helium to a vacuum of 23 inches, and finally, hydrogen was admitted to a vacuum of 5 inches. The light was then turned on, and the leaves permitted to photosynthesize for 60 minutes. At the end of the experiment the chamber was slowly evacuated, the remaining CO_2 being caught in the spiral and NaOH bubbler. Six flushings with hydrogen to remove as much CO_2 as possible, the final evacuation being to ca. 29 inches, consumed an additional hour. The respiration time was therefore two hours.

The plants taken from the chamber were placed immediately into liquid nitrogen and ground to a powder. The frozen powder was then treated as shown in Fig. 2.

The crystalline glucose was obtained by evaporating the hydrolyzed sucrose mixture to a syrup, dissolving this syrup together with several hundred milligrams of carrier glucose in the minimal amount of 95 per cent alcohol, and then adding ether to produce a slight cloudiness. Upon cooling, glucose crystallizes out.

It should be remembered, however, that the above distribution of activity is valid only for the specific set of conditions for photosynthesis given. It may be expected to be different for other conditions, *i.e.* time, O_2 pressure, CO_2 pressure, etc.

The activity measurements of the various fractions shown in the foregoing chart are given in Table 1.

IN THE LABORATORY

A Micromethod for the Determination of 1−(+) Lactic Acid[1]

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Lehmann (*2*) has presented a detailed description of a simple micromethod for determining 1−(+) lactic acid which appears to have been largely overlooked. We have found this basic method very useful and have devised certain modifications which eliminate interfering substances and permit final measurement to be made colorimetrically.

The method depends on the quantitative oxidation of lactic acid to pyruvic acid by means of the yeast enzyme, lactic dehydrogenase, in the presence of potassium ferricyanide, which acts as hydrogen acceptor. Lehmann titrated the resulting potassium ferrocyanide with ceric sulfate, using 1 per cent "setoglaucin-o" as the indicator. Diphenylamine sulfonic acid may likewise be used (*3*) as the indicator. According to Lehmann, the method is quite specific. Of a group of 52 substances investigated, which included carbohydrates, amino acids, α- and β-hydroxybutyric acids, intermediary metabolites, and substances related structurally to lactic acid, the only compounds which interfered with the determination were glycolic, α-hydroxybutyric, and glyceric acids, α-glycerophosphate, hexosediphosphate, and hexosemonophosphate. The first two compounds are not present in sufficient concentration to interfere in biological fluids. The last four can be removed by treatment with $CuSO_4$-$Ca(OH)_2$ according to the method of Friedemann and Kendall (*1*). Ascorbic acid which interferes by reacting directly with potassium ferricyanide can likewise be removed by this procedure. Preliminary treatment with $CuSO_4$-$Ca(OH)_2$ does not interfere with the enzymatic reaction providing the pH is properly adjusted before incubation with the enzyme.

[1] This work was supported by a grant from the Mallinckrodt Chemical Works.

Thus, the procedure used in this laboratory is similar to that described by Lehmann except for the use of $CuSO_4$-$Ca(OH)_2$ for the removal of interfering substances and the determination of potassium ferrocyanide by a colorimetric instead of a titrimetric method. A brief description follows.

Reagents necessary for the method are M/15 Na K phosphate buffer, pH 7.4; 5M/1,000 potassium ferricyanide in water; and ferric salt prepared as follows: Twenty grams of gum ghatti in a cheesecloth bag is soaked in 1 l. of water for 24 hours, and 5 grams of anhydrous $Fe_2(SO_4)_3$ and 75 cc. of 85 per cent H_3PO_4 + 100 cc. of water are added. After mixing, 15 cc. of 1 per cent $KMnO_4$ is added in order to destroy reducing materials in the gum ghatti. The solution is allowed to stand a few days before using.

Fleischmann's baker's yeast is powdered and dried at room temperature. It is then washed several times with distilled water (100 grams in 1 l. of water), following which it again is dried at room temperature and powdered. This dried yeast is the source of the enzyme, lactic dehydrogenase, and keeps indefinitely in the refrigerator. Before using, the yeast is washed once with phosphate buffer (50–100 mg. yeast, depending upon the activity, in 10 cc. of buffer), centrifuged, and resuspended in the same volume of buffer. The activity of the enzyme as well as the removal of intracellular substrate may be tested as suggested by Lehmann by incubating 1-cc. aliquots of the yeast suspension alone, and with 180 γ lactate, in the presence of 1 cc. of a solution of o-chlorophenol-indo-2:6-dichlorophenol (sodium 2,6-dichlorobenzenone-indo-3-chlorophenol (ortho), Eastman P 3467) (1:50,000) in an open test tube. In the presence of lactic acid the indicator should be decolorized in 3 minutes or less. The control should not be decolorized in 10 minutes and usually is not in an hour.

Procedure: The sample containing lactic acid is deproteinized with trichloracetic acid and centrifuged. The supernatant fluid is neutralized and diluted so that 1 cc. contains not more than 200 γ of 1-lactic acid. If ascorbic acid or phosphorylated hexoses be present, the sample is treated with $CuSO_4$-$Ca(OH)_2$ as described by Friedemann and Kendall. Treatment with trichloracetic acid is unnecessary when the $CuSO_4$-$Ca(OH)_2$ precipitation is used in unknowns with small amounts of

proteins. After removal of the $CuSO_4$-$Ca(OH)_2$ precipitate by centrifugation, the sample containing the lactic acid is neutralized. One cc. (containing 200 γ or less) is incubated with 1 cc. of yeast suspension in phosphate buffer and 1 cc. of potassium ferricyanide for $\frac{1}{2}$ hour at 30° in open test tubes. Air does not interfere with the determination. At the end of the incubation period, the mixture is centrifuged. To 2 cc. of the supernatant fluid ferric sulfate solution is added, the mixture is diluted, and the amount of resulting Prussian blue determined using a photoelectric colorimeter with a yellow-green filter. In our experiments the Cenco-Sheard-Sanford photelometer was used. For this instrument we found it best to use two standard curves—one for samples containing less than 75 γ of l-lactic acid/cc., the other for samples containing 75–150 γ. If the sample contains less than 75 γ/cc., 2 cc. of the supernatant fluid from the enzyme mixture are placed directly in a cuvette, 1 cc. of ferric salt solution and 2 cc. of water are added, and the reading made immediately. For samples containing 75–150 γ, 2 cc. of the supernatant fluid from the enzyme mixture are placed in a 10-cc. volumetric flask, 2 cc. of Fe^{+++} salt added, the mixture diluted to volume, and the Prussian blue determined.

A practically straight-line relationship is obtained when per cent transmission is plotted against concentration. The curve is completely reproducible with the same yeast preparation and when incubated for the same length of time. We found it best, however, to make a standard curve with each set of analyses by setting up one blank determination and one with a known amount of lactic acid, in this way correcting for any variation in the yeast suspension or in the procedure.

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Histochemical Demonstration of Alkaline Phosphatase in Decalcified Dental and Osseous Tissues[1]

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Histochemical studies on alkaline phosphatase in bones and teeth have been restricted to the incipient stage of mineral deposition, due to the fact that it very soon becomes impossible to section these structures without first decalcifying them. Employment of acids to remove the mineral has resulted in destruction of this enzyme. In rats, therefore, it has not been possible to follow the alkaline phosphatase in odontogenesis later than 4 days past birth (*1*).

It is obviously desirable to learn the localization and relative activity of this enzyme in bones and teeth throughout their developmental history. It would be expected that such information might have functional significance. The dental tissues are of extraordinary interest in this regard, since they are highly specialized for mineral deposition.

[1] Aided by a grant from the Milton Fund, Harvard University.

In the development of a method for the successful demineralization of the hard structures without at the same time destroying the alkaline phosphatase, we have been guided by a study of the activity of this enzyme *in vitro* under a variety of environmental and chemical factors (*2*).

The heads of 28-day-old rats were split in the midsagittal plane, fixed in cold, 80 per cent alcohol, and placed in the following solutions of specified pH:

I. (a) equal parts of 20 per cent sodium citrate and 2 per cent formic acid ..... pH 4.9
   (b) equal parts of 20 per cent sodium citrate and 5 per cent formic acid ..... pH 4.2
II. (a) 5 per cent aqueous ammonium citrate ..... pH 4.8
   (b) 10 parts 5 per cent ammonium citrate and 1 part 15 per cent citric acid ..... pH 4.2

Completion of decalcification, as shown by the needle test, required 1–5 days. During dehydration of the tissue blocks all alcoholic solutions were buffered to pH 9.3 ± 0.1, a step important in preserving the maximum phosphatase activity. In line with this objective it was found necessary to use a minimum of heat to expand the paraffin ribbon; we allowed 10–15 seconds at 45°–50°C. when using paraffin with m.p. 56°–58°C.

The upper incisors and molars with bony encasements in each case were sectioned longitudinally. The sections were uniformly incubated for 3 hours at 37°C., using sodium glycerophosphate as a substrate. Visualization of the enzymatic action was brought about by the generally accepted method of Gomori (*3*), which results in the formation of black cobalt sulfide at the site of enzyme activity.

The range in hydrogen-ion concentration over which decalcification could be accomplished without destroying alkaline phosphatase was found to be very narrow, *i.e.* pH 4.8–5.0. When this range was exceeded on the alkaline side, the rate of decalcification became unduly slow; with a slight increase in acidity the alkaline phosphatase activity was irreversibly lost. This is illustrated by the observation that little or no phosphatase activity was demonstrable in the tissues decalcified in solutions Ib and IIb at pH 4.2. The phosphatase activity was well preserved, however, in comparable tissue blocks decalcified at pH 4.8 and 4.9 (solutions Ia and IIa, respectively). The purpose in buffering all the alcoholic solutions used subsequent to decalcification was to provide immediately an optimum condition for the phosphatase to retain and perhaps to regain activity. That this may be an important factor was further demonstrated by allowing sections to stand 24 hours in an aqueous medium buffered at pH 9.3 ± 0.1 prior to incubation; there was a definite increase in phosphatase activity in comparison with adjacent sections not so treated and which contained demonstrable phosphatase activity. The reaction was also accentuated by the addition of magnesium chloride to the substrate. In fact, when sections were pretreated in the manner just described and subsequently incubated with magnesium ions in the substrate, an amount of precipitate was deposited in the odontogenic and osteogenic tissues which was considered to represent a maximum alkaline phosphatase reaction.

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