TABLE III. Effect of Increasing Food Consumption and Body Weight Gains on Mammary Gland Growth.

Mean daily food consumption during pregnancy (g)	No. of animals	Total DNA (mg/100 g of body wt.; mean)	Increase of body wt. (g)	No. of animals	Total DNA (mg/100 g of body wt.; mean)
17	5	6.02	under 29	4	5.81
18	4	6.17	30–39	5	6.43
19	4	6.33	40–49	5	6.55
20	1	6.69	50–59	2	6.79
21	3	8.05	60–69	2	7.88
22	—	—	Total	18	6.50
23	—	—			
24	1	6.13			
Total	18	6.50			

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Enzymatic Determination of Small Quantities of Hydrogen Peroxide* (33468)

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Avi-Dor *et al.* described a method for the determination of minute amounts of H_2O_2 that is based on the use of horseradish peroxide and hydrogen donors. With this they measured the H_2O_2 formed by purified D-amino acid oxidase from D-alanine and O_2 (1). However, the detection of the small amounts of H_2O_2 formed during such oxidative dehydrogenation is complicated by decomposition of the H_2O_2 by catalase or by further oxidation of the metabolites. To avoid these problems, Keilin and Hartree (2) attempted direct estimations of H_2O_2 based on the oxidation of ethanol by H_2O_2 in the presence of an excess of catalase. This reaction proceeds more rapidly than the catalytic decomposition of H_2O_2. Measurements of O_2 consumption in their system have established that certain enzymes [mammalian L-amino acid oxidase (3) and L-α-hydroxy acid oxidases (4)] do produce H_2O_2 during the reaction. The work herein reported described the pho-

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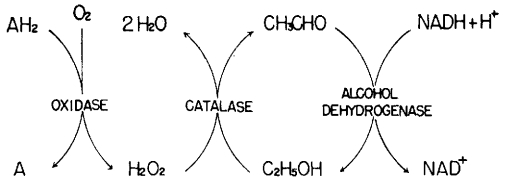

FIG. 1. Principle of the assay for hydrogen peroxide produced during enzymatic oxidation: AH_2 represents the hydrogen donor.

tometric determination of the small amounts of H_2O_2 generated by L-α-hydroxy acid oxidase, L-amino acid oxidase, or uric acid oxidase in the presence of ethanol, excess catalase, alcohol dehydrogenase (yeast), and NAD (Fig. 1).

Materials and Methods. Enzyme preparations. The L-α-hydroxy acid oxidase was obtained from rat livers by the method of Nakano *et al.* (4). Crystalline mammalian L-amino acid oxidase was prepared from rat kidneys as previously described (5, 6). Uric acid oxidase derived from *Candida utilis* was supplied by Seikagaku Kogyo. Crystalline beef liver catalase (in a 0.5% solution of thymol in water) and crystalline yeast alcohol dehydrogenase (in a 2.0 *M* solution of $(NH_4)_2SO_4$) were purchased from Sigma and from Boehringer, respectively.

Enzyme assays. The L-α-hydroxy acid oxidase and L-amino acid oxidase were measured by the 3-hydroxyisoquinoline or the 2, 4-dinitrophenylhydrazone method (4, 5). L-Hydroxyisocaproate was the usual substrate for both L-amino acid oxidase and L-α-hydroxy acid oxidase. One unit represented that amount of enzyme which at 37° and other specified conditions produced 1 mμmole of the α-keto acid in 20 min (4). In some experiments glycolate was used in place of L-α hydroxyisocaproate as the substrate for L-α-hydroxy acid oxidase. The 2, 4-dinitrophenylhydrazone method was employed in measuring α-keto acid formed from the corresponding hydroxy acid in the experiments conducted at 23° with relatively large amounts of enzyme. Uric acid oxidase was assayed by a modification of the method of Schneider and Hogeboom (7). The incubation medium consisted of 0.1 ml of 0.00125 *M* uric acid, 2.9 ml of 0.1 *M* sodium phosphate buffer (pH 7.9), the enzyme, and water in a total volume of 3.13 ml. The rate of degradation of uric acid was determined spectrophotemetrically at 293 mμ at room temperature (23°). The amount of uric acid consumed was calculated as follows:

$$\frac{5.95 \times 3.13 \times (E_0 - E_t)}{0.76} = \text{m}\mu\text{moles of uric acid consumed during t min,}$$

where E_0 and E_t represent the optical density at zero time and after t min of incubation, respectively.

Results and Discussion. The L-amino acid oxidase, L-α-hydroxy acid oxidase, and uric acid oxidase were chosen as hydrogen peroxide generating enzymes. Consumption of NADH in the oxidase–catalase–alcohol dehydrogenase system was measured in a Beckman cuvette containing 2.8–3.0 ml of 0.1 *M* sodium phosphate buffer (pH 7.9), 0.1 ml of 3.0×10^{-3} *M* NADH in 1% $NaHCO_3$, 0.1 ml of suitable substrate (0.1 *M* hydroxy acid or 0.00125 *M* uric acid), 0.01 ml of diluted ethanol (1:3 v/v), 0.2 ml of the solution containing about 200 μg of crystalline beef liver catalase, 0.01 ml of alcohol dehydrogenase (30 mg/ml), and 0.1 ml of a suitable dilution of the enzyme. The final volumes of the reaction mixtures were 3.13 and 3.33 ml in the experiments with uric acid oxidase or with L-amino acid oxidase (or L-α-hydroxy acid oxidase), respectively. The reaction was initiated by the addition of the enzyme and continued for a specified time at 23°. Absorbancy was measured at 340 mμ at 1 min intervals, with distilled water as the control. Results obtained with L-α-hydroxy acid oxidase and L-amino acid oxidase are shown in Fig. 2. The absorbancy at zero time (E_0) was extrapolated graphically and the amount of NADH consumed was calculated according to the following formula (8):

$$\frac{(E_0 - E_t) \times V}{E} = \text{μmoles of NADH consumed/assay mixture,}$$

where V and E represent the assay volume and the extinction coefficient of NADH (E_{340} = 6.22 $Cm^2/\mu mole$), respectively; E_t rep-

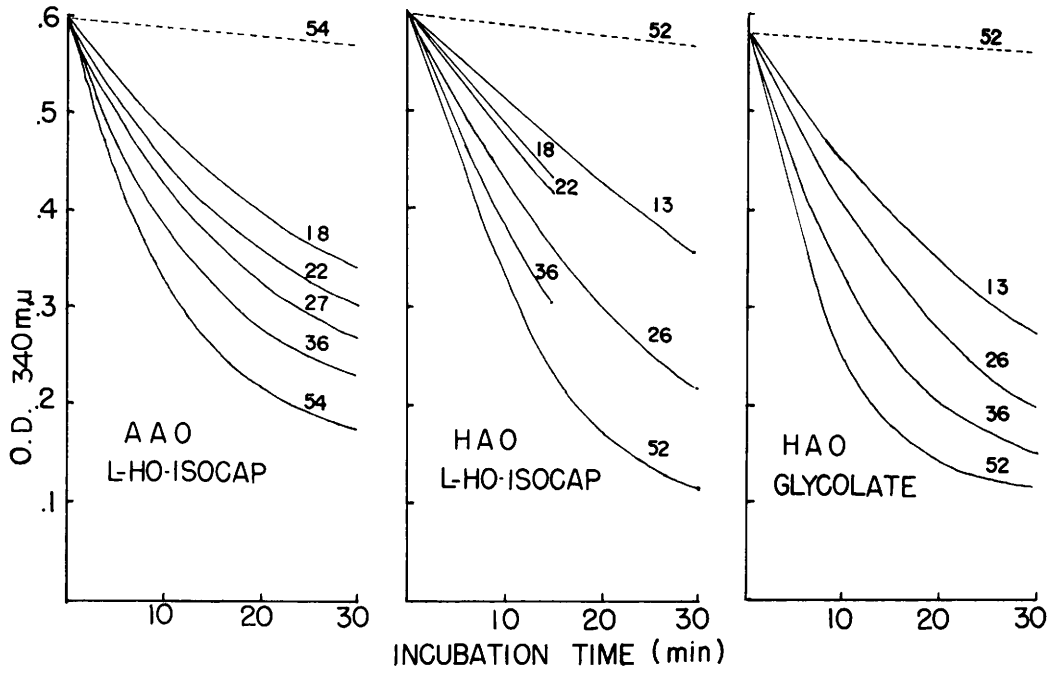

FIG. 2. Relation of incubation time and change of optical density at 340 mμ.

L-Amino acid oxidase with a specific activity of 12,000 units/mg protein (AAO) and L-hydroxy acid oxidase with specific activity of 16,000 units/mg protein (HAO) were used as hydrogen peroxide generating enzymes and the experiments were carried out in the presence of NADH, excess of catalase, ethanol, alcohol dehydrogenase and L-hydroxyisocaproate (L-HO-Isocap) or glycolate (see text). (- - -), the results obtained in the absence of added ethanol; the numbers over the curves indicate the amount of enzyme in μg.

resents the optical density obtained after t min incubation.

The keto acid was assayed at 23° in media without added ethanol, alcohol dehydrogenase, or NADH. At the end of incubation, 1.6 ml of 30% trichloroacetic acid were added to the reaction mixture. An aliquot of the deproteinized supernatant was used for each determination of the keto acid; the calibration curves were constructed with the 2, 4-dinitrophenylhydrazones of authentic keto acids. When the amount of keto acid formed in 10 min from the corresponding hydroxy acid was less than 150 mμmoles, the ratio of mole of NADH consumed to mole of keto acid formed was approximately 1:1.2 (Fig. 3). The NADH does not decrease appreciably in the absence of ethanol (Fig. 2). When approximately 50 mμ moles of hydrogen peroxide were added to the catalase–alcohol dehydrogenase system free of oxidase, the

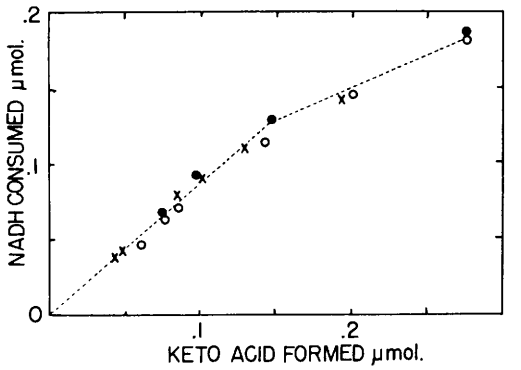

FIG. 3. Relation of NADH consumed and keto acid formed from L-hydroxyisocaproate or glycolate: L-hydroxyisocaproate was used as substrate for L-amino acid oxidase (X) and for L-hydroxy acid oxidase (○); glycolate was also used as the substrate for L-hydroxy acid oxidase (●); the incubations were carried out under the condiions described in the legend to Fig. 2. The keto acid was determined by the 2,4-dinitrophenylhydrazone method.

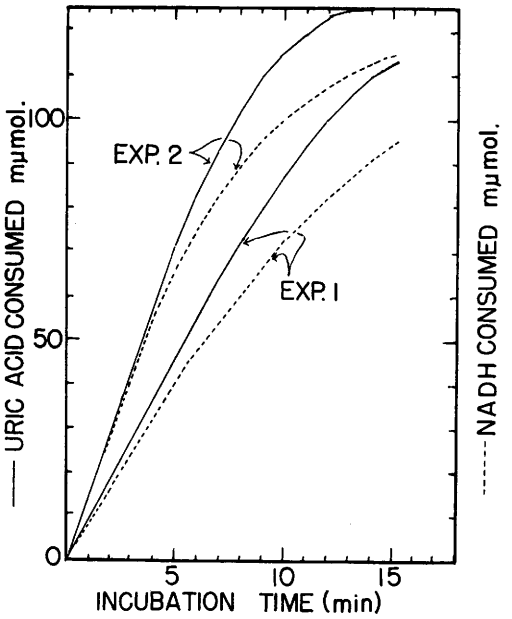

FIG. 4. Time course studies of uric acid oxidation and NADH consumption: consumption of uric acid (—), and of NADH (- -) was measured photometrically in the assay system and in the uric acid oxidase-catalase-alcohol dehydrogenase system, respectively (see text).

consumption of NADH (40–50 mμmoles) was almost complete within 2 min. In the experimental procedure the H_2O_2 formed by action of an oxidase reacts with ethanol to produce acetaldehyde; the aldehyde is then converted to ethanol with simultaneous consumption of NADH. Even when the amount of the alcohol dehydrogenase employed in the assay was as small as 0.006 mg, the consumption of NADH still approximated 95% of that recorded under the standard assay conditions. Such agreement between NADH consumption and keto acid formation is probably attributable to the extremely small turnover numbers of L-amino acid oxidase and L-α-hydroxy acid oxidase compared with those for catalase and alcohol dehydrogenase.

The H_2O_2 formed during uric acid oxidation by uric acid oxidase under similar conditions can also be estimated indirectly from the consumption of NADH in the uric acid oxidase–catalase–alcohol dehydrogenase system. The consumption of NADH and of uric acid is shown in Fig. 4. The data obtained during the first 5 min of the reaction indicate that with uric acid oxidase of high enzyme activity the consumption of NADH coincides with that of uric acid.

Summary. Two reactions, (a) H_2O_2 with ethanol in the presence of an excess of catalase, and (b) acetaldehyde with NADH in the presence of yeast alcohol dehydrogenase have been employed in assays of H_2O_2 during the oxidation of a suitable substrate with an H_2O_2 generating enzyme. A method is described for the accurate measurement of minute amounts of H_2O_2. L-α-hydroxy acid oxidase from rat liver, L-amino acid oxidase from rat kidney, and uric acid oxidase from *Candida utilis* were used as H_2O_2 generating enzymes. The amount of H_2O_2 produced was determined photometrically from the change in optical density at 340 mμ. Recoveries with this method exceeded 80%.

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