

Evidence that Molybdenum Is a Nondialyzable Component of Xanthine Oxidase^{1, 2}

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Isolation and purification of xanthine oxidase from the milk of cows that have received Mo⁹⁹ has provided evidence that molybdenum is an integral part of the xanthine oxidase of milk and, presumably, of other sources. This finding is in accord with the evidence of Westerfeld and Richert (1) for the existence of a nutritional factor essential for the production and maintenance of rat intestinal xanthine oxidase and the identification of this factor as molybdenum by De Renzo *et al.* (2).

Labeled sodium molybdate was injected intravenously into a dairy cow and xanthine oxidase was isolated by the method of Ball (3) from milk collected one to several days thereafter. This procedure was repeated with the same cow 2 wk later. In all cases after the initial removal of excess molybdenum by one or two treatments with 25–40% ammonium sulfate, the ratio of Mo⁹⁹ to xanthine oxidase activity remained essentially constant despite rigorous purification procedures, as indicated below for a typical sample. Xanthine oxidase was estimated by Kalckar's method (4) and Mo⁹⁹ by radioassay procedures (Table 1).

That the Mo⁹⁹ was not removed from, and probably did not enter, the xanthine oxidase merely by exchange was demonstrated by addition of 2 g of ammonium molybdate to a sample of milk from the treated cow and subsequent isolation of the enzyme. This treatment with inert molybdenum did not change the ratio of Mo⁹⁹ to xanthine oxidase. In another dilution experiment, nonradioactive milk (126 units of xanthine oxidase) was mixed with an equal amount of radioactive milk (46 ct/sec/unit of xanthine oxidase; 10.6 ct/sec/unit of xanthine oxidase after purification; 91 units of xanthine oxidase). After isolation of the enzyme and dialysis against water for 40 hr, the xanthine oxidase had a value of 4.23 ct/sec/unit in good agreement with the value of 4.43 expected if there were no exchange of the excess Mo⁹⁹ with the inert molybdenum of the nonradioactive xanthine oxidase.

Spectrophotometric data suggest a molar ratio of flavin to molybdenum of 2:1.

Molybdenum has been recognized for a long time as an essential nutrient for plants, serving metabolic functions involved in the utilization of nitrogen. It

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TABLE 1

| | Mo ⁹⁹ Ct/sec/unit xanthine oxidase |
|---|--|
| Milk | 1095 |
| Cream | 71 |
| Na ₂ HPO ₄ extract | 67 |
| Na ₂ HPO ₄ extract after CaCl ₂ treatment | 88 |
| Xanthine oxidase after 1st (NH ₄) ₂ SO ₄ ppt | 12.1 |
| Xanthine oxidase after 2nd (NH ₄) ₂ SO ₄ ppt | 12.6 |
| Xanthine oxidase after 20-hr dialysis | 10.9 |
| Xanthine oxidase after 40-hr dialysis | 10.6 |
| Average for 4 different purified samples (7 milkings) | 10.61 (range 10.4–10.78) |

would now seem that molybdenum also plays an essential role in animals, at least to the extent that xanthine oxidase is concerned with necessary metabolic processes. In addition, the toxic behavior of excess molybdenum in the copper-molybdenum imbalance may possibly be explained in terms of oxidase activity.

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Intracellular Localization of Labeled Nucleic Acid Determined with Autoradiographs¹

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Autoradiographs have been used to detect incorporation of phosphorus (P³²) into the deoxyribose nucleic acids (DNA) of individual nuclei (1, 2). In this way the time of synthesis of DNA in relation to the division cycle during mitosis and meiosis has been determined. The method is also applicable to the study of phosphorus incorporation into the ribonucleic acids (RNA) of single cells. By applying a thin photographic emulsion layer to serial sections of large cells and making autoradiographs as previously described (2), the radioactive element can be located with sufficient precision to determine its intracellular distribution. For example, in the large cells of the gastric caeca and salivary glands of the third instar larvae of *Drosophila*, the relative rates of incorporation of P³² into the RNA of cytoplasm, chromatin, and nucleolus can be followed.

Early third instar larvae of *Drosophila repleta* were

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fed P^{32} in the form of inorganic phosphate (120 $\mu\text{C}/\text{ml}$ of water containing 30 mg of gelatin and 30 mg of brewers' yeast). Incorporation occurs most rapidly in the cells of the salivary glands and the gastric caeca, and the comments here refer to the behavior of these cells. At intervals of 1 hr after beginning to feed on the labeled food, larvae were fixed in Carnoy's fluid, and prepared as paraffin sections (7 μ) on slides.

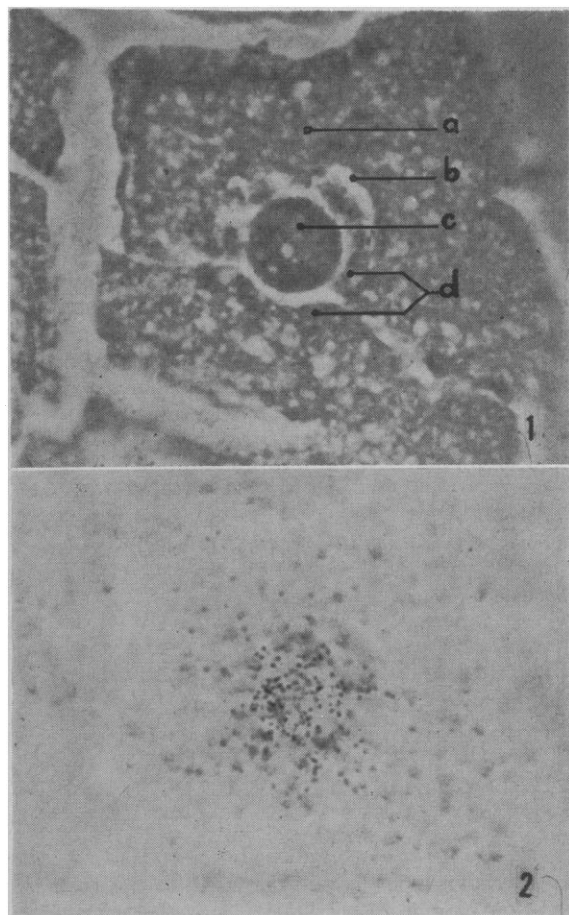


FIG. 1. Autoradiograph of a cell of the gastric caecum fixed 1½ hr after feeding food containing P^{32} . 1. Phase photograph of the cell; the structures indicated are (a) cytoplasm, (b) nuclear membrane, (c) nucleolus, (d) chromatin. 2. Bright field photograph showing distribution of silver grains above the same cell. $\times 1220$.

After passing these slides through hot ether-alcohol, washing in lower grades of alcohol, cold 5% trichloroacetic acid, and water, one of a pair of slides containing tissues of the same larvae was coated with autoradiographic stripping film. The other slide was placed in a solution of protease free ribonuclease² (0.2 mg/ml of water at pH 6.0 for 2 hr at 37°) (3). After this digestion, or in some experiments after hydrolysis and staining by the Feulgen procedure, the second slide of the pair was coated with stripping film.

A study of the autoradiographs, from those larvae

² Prepared by Worthington Biochemical Co.

fixed 1 hr after beginning to feed on the labeled food, shows that the highest concentration of P^{32} that remains after fixation and the subsequent washings is in the nucleolus (Fig. 1). About one-tenth this concentration is present in the cytoplasm. Some cells at this early period also show a relatively high concentration in certain regions of the chromatin. The concentration may exceed that in the nucleolus.

Those larvae fixed 2 or 3 hr after beginning to feed show an increasing concentration of P^{32} in all parts of the cell. In 2 hr the concentration of P^{32} in the nucleolus is higher than in the chromatin and the cytoplasm has one-fifth to one-third the concentration present in the nucleolus. If the larvae are removed to nonradioactive food enriched with yeast after the first 2 hr, the P^{32} is still higher in the nucleolus than the cytoplasm 1 hr later, but in 2–3 hr (4–5 hr after beginning to feed on the radioactive food) the P^{32} is about equally distributed in various parts of the cell. Nearly all the P^{32} that remains in the nucleolus and most of that in the cytoplasm and chromatin is removed from the cells on the slides digested in ribonuclease or subjected to the Feulgen hydrolysis (1N HCl for 10 min at 60°). This is interpreted to indicate that most of the P^{32} is incorporated into ribonucleic acids. The P^{32} remaining after digestion or hydrolysis is usually one-tenth or less of the total and its distribution is rather uniform throughout the cell.

Resolution of structures 2–3 μ apart is theoretically possible (4) with this type of autoradiograph. In practice one has the added advantage that adjacent structures may be separated in serial sections. If a nucleolus with surrounding chromatin and cytoplasm of a cell is included in one section, a part of the chromatin of that cell with surrounding cytoplasm in the succeeding section, and only cytoplasm in a third section, estimation of the isotope in each of these three parts can be made with greater precision than would otherwise be possible. In this way, with thinner sections, one should be able to resolve structures in smaller cells. In the cells of *Drosophila*, which are 40–50 μ in diameter with a nucleus 18–20 μ in diameter and a nucleolus 8–12 μ in diameter, resolution is not a problem.

A large proportion of the nuclear RNA of higher specific activity than cytoplasmic RNA previously reported (5–7) may be contributed by nucleolar RNA. The nucleolus may be a center of RNA synthesis as suggested by Pollister and Leuchtenberger (8) or a reservoir of RNA produced in other parts of the nucleus. The initial high specific activity of chromatin observed in these autoradiographs suggests the latter. Although one cannot definitely answer questions of sites and rates of synthesis yet, the technique provides a tool by which variations in turnover rates may be studied at the intracellular level. Perhaps the role of RNA can eventually be evaluated by application of this technique along with photometric techniques that allow measurement of relative concentration of certain cellular constituents.

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Gastric Ascorbic Acid in the Gastritic Guinea Pig¹

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Among other things, the rat differs from the human in having a squamous forestomach and an endogenous supply of ascorbic acid. When it was found (1) that a chemically induced gastritis in the rat resulted in a decrease of ascorbic acid in the stomach and adrenals, it became of considerable interest to determine if a similar relation exists in an animal having an entirely glandular stomach and lacking endogenous ascorbic acid. The present study, therefore, was carried out on the guinea pig.

TABLE 1
EFFECT OF EUGENOL ADMINISTRATION ON GASTRIC AND ADRENAL ASCORBIC ACID IN THE GUINEA PIG

| No. of guinea pigs* | Treatment | Chem. form. | Mean ascorbic acid | |
|---------------------|---------------------------------|-------------|--------------------|----------------|
| | | | Stomach (mg %) | Adrenal (mg %) |
| 11 | H ₂ O controls (st)† | Oxidized | 1.5 ± 0.69 | 5.3 ± 3.0 |
| | | Reduced | 14.4 ± 0.42 | 117.7 ± 7.2 |
| | | Total | 15.9 ± 0.52‡ | 123.0 ± 7.5 |
| 10 | Eugenol (st)‡ | Oxidized | 1.0 ± 0.25 | 3.4 ± 2.9 |
| | | Reduced | 7.9 ± 1.8 | 84.4 ± 4.4 |
| | | Total | 8.9 ± 1.9 | 87.8 ± 2.6 |

* Weight loss not observed in any animal.

† By stomach tube.

‡ Differences between corresponding totals from the two groups are statistically significant. $P < 0.01$.

Male Rockland Farms guinea pigs weighing 350–450 g received Purina Rabbit Chow Checkers³ and tap water ad libitum as well as daily intramuscular injections of 25 mg sodium ascorbate in 1.0 ml physiological saline. The guinea pigs were divided into 2 groups. The first group, controls, received 3-ml oral

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³ Preliminary analysis of our stock of Purina Rabbit Chow Checkers indicated that the concentration of ascorbic acid was < 0.08 mg %.

doses of water daily for 7 days. The second group received the same amount of a 1.0% emulsion of eugenol. The ascorbic acid was determined by the method of Roe *et al.* (2).

Introduction of the eugenol emulsion to the gastric lumen by stomach tube brought about a grossly evident gastritis which was absent in the water-fed controls. The total ascorbic acid concentration in the stomachs of the gastritic guinea pigs was significantly decreased by approximately 44%. In the same animals there was a simultaneous decrease of about 29% in the adrenals, which was probably associated with a systemic stress response. The ratio of oxidized to reduced ascorbic acid in the stomachs and adrenals of the gastritic animals was not significantly different from that in the controls (Table 1). The decrease in gastric tissue ascorbic acid during an induced gastritis is more pronounced in the guinea pig (44%) than in the rat (13%) (1). In the rat a smaller decrease of gastric ascorbic acid resulted, apparently because part of the loss was simultaneously replenished from biosynthetic sources.

The data suggest that the ascorbic acid decrease in the stomach of both species during gastritis is a result of rapid utilization of vitamin C at a site of regeneration. The rapid utilization of ascorbic acid at sites of regeneration also has been suggested by the work of Leise *et al.* (3), who reported that the percentage of takes in transplantation of a C 954 hepatoma is increased from 36 to 52% in C57L(Fx) mice when supplementary ascorbic acid is supplied. Moreover, Minor and Ramirez (4) have reported that cancer patients utilize more ascorbic acid than patients having nonmalignant disease, as determined by daily measurement of ascorbic acid intake and excretion. The present work suggests a similar rapid utilization of ascorbic acid in the gastritic mucosa.

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Chromosomal Interchanges as a Basis for the Delimitation of Species in *Oenothera*¹

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The delimitation of two species upon the basis of their failure to form a hybrid is untenable wherever

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