events occurring at some point proximal to the urinary tract. To define a causal relationship will entail a greatly expanded knowledge of the tissue mucoproteins and their breakdown products. Meanwhile this study suggests that quantitative urinary mucoprotein studies might profitably be undertaken in a variety of diseases involving connective tissues, including especially the acute and chronic bone dystrophies.

Summary. Control rats excrete, in a 72-hour period, 0.35-1.92 mg (mean: 1.08 mg) urinary mucoprotein determined as mannose-galactose standard. Following injection of 1000 units of parathyroid hormone, mucoprotein excretion increased significantly to 0.52-4.52 mg (mean: 2.84 mg). This increase is correlated in time with a dissolution of bone matrix, with an increase in plasma mucoprotein, and with the appearance of mucoprotein granules in kidney tubule cells and mucoprotein-containing casts in tubules.

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Use of an Organic Chelating Agent in Histochemical Study of Alkaline Phosphatase Activation.* (20640)

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Ethylene diamine tetraacetic acid (EDTA), by virtue of its capacity to form stable chelate complexes with many bivalent metals, will inhibit a number of enzymes *in vitro* presumably through competition with the enzyme for the activating metallic component(1-4). Histochemical application of this property was suggested by the solubility of the chelates formed, and alkaline phosphatase was chosen as a test enzyme for several reasons. 1) It has an ionically available activating metal (magnesium). 2) The enzyme has been extensively studied *in vitro* and considerable data has been accumulated with respect to its activation and inhibition. It provided, therefore, a reasonably satisfactory baseline for the method. 3) At least one component of the system is relatively resistant to alcohol fixation and paraffin embedding. 4) It can be readily demonstrated by 2 different histochemical technics which give identical localizations. 5) Alkaline phosphatase (phosphomonoesterase I) is part of an isodynamic enzyme system and there is reason to suspect that it may actually represent a group of enzymes with a similar pH optimum rather than an entity. There was a possibility, therefore, that these might be separable histochemically on the basis of their potential metallic activators and anionic inhibitors as had been demonstrated *in vitro* in the case of members of the system having different pH optima(5,6).

Method. Rabbit kidney which contains large amounts of alkaline phosphatase in characteristic distribution was used as a test tissue. Thin pieces of fresh kidney were fixed in cold 80% alcohol for 24 hours, dehydrated in cold

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graded alcohol and n-butanol in a period of 6 hours, and then left in cold n-butanol overnight. The tissue was then infiltrated in several changes of paraffin within a period of 2 to 3 hours at a temperature not exceeding 56°C. After embedding, the blocks were refrigerated until ready to cut. Sections were cut evenly at 7 μ and heating was omitted. Such sections, when stored in the refrigerator, retained their activity for many months. Following rapid removal of paraffin in xylol and rehydration in graded alcohols to water, the sections were washed in running water and exposed for 24 hours at room temperature to a solution containing 10 mg per ml of chelating agent adjusted with sodium hydroxide to pH 6.0 using the glass electrode. The concentration of active chelating agent was determined following adjustment of pH by means of a modified Schwarzenbach procedure using standard 0.01 molar calcium acetate with borate buffer at pH 9.2 and eriochrome black T as an indicator (7-9). Appropriate dilution was then made and the final pH rechecked. Following exposure to EDTA, the sections were washed in running water for 5 minutes in order to free the tissue of soluble chelates and excess chelating agent. They were then rinsed in distilled water and placed for one hour, together with active control sections, in a glycerophosphate substrate containing 0.3% sodium glycerophosphate (Eastman) and 1% calcium chloride in 0.05 molar sodium barbital buffer at pH 9.2. Magnesium salts were omitted from the substrate. The sites of alkaline phosphatase activity were then made visible by means of the Gomori cobalt sulfide technic(10), a method which lends itself rather readily to rough semiquantitative standardization. Although the calcium ion present in the substrate is capable of reactivating the enzyme, Ca^{++} is not a strong activator and no discernible evidence of reactivation was noted in less than 2 hours of incubation on numerous occasions. The alternative diazo method(10, 11) was frequently used for confirmation. Reactivation of the system could be readily accomplished by exposure of the slides before incubation in substrate to solutions of various activating ions for 2 to 6 hours or longer. The optimal concentration of activating ion varies widely with the metal used, but, in the case of rabbit kidney, little difference could be detected in concentrations from 0.1 to 0.01 molar, and the former concentration was used routinely in these preliminary experiments. Since the laboratory distilled water contained significant quantities of contaminating ions capable of reactivating or inhibiting the enzyme, all the distilled water used in these experiments was passed through an anion-cation exchange resin. In addition, relatively high concentrations of the test ions were used in order to reduce to a minimum the possible effects of any contaminating cations that might remain in the water or be present in the samples of metallic salts available. The metallic salts used were chlorides or, in a few instances, nitrates of the highest grade of purity available. With few exceptions (*e.g.* beryllium nitrate about pH 4.0) the pH of the 0.1 molar solutions ranged from 5.9 to 6.9. Inactivated and untreated active control sections were run with each experiment.

Observations. Preliminary studies were aimed at determination of optimal levels of pH, temperature, concentration and time of exposure necessary for EDTA inactivation of rabbit kidney alkaline phosphatase.

pH. Ethylenediamine tetraacetic acid, although itself highly insoluble, is capable of reacting with up to 4 equivalents of base to form increasingly soluble salts over a pH range from 2.2 to 10.6. By the use of increasing amounts of sodium hydroxide, therefore, it was possible to adjust the pH of the chelating agent to any desired level. Buffering was not ordinarily necessary since the amount of ion available for chelation in the sections as compared with the large excess of chelating agent present was insufficient to produce significant pH alteration. The efficiency with which any given cation is complexed varies with the pH, and calcium and magnesium are most effectively bound at alkaline pH levels. In view of the fact, however, that effective complexing of these and many other cations will occur on the acid side as well, and that alkaline phosphatase is much more stable on the acid than on the alkaline side, a pH of 6.0-6.5 was found to be optimal in the presence of a considerable excess of the chelating agent. Sections exposed to

TABLE I. Effect of EDTA Concentration on Speed of Inactivation of Alkaline Phosphatase.

EDTA, mg/cc	24 hr		90 hr	
	pH 5.9	pH 8.	pH 5.9	pH 8.
.1	2+	4+	0	2+
.2	+	3+	0	+
.4	±	2+	0	±
.6	0	+	0	±
1.	0	0	0	0
2.	0	0	0	0
5.	0	0	0	0
10.	0	0	0	0
Mg^{++} reactivated	3+	3+	3+	3+
Untreated control	4+	4+	4+	4+

1% EDTA for as long as one month and then reactivated by Mg^{++} showed no appreciable loss of activity on the acid side of neutrality above pH 4.6. Irreversible loss of activity occurred on the alkaline side, however, increasing as the pH optimum of the enzyme was approached.

Temperature. The rate of inactivation was slowed at refrigerator temperatures. Since warming for prolonged periods was undesirable, room temperature (22-24°C) was arbitrarily adopted as a safe and convenient level for these experiments. Active control sections kept at room temperature in buffer solutions of pH comparable with that of the chelating agent, and for comparable periods of time, showed no significant loss of alkaline phosphates activity.

Concentration of chelating agent and period of exposure. As might be expected, the rate of inactivation at any given pH level and temperature was found to be dependent on the concentration of active chelating agent and the period of exposure. Inactivation could be effected in a 24-hour period by concentrations considerably less than 1% and was actually much more rapid at an acid pH (Table I). All these slides could be readily reactivated, however, and tenfold increases in concentration and exposure time had no demonstrable deleterious effect. As a matter of fact, slides exposed at room temperature to the chelating agent in concentrations of 100 mg per ml (10%) and at the non-optimal pH of 7.2 for a period of 6 weeks showed only slight diminution of activity on reactivation with magnesium

Reactivation of EDTA-inactivated rabbit kidney alkaline phosphatase by metallic ions. The slides in each set of more than 10 experiments were cut serially from the same block, inactivated at the same time, and exposed to 0.1 molar concentrations of the various metallic ions for 6 hours under identical conditions of temperature and concentration. Although there were minor individual variations in the different series, Mg^{++} proved to be the most consistently effective ion capable of reactivating the system. Co^{++} and Zn^{++} were almost equally active, however, with Ca^{++} rather less so. These results correspond with those obtained in *in vitro* studies(5,6). Ni^{++} showed slight but consistent activating potential. Sr^{++} and Ba^{++} which have been considered inhibitory by some investigators (12) produced weak to moderate activation. Active control slides in this group showed no evidence of inhibition. Mn^{++}, Pb^{++} and Cd^{++} in 0.1 molar concentrations showed no tendency to reactivate the system but also failed to inhibit it. Hg^{++}, Be^{++} and Cu^{++} were constant strong inhibitors. The inability of Mn^{++} to reactivate alkaline phosphatase by this method is noteworthy, since manganese is reported to be capable of activating the purified enzyme *in vitro*(5,6).

Preliminary studies on kidney tissue from other animals have shown variations in the levels at which certain inhibitory ions can compete for the enzyme under these conditions. In contrast to rabbit kidney alkaline phosphatase, that of guinea pig or monkey kidney is markedly to completely inhibited by Cd^{++} and Pb^{++} in 0.1 molar and slightly inhibited in 0.01 molar concentrations. As might be expected, neither of these ions will activate the EDTA-inactivated system in these tissues. On the other hand, Mn^{++} in 0.1 molar concentration shows no tendency to inhibit the enzyme of any of these tissues and also consistently fails to activate it.

Microscopic examination of the control sections and of those EDTA-inhibited sections reactivated by exposure to the various metallic ions thus far tested has shown no significant variation in localization of alkaline phosphatase activity. It is possible, however, that important components of the alkaline phospha-

tase system are lost by the method of fixation and embedding used since there is no question that such treatment considerably reduces the intensity of the reaction(10,11). Studies using more efficient methods of tissue preparation such as freezing-drying are continuing. Other components of the phosphatase system are also being studied, and the reversible inactivation by EDTA of 5-nucleotidase has already been demonstrated by histochemical means.

Summary. A method of controlled inactivation of alkaline phosphatase in tissue sections by removal of the activating metallic component through chelation is described. The enzyme activity of alcohol-fixed, paraffin-embedded rabbit kidney can be completely inhibited by EDTA and subsequently reactivated by a variety of metallic cations in 0.1 molar concentration, including Mg^{++}, Co^{++}, Zn^{++}, Ca^{++}, Sr^{++} and Ba^{++}. Ni^{++} is a weak reactivator. The localization of enzyme activity in the tissue is the same regardless of the reactivating ion. Hg^{++}, Be^{++}, and Cu^{++} are constant strong inhibitors. Mn^{++}, Pb^{++} and Cd^{++} fail to reverse EDTA inhibition but will not themselves inhibit the active enzyme in the concentrations used. Preliminary experiments indicate, however, that the alkaline phosphatase of guinea pig and monkey kidney is inhibited by Pb^{++} and Cd^{++} in much lower concentrations.

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Effect of Colchicine on Synthesis of Desoxyribonucleic Acid in Tissue Cultured Rat Fibroblasts.* (20641)

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Early observations on the effect of colchicine on growing tissue (Dustin(1)) suggested that this substance exerts a stimulatory effect on the process of cell division. In later analyses it was shown by Ludford(2) and others that the increase in mitotic figures was the result of an inhibition of mitosis at metaphase. Ludford postulated an inhibitory effect on the mitotic spindle, which has recently been convincingly illustrated by the work of Inoue(3) on the birefringence of the spindles of normal and colchicine-treated sea urchin eggs. The suggestion of a specific effect led to experiments which demonstrated that at low col-

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