is associated with the microsomes. On the other hand, in contrast to the rat, guinea pig liver p-nitrophenylphosphatase is located predominantly in the microsomal fraction(5). This suggests a basic difference between the two species and generalizations on the comparative intracellular distributions of acid phosphatase may not be warranted at this time.

In view of the present findings, it is suggested that further studies on possible separate physiological roles for the two enzymes should be studied, particularly as they are related to the process of tissue autolysis in which acid phosphatase is known to be important(1).

Summary. Two acid phosphatases, a lysosomal and supernatant form, have been demonstrated in rat liver by differences in their heat stabilities at 50°C, pH activity curves and sensitivities to tartrate and fluoride inhibition.

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Separation of the Antihemophilic Factor (F. VIII) from Fibrinogen With Thrombin and Manganese Chloride.* (30948)

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When blood is allowed to clot, prothrombin converts to thrombin and fibrinogen to fibrin, while the antihemophilic factor (AHF, Factor VIII) is "consumed" in some manner. AHF disappears during the clotting process and cannot be measured in serum(1). The addition to plasma of thrombin, a proteolytic enzyme, causes the fibrinogen to clot and AHF activity to disappear(2). The inference has been that the thrombin produced during clotting destroys AHF enzymatically. Trypsin, another proteolytic enzyme, also destroys AHF activity(3) and alters fibrinogen, rendering it nonclottable(4). Since hydrolysis of some proteins by trypsin can be prevented by the chlorides of certain metal ions, notably manganese(5), we have investigated the effect of these cations on the destruction of Factor VIII by thrombin and trypsin. As a result, we have discovered conditions under which

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