missing and others had a "gnawed" (*15*) appearance. The external limiting membrane had nearly disappeared, and only traces remained. Recent electron-microscopic studies (*16*) have summarized the ultrastructural pathology of the rods in rats deficient in vitamin A.

In moths deficient in vitamin A, segments of the outer retinula border were missing (Fig. 1G), presenting a "gnawed" appearance. Certain retinular cells had a retrograde change with a loss or resorption of acidophilic material. The cell contours may have been maintained, but many nuclei appeared suspended by trabeculae. Remaining cytoplasm was granular, and the total appearance seemed similar to the parenchymatous degeneration of vertebrate tissue (*17*). The rhabdom in cross section (Fig. 1H) was diffuse, swollen and more lightly stained. Its overall semblance was more flexuous than that of rhabdomes from rats given two treatments. The basement membrane had disappeared in certain areas, allowing both tracheolar and retinular tissue to herniate into the subretinular area (Fig. 1I). The presence of convoluted and flaccid postretinal axonal fasciculae suggested that degeneration of the nervous tissue had also occurred. This observation tends to be substantiated on a functional basis by records of meager postsynaptic response and by the absence of a retinal action potential in the electroretinograms of these moths (*8*).

A deficiency of vitamin A does not appear to inhibit growth or limit the vitality of these moths. Such deficient moths do, however, show little if any orientation to light and usually do not become successfully adapted to the dark. The ommatidial disks in deficient moths are milky-white in color, while those of animals reared on tobacco are golden. This tuft of retinulae in the moths given diets supplemented with β-carotene or vitamin A is white or very pale yellow. When these latter moths become adapted to the dark a white glow is noted when the cornea is reilluminated, while an orange glow is noted in moths fed tobacco. Dark-adaptation curves reveal that moths given either carotenoid have a higher sensitivity to light than moths reared on tobacco do (*8*).

Apart from our brief remarks on the abnormality of electroretinograms of deficient moths, other studies (*18*) indicate that houseflies can be reared without dietary intake of vitamin A and that they suffer a partial loss of photosensitivity (over a 4-log-unit increase in visual threshold). It has been suggested that a lack in dietary carotenoid can be offset to a degree by storage of the provitamin in the egg or by the presence of microorganisms that produce vitamin A, or by both (*18*). Our results suggest, however, that *Manduca sexta* does not have these sources and that carotenoid intake during larval life is crucial for normal vision ("normal" in this sense referring to night vision) in the adult moth.

Because metaplasias that cause squamatization and keratinization of epithelium (*17*) arise in vertebrates with severe vitamin A deficiency, such animals serve as relatively poor experimental subjects for studies of nutritional night blindness. This problem in rats has recently been overcome (*19*) through use of vitamin A acid (in this case the only metaplasia was that associated with the retinal epithelium). In the moth, however, no such analogs are required, and thus this species might serve well as a laboratory animal in the study of nutritional night blindness as well as other dystrophic diseases of the eye.

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Reagentless Substrate Analysis with Immobilized Enzymes

Abstract. *By coupling an immobilized enzyme system with an electrochemical sensor, the reagent requirement for this glucose method is eliminated. Miniaturization and a further simplification of the instrumentation for the continuous analysis of glucose is achieved.*

Techniques employing immobilized enzyme material for chemical analysis have been reported (*1*, *2*). These techniques eliminate the need for soluble enzyme reagents in analyses based on enzyme-catalyzed reactions. Furthermore, when a soluble enzyme is the only reagent required, a reagentless system of analysis can be achieved. Such a system is demonstrated for the continuous determination of glucose with glucose oxidase.

$$O_2 + \text{glucose} \xrightleftharpoons{\text{G.O.}} H_2O_2 + \text{gluconic acid}$$

In the above reaction, glucose oxidase (G.O.) catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide. In this method of glucose determination oxygen uptake is taken as a measure of the reaction. Since the oxygen is always contained in the samples, and glucose is the sought-for constituent, the glucose oxidase is the only reagent which must be added to the system for analysis. By immobilizing this enzyme requirement and using an electrochemical method for measuring oxygen tension (*3*), a "reagentless" determination of glucose is achieved.

The preparation and characterization of the immobilized enzyme material used in these studies has been reported (*2*). In the enzyme immobilization procedure, the enzyme is entrapped in a gel matrix by photocatalytic polymerization of acrylamide and *N,N*-methylenebisacrylamide. The copolymeriza-

tion reaction is catalyzed by riboflavin and potassium persulfate at room temperature. The gel material is then mechanically fragmented, lyophilized, and sieved to give a uniform particle size between 20 and 40 mesh. The pore size and other physical properties of the polyacrylamide gel particles is a function of the concentration of the monomer (acrylamide) and the cross-linking agent (*N,N*-methylenebisacrylamide) used in the copolymerization reaction and has been studied in detail as reported elsewhere (*2*, *4*).

Enzyme gel particles with initial concentrations of 750 mg and 80 mg of purified glucose oxidase (Fermco, Chicago, Ill., 135,000 units/gram) per 100 ml of acrylamide gel were prepared. The apparatus used in these experiments is illustrated in Fig. 1. Glucose oxidase gel particles were placed in a miniature chromatographic column made from a 4-cm length of capillary tubing 1.5 mm in diameter. A nylon net cemented with epoxy resin to the downstream end of the capillary tube holds the immobilized enzyme gel particles in the flowing stream. Samples were pumped through the column at 0.4 ml/min with a peristaltic pump. An automatic sample turntable changed the samples at the rate of 20 per hour. An oxygen electrode (Beckman No. 325812) was used with a recording electrometer (Heath Co., Benton Harbor, Mich.) to monitor the oxygen content of the enzyme gel effluent stream.

In Fig. 2 oxygen tension is plotted as a function of glucose concentration. Glucose solutions were made up in 0.1*M* phosphate buffer, *p*H 7.4, and allowed to equilibrate with the oxygen tension of room air before being placed in the sample cup. The various response curves in Fig. 2 were obtained with capillary columns packed with from 8 to 100 enzyme gel particles (750 mg of glucose oxidase per 100 ml of gel). Each glucose response curve was initially linear with respect to oxygen tension up to 40 mg/100 ml of glucose. Increasing the number of enzyme gel particles in the column does not extend the linear range but does produce a greater change in oxygen tension for a given change in glucose concentration. However, if the amount of enzyme in the gel is decreased, then the linear portion of the response curve is extended. The data from experiments using particles of different intragel enzyme concentrations are presented in Fig. 3.

The data in Figs. 2 and 3 describe

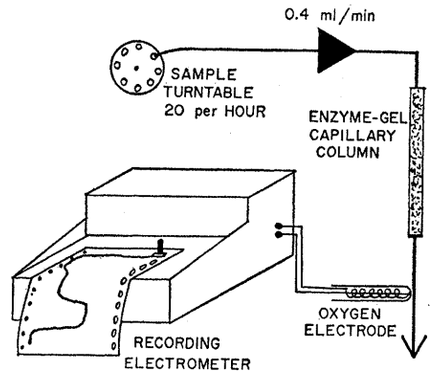

Fig. 1. Instrumentation system.

the parameters which affect the useful range and sensitivity of a continuous analysis system based upon immobilized enzyme activity. When given the change in oxygen tension and the flow rate of the system, then oxygen consumption can be calculated. Within the limits of excessive oxygen consumption which would affect the rate of the immobilized enzyme reaction, increased sensitivity can be obtained by placing more enzyme gel particles into the capillary column.

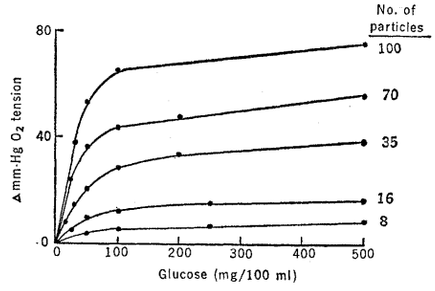

Fig. 2. The dependence of oxygen uptake on the number of gel particles with varying glucose concentrations.

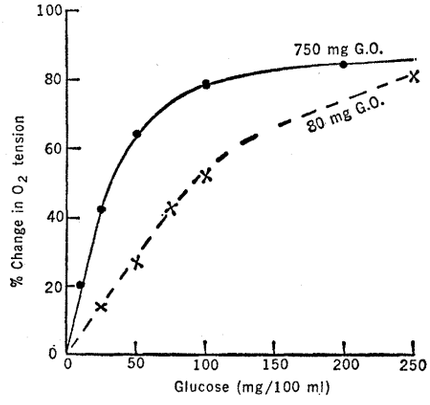

Fig. 3. The dependence of oxygen uptake on the enzyme concentration in the gel with varying glucose concentrations. The percent of change in oxygen tension is relative to 500 mg of glucose per 100 ml.

The extent of the linear range, on the other hand, is dependent upon the characteristics of the enzyme gel material and cannot be extended by increasing or decreasing the number of gel particles in the column. To change the linear range, the amount of enzyme contained in the enzyme gel material must be changed. These data suggest that at sufficiently high enzyme gel concentrations all of the substrate which diffuses into the gel is consumed and the rate of reaction is diffusion limited. As the amount of enzyme in the gel is decreased, so that all of the glucose which diffuses into the gel is not consumed by the reaction system, the rate of reaction is also limited by the enzyme in the gel, and the linear range of the analysis system is extended. Thus, an apparent Michaelis constant for an enzyme gel material (see *2*) which depends upon the concentration of the enzyme in the gel matrix can be established.

Using immobilized enzyme activity for continuous chemical analysis affords several advantages. The primary reagent requirement, the enzyme, is not consumed during the substrate analysis, and in this sense is eliminated as a reagent requirement. By the use of an oxygen electrode as the sensing device, a "reagentless" analyzer is achieved. The hydrophobic membrane (Teflon) overlying the platinum cathode of the Clark-type oxygen electrode gives this sensing device a remarkable specificity for oxygen despite the presence of numerous other electroactive substances in biological fluids. Also, by entrapment in a gel the enzyme reagent is theoretically protected from interferences of high molecular weight, such as proteolytic enzymes that may be present in the biological solution for analysis. Finally, when enzymatic analysis requires the separation of substrate from enzyme after a given incubation time, then this separation is easily automated if the enzyme reagent is immobilized in a flowing stream configuration of analysis. A modification of this method aimed at eliminating the dependence on a flowing stream configuration of analysis has been reported (*5*). This method gives results on whole blood and plasma. These results are comparable, but average about 5 percent less than results obtained by the use of the Autoanalyzer ferricyanide method.

Miniaturization and further simplification of the instrumentation for continuous analysis should facilitate in vivo

analysis. It is hoped that an awareness of the advantages of immobilized enzyme activity as an analytical tool will lead to applications in clinical investigations or wherever there is a need for miniaturized continuous chemical analyzers.

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Regeneration of Rat Liver: Transfer of Humoral Agent by Cross Circulation

Abstract. *Carotid-to-jugular cross circulation between partially hepatectomized and normal rats, via polyethylene cannulas, stimulated incorporation of ^{14}C-thymidine into hepatic DNA in the normal partners when it was maintained for 19 hours at a flow rate of about 2 milliliters per minute. Cross circulation for 7 hours or less was ineffective.*

When part of a rat's liver is excised, the remaining hepatocytes proliferate until the original mass of the liver is regained. The signal for this response has been variously proposed to be overloading of the excretory function of the liver, changes in liver hemodynamics, or changes in the concentration of a hypothetical blood-borne ("humoral") agent that regulates growth of the liver (*1*). As a result of reports that partial hepatectomy in one member of a parabiotically united pair of rats stimulated mitosis in the normal partner, numerous attempts were made to stimulate liver growth by injecting normal rats with blood, serum, plasma, or liver preparations from partially hepatectomized donors, or to inhibit regeneration by injecting partially hepatectomized rats with similar materials from normal donors (*1*). Reports of success generally eluded confirmation, however.

The recent demonstrations (*2*) that partial hepatectomy stimulates mitosis and DNA synthesis in liver autografts remote from the site of hepatectomy have reinvigorated the search for a humoral agent. Believing that such an agent might be metabolized so rapidly that only its prolonged and continuous introduction into the test animal would demonstrate it, we reinvestigated the problem with cross circulation at high rates of flow between normal and partially hepatectomized rats.

We used Charles River COBS female rats, 37 to 42 days old, weighing 112 to 160 g, denied food from the start of the experiments, and kept in restraining cages during cross circulation. Fractions of liver excised were 34 percent (left lateral lobe) (*3*), 68 percent (median plus left lateral lobe) (*3*), or about 85 percent (all the liver except the caudate lobe and about one-fifth of the right lateral lobe). Cross circulation was started within 90 minutes of hepatectomy except that the 85-percent hepatectomized rats were permitted about 2 hours to recuperate. Rats were selected such that the partner to be hepatectomized was slightly heavier than the normal partner.

Blood circulated from the left carotid artery of each rat to the right jugular vein of its partner. The arterial cannula consisted of 2 cm of PE-50 polyethylene tubing (Clay-Adams; internal diameter, 0.058 cm) inserted into the artery; it led into 1.5 cm of Vivosil tubing (Becton-Dickinson; internal diameter, 0.064 cm; used for flexibility) which in turn was connected to another 6.5 cm of PE-50. The venous cannula was a 10-cm length of PE-50 tubing. The cannulas were filled with heparinized saline (100 units per milliliter) before insertion, and an additional 0.35 ml of heparinized saline was injected through the arterial cannula after its insertion. Each rat received 7 ml of isotonic saline subcutaneously before cannulation, and 1.5 to 2.0 ml of saline through the venous cannula immediately before cross circulation was established by joining the arterial and venous cannulas of the partners with 15-cm lengths of PE-90 polyethylene tubing (internal diameter, 0.086 cm). This circuit permits a flow rate of approximately 2 to 2.5 ml/min. At the end of the cross circulation, we checked the flow by placing the partners on a double-pan trip balance and timing the change in weight following brief clamping of one of the cannulas. Except as noted, cross circulation was maintained for about 19 (18 to 20) hours before the partners were separated.

To study DNA synthesis, we injected thymidine-2^{14}C (0.80 μc; specific activity, 30 μc/μmole) into the tail veins of the separated animals 20 hours after operation in the hepatectomized partner and 20.5 hours after the start of cross circulation in the normal partner; we killed the rats 1 hour later. DNA was extracted from the livers with hot 5 percent trichloroacetic acid (*4*), and the extract was assayed for DNA content by the diphenylamine procedure (*5*) and for radioactivity in a Packard Tri-Carb scintillation counter, model 314 EX.

Table 1 shows that labeling of hepatic DNA remains at a low level in normal rats when they are cross-circulated with each other, but rises appreciably in normal rats cross-circulated with 68- or 85-percent hepatectomized partners. The effect is dose-related; labeling is not increased in partners of 34-percent hepatectomized rats compared with members of normal pairs, whereas it increases threefold in partners of 68-percent hepatectomized rats and more than sevenfold in partners of 85-percent hepatectomized rats. The latter increase is in the range obtainable in single rats by excision of 30 to 40 percent of the liver (*3*).

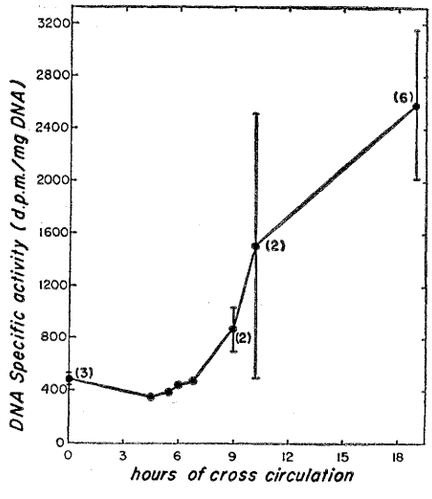

Fig. 1. Specific activity of hepatic DNA from normal members of 85 percent hepatectomized–normal pairs as a function of the duration of cross circulation. Rats were killed 1 hour after intravenous injection of thymidine-2-^{14}C in separated animals 20.5 hours after the start of cross circulation. Hepatectomies were done 2 hours before cross circulation began, except for the 4.5- and 5.5-hour points on the graph, for which operation preceded cross circulation by 7.5 and 6.5 hours, respectively (see text). For points covering more than one rat, vertical bars show standard errors of the means; numbers of rats appear in parentheses.