podermic syringes in sizes ranging from 0.1–5.0 cc. For the insertion of various sizes of pipets, the rubber grommet of a Caulfield pipetting bulb is used. The plunger of the syringe is fastened to a long nut that is keyed against turning in the aluminum tube. A finely threaded lead screw engages the nut to impart the plunger motion. The lead screw, in turn, is rotated by a synchro motor that is housed in the large-diameter portion of the instrument and is electrically connected to a synchro generator (pipettor control) located next to the control box. Synchro instruments of the No. 3 frame size develop sufficient torque for this application.

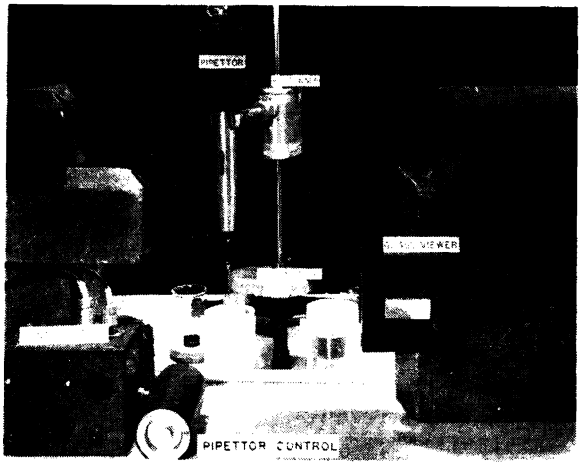

FIG. 2. A remotely controlled pipetting apparatus for the dispensing, diluting, and sampling of radioactive solutions.

These synchro instruments, which are sometimes available at low cost through electronic surplus houses, are not motors or generators in the usual sense. Connected by five wires of any reasonable length and excited with 110 v, ac, the motors of the two instruments follow each other's motions as if connected by flexible shafting. Turning the knob of the pipettor control in this application, thereby turns the lead screw of the pipettor an equal amount and can control the plunger position to within 0.0002 in. A small window is cut through the side of the tube, through which the gross motion of the plunger may be observed. When the plunger "bottoms" in the syringe the resistance to continued rotation is readily perceived at the control knob.

Figure 2 shows the pipettor,[2] rod runner, and rotating ringstand behind a lead shield in a typical laboratory experiment in which an experimental injection solution is being prepared from a "hot" source such as the stock isotopes received from Oak Ridge.

The 360° rotation of the rotating ringstand, the vertical motion of the rod runner, and the automatic pipettor provide all the necessary motions needed for the dispensing, diluting, and sampling of radioactive solutions. The accuracy of the delivered volumes with this apparatus compares favorably with that obtained by hand pipetting with the same pipets. Although the function of this apparatus has been described primarily for pipetting radioactive solutions, it can be advantageously applied where more complete manipulative facilities are not available, or where the required motions are sufficiently simple and repetitive to make its use desirable.

[2] The authors wish to thank Atlee S. Tracy for photographing this apparatus.

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The Effect of Progesterone on Rate of Phosphate Release from Adenosine Triphosphate by Rat Liver Homogenates[1]

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In a previous paper we have described the variation of phosphate release from adenosine triphosphate (ATP) by homogenates of human endometrium during the menstrual cycle (*1*). The ATP phosphate-releasing enzymes present in the endometrium show an increase of about 50% in activity from the fourteenth to the twenty-second day of the normal cycle, dropping off slightly in activity thereafter. Since the urinary pregnanediol curve has a peak at this same time, it was of interest to investigate further the influence of progesterone on the enzymes of the adenylic acid system. Rat liver homogenates were chosen as a source of the enzymes, as the alcohol used to dissolve the progesterone proved to inactivate partially the endometrial enzyme but had no detectable effect on the rate of ATP dephosphorylation by the liver homogenate. It is of course realized that the rat liver enzyme might be quite different from the human endometrial enzyme, but the results are nevertheless considered of sufficient intrinsic interest to record.

Methods. For the determination of the rate of phosphate release from ATP the method resembles that of DuBois and Potter for ATPase (*2*) except for the volumes employed. One-half molar barbituate buffer (*3*), 0.04 *M* calcium chloride, and distilled water were pipetted into a small beaker in the ratio of 3:1:2, respectively. When progesterone was to be added, it was first dissolved in absolute ethanol, and this solu-

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[2] The authors acknowledge with thanks the courteous assistance of Dr. Leslie Hellerman.

tion was then substituted for part of the water so that the final concentration was 10% ethanol. Control studies showed the ethanol had no effect on the amount of phosphate released from ATP in rat liver. The pH was adjusted to 7.4, and 0.9 ml of this solution was pipetted into each tube with 0.45 ml of 0.015 *M* ATP (pH 7.4) and 0.6 of a 1% rat liver homogenate. Incubation time was 15 min at 37° C. The reaction was stopped by the addition of 0.3 ml of 50% trichloroacetic acid. The tubes were placed in the water for a few minutes and then centrifuged; 0.5 ml of the supernatant was used for inorganic phosphate determination by the Fiske and SubbaRow method (*4*). Tissue, reagent, and substrate blanks were made with all experiments, and the results subtracted from the experimental values.

The ATP used in the experiments was obtained from Schwartz laboratories in New York. It was stored in a desiccator in a refrigerator and separately weighed for each experiment. When the acid ATP preparations were fresh, increasing the concentration above 34.9×10^{-4} *M* had no effect on the rate at which phosphate was released. However, after storage for as little as 6 weeks, but mostly longer, the rate at which phosphate was released in 69×10^{-4} *M* ATP was sometimes irregularly greater. Also ethanol had no effect on newly ordered preparations but decreased the releasing rate of the older preparations considerably. We do not know the cause for this change. In this paper fresh acid ATP was used.

Rats from an inbred hooded strain from the Johns Hopkins Hospital were used in the experiments. No differences were found in the animals, although year-old female animals were usually chosen. Rats were killed by a blow on the head, and the tissue was frozen in isopentane cooled by liquid nitrogen. Tissues were stored at −30° C until they were to be used. Experiments showed that no ATP phosphate-releasing activity was lost during such storage. At the initiation of each experiment a sample of the liver was weighed on a torsion balance, and a 1% homogenate made with distilled water.

Experiments. When progesterone was added to the incubation medium in the above manner, the rate of phosphate released from ATP by rat liver increased up to 65%, the increase depending upon experimental conditions (Fig. 1). The question immediately arises as to whether the increase is a direct effect, such as an enzyme activator, or the result of other reactions in which ATP participates in the metabolism of the hormone. In attempting the resolve this problem, several experiments were carried out as detailed below.

Variation of progesterone concentration. A series of experiments measuring the increase in the rate of phosphate release at various incubation times and progesterone concentrations was carried out. One of these experiments, representative of a group, is presented in Fig. 1. From these data it can be seen that the amount of "activation" with progesterone depends on the quantity of the hormone in solution. Ethanol

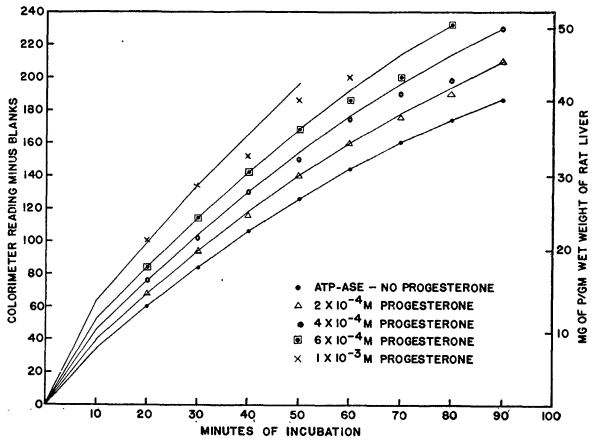

FIG. 1. Milligrams P/g liver in relation to time for various concentrations of progesterone. ATP 34.5×10^{-4} *M*; homogenate, 0.3%; pH, 7.4.

control samples corresponded with the experimental values for ATP phosphate-releasing activity without added progesterone. The solubility of progesterone in 10% ethanol is less than 2×10^{-4} *M*. In some experiments, crystals of progesterone could be seen forming in the tubes before addition of homogenate, and if allowed to come to equilibrium, no increase in the rate at which phosphate was released was found. The crystallization of progesterone at lower concentration does not occur after the homogenate is added. Therefore, the time required for adding the homogenate to each tube is critical. Because of this difficulty with progesterone solubility, higher concentrations than reported were not satisfactory. Therefore, the point of highest augmentation could not be experimentally determined. Because of solubility difficulties it is believed that the points of 1×10^{-3} *M* progesterone observed in Figs. 1 and 6 are irregular.

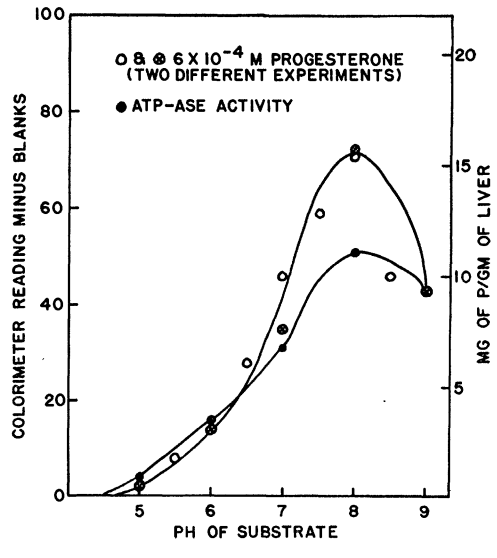

FIG. 2. Milligrams P/g liver in relation to pH with and without added progesterone. Time, 15 min; ATP 34.5×10^{-4} *M*; homogenate, 0.3%; progesterone 6×10^{-4} *M*.

Stability and inactivation. It is known that ATP phosphate release is stable in ice water for a number of hours, and heating the homogenate to 60° C for 15 min completely destroys the enzyme activity. The progesterone effect proved to be stable in ice water also, and when the enzyme activity was destroyed by heat, no progesterone effect could be noted.

Variation of pH. Data illustrating the findings of pH variation are presented in Fig. 2. The optimum pH for both ATP phosphate-releasing activity and the progesterone augmentation was pH 8. If reference is made to Fig. 1 for values of ATP phosphate-releasing activity between pH 7 and 8.5, it will be found that the rate increase by progesterone depends upon the ATP phosphate-releasing activity and is the same whether the ATP phosphate-releasing activity is limited by time or pH.

TABLE 1

EFFECT OF CALCIUM AND MAGNESIUM IONS ON PROGESTERONE AUGMENTATION OF PHOSPHATE RELEASE FROM ATP
(colorimeter readings)

Incubation medium	No added ions	Calcium		Magnesium	
		3×10^{-3}	6×10^{-3}	1.5×10^{-3}	3×10^{-3}
1. Barbiturate buffer 34.5×10^{-4} *M* ATP	56	27	21	62	46
2. Barbiturate buffer 10% ethanol 34.5×10^{-4} *M* ATP	58	34	25	62	50
3. Barbiturate buffer 10% ethanol 6×10^{-4} *M* progesterone 34.5×10^{-4} *M* ATP	81	57	31	82	58

Effect of calcium and magnesium ions. There has been much discussion in the literature concerning the effect of calcium and magnesium ions on ATPase (*5, 6*). Although calcium is a well-known activator of myosin ATPase, investigators have reported quite different results with the liver enzyme. DuBois and Potter (*2*) report an activation by both calcium and magnesium ions at 3×10^{-3} *M*. Our experiments indicate both to be inhibitory at this concentration. Swanson found the discrepant effect to depend on the ATP preparations used (*7*). All experiments reported here were done with the acid form of ATP from Schwartz laboratories in New York. Although this form is not of the highest purity, it was found to be satisfactory in this work, provided fresh samples were used. The detailed data of a single experiment with calcium, magnesium, and progesterone are noted in Table 1. These values are expressed as colorimeter readings, but the actual amount of phosphate released can be found by referring to Fig. 1. If the colorimeter readings in Table 1 for barbiturate buffer and ATP (row 1) are referred to the ATPase–no progesterone curve of Fig. 1, and the activity is found with reference to

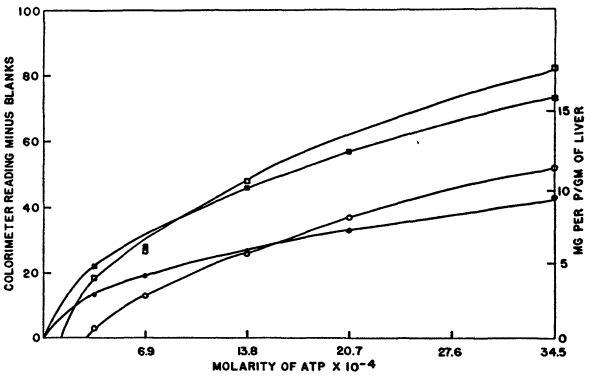

FIG. 3. Milligrams P/g liver in relation to molarity of ATP for 2 homogenate concentrations with and without added progesterone. Time, 15 min; pH, 7.4; ○ and □ progesterone 2×10^{-4} *M*; ● 0.3% homogenate; ■ 0.6% homogenate.

time, the progesterone augmentation of the rate is approximately the same for both experiments at 6×10^{-4} *M* progesterone (row 3, Table 1). Therefore, the data indicate that, regardless of whether the activity of the enzyme is controlled by calcium or magnesium, or pH, or time, the augmentation in rate depends upon the level of enzyme activity without progesterone.

ADP and AMP as substrates. In an effort to establish whether the influence of progesterone is specific for ATP, ADP, and AMP were substituted as substrates, using the same conditions. The 10% ethanol necessary for dissolving the progesterone reduced ADPase activity by more than 50% but had no effect on 5-nucleotidase. Progesterone had no detectable effect on the phosphate-releasing rate by either of the 2 enzymes.

ATP concentration. For further evidence as to the mechanism by which progesterone catalayzes the rate of phosphate released from ATP, experiments in which this substrate was a limiting factor were performed. At low ATP concentrations and 2×10^{-4} *M* progesterone, the hormone appeared to inhibit, rather

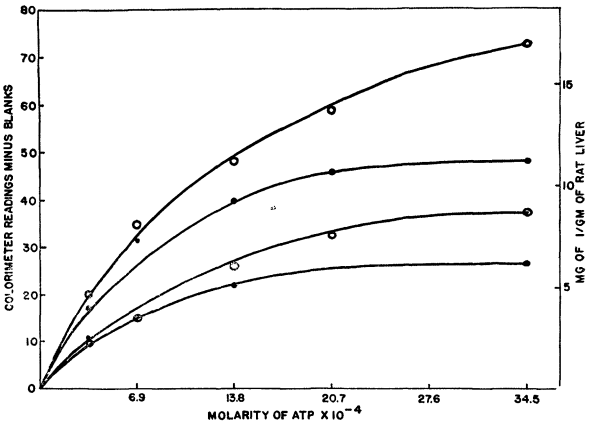

FIG. 4. Milligrams P/g liver in relation to molarity of ATP for 2 homogenate concentrations with and without added progesterone. Time, 15 min; pH, 7.4; ○ progesterone 6×10^{-4} *M*; ● 0.15% and 0.3% homogenate.

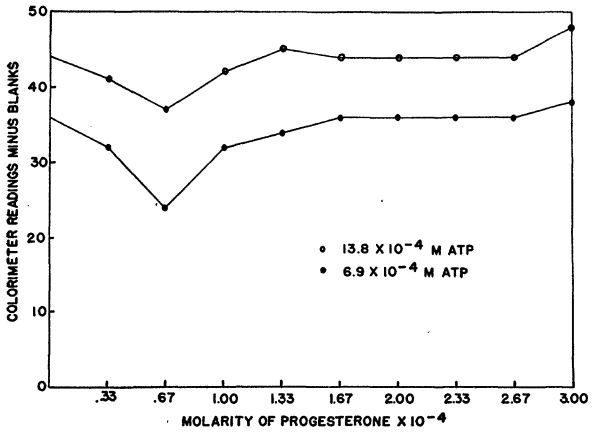

FIG. 5. Milligrams P/g liver in relation to molarity of progesterone for two ATP concentrations. Time, 15 min; pH, 7.4; homogenate, 0.03%.

than augment, the rate of the reaction. The point where no net effect was observed depended on the concentration of the homogenate. Data illustrating these findings are summarized in Fig. 3 at two different enzyme concentrations. However, when the progesterone concentration was increased to 6×10^{-4} *M* no inhibitory effect was found (Fig. 4). A third experiment in which a limiting amount of ATP was held constant and progesterone concentration was varied further supported the inhibiting effect of progesterone at low concentration. As the amount of progesterone was increased, the inhibiting effect was overcome, and an augmentation was noted at higher concentrations (Fig. 5).

Discussion. Under conditions of low substrate concentration, progesterone reduces rather than accelerates the amount of phosphate released. The amount of reduction is dependent not only upon the ATP concentration but also on the amount of enzyme present; thus 0.15% rat liver homogenate shows a much greater deceleration effect with progesterone than does 0.30% homogenate under the same conditions. If the same experiment is repeated at a higher progesterone concentration, 6×10^{-4} *M*, no reduction in the amount of phosphate released is observed (Fig. 4). The net progesterone effect is that of an activation of phosphate release (Fig. 6). It may, therefore, be concluded that the influence on phosphate release from ATP by progesterone is dependent upon the relative concentrations of ATP, progesterone, and enzyme in such a way that there must be more than a minimum concentration of any one of these 3 variables to cause a net acceleration.

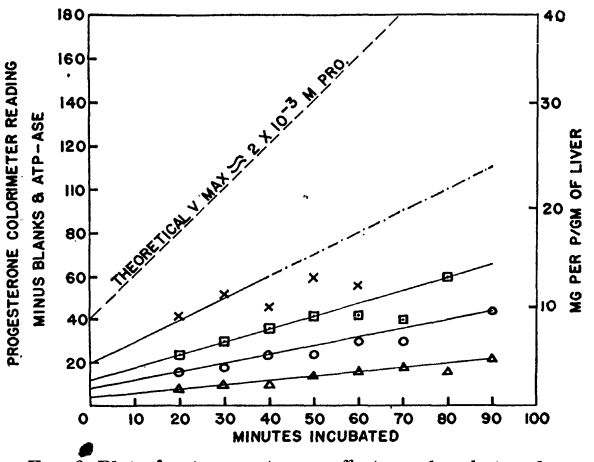

FIG. 6. Plot of net progesterone effect on phosphate release from ATP according to time.

These data lead us to believe that, although progesterone and ATPase of rat liver have a direct relationship, it is not simply that of an enzyme and its activator. This theory is further supported by the fact that, if in Fig. 1 the experimental ATP phosphate-release values are subtracted from those of progesterone, a series of straight lines results (Fig. 6). The Michaelis-Menten equation then becomes applicable.

$$\frac{V_{max}(S)}{V = K_s + (S)}$$

or

$$1/V = 1/V_{max}\ K_s/V_{max}\ 1/(S) \qquad (1)$$

V is the overall velocity of the enzymatic reaction; V_{max}, the maximum value of this velocity; (S), the substrate concentration (progesterone); and K_s the dissociation constant of the enzyme-substrate complex. It will be noted that Eq. (1) is a straight line in which $1/V_{max}$ is the y intercept and K_s/V_{max} is the slope of the line formed when $1/V$ is plotted against $1/S$. In this way the maximum velocity may be calculated. This velocity is found to be approximately 2×10^{-3} *M* progesterone.

The effect of progesterone may be represented by the following scheme:

$$\begin{array}{ccc} ADP + PO_4 + Pr(Pr') & & ADP + E + PO_4 \\ \Downarrow\Uparrow (5) & & (2) \Downarrow\Uparrow \\ ATP \cdot Pr \cdot E & \overset{Pr}{\rightleftharpoons} & ATP + E \rightleftharpoons ATP \cdot E \\ \Downarrow\Uparrow (6) & (4) & (3) \Downarrow\Uparrow \quad (1) \\ AMP + E + 2PO_4 + Pr(Pr') & & AMP + E + 2PO_4 \end{array}$$

Our data indicate that the net velocity of the reactions by pathway (4) is more rapid than by (1), provided progesterone, ATP, and enzyme are present in sufficient concentration. However, with limited concentration of these 3 components, the combined net velocities of (4) and (5) or (4) and (6) are less than (1) and (2) or (1) and (3). This may be accounted for by assuming that there is a greater affinity for E for the ATP · Pr complex than for ATP alone, and that there is an appreciable accumulation of ATP · Pr · E.

It is to be noted that in this scheme alternate pathways: (2) and (5) on one hand, and (3) and (6) on the other, are suggested. Novikoff, Hecht, Podber, and Ryan (*5*) recently indicated that the enzyme in question is probably ATPase, but, in view of the possibility of an apyrase, the alternate pathway is shown.

It is further indicated that progesterone may be obtained from the reaction in an alternate state Pr′. Preliminary studies indicate that Pr′ is water soluble, but further studies are in progress on the nature of this change.

One of the most meaningful aspects of this study may be the *in vitro* demonstration of the effect of a hormone on an enzymatic reaction at a subcellular level. It is furthermore indicated that the participation of the hormone in the enzymatic reaction results in its alteration (metabolism!). This specific example may, therefore, illustrate a concept of wide application and significance.

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A Simplified Method for Cultivation of Poliomyelitis Virus in Tissue Culture

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Roller tube tissue culture methods, as described by Enders and associates (*1, 2*) and extended by others (*3–6*), have opened new vistas in poliomyelitis research by providing techniques more economical than previous methods, requiring the use of monkeys, for poliomyelitis virus isolation, virus typing, and the assay of specific antibodies.

However, there still remain a number of practical difficulties and limitations, and foremost among them are the ingredients of the medium. Tissues such as human embryo and monkey testes either are not always readily available or are expensive. Horse serum is frequently inhibitory to virus, and chick embryo extracts are at times sources of contamination. While search is continuing for a suitable substitute for monkey testes, currently the most commonly employed tissue, we wish to report briefly on some changes in technique. These modifications permit the rotary drum to be dispensed with, and, when monkey testis tissue is used, the chick embryo extract and horse serum to be replaced by plasma hydrolyzate with rather satisfactory results.

Shortly after tissue culture study with poliomyelitis virus was instituted at this laboratory one year ago, it was observed that rotation of the tissue cultures is not essential for adequate cell or virus multiplication. If the culture tubes are kept stationary in slanted position so that the fluid just covers the tissue embedded at the lower end of the test tube, the cells as well as the virus grow very well. Other workers have had similar experience (*7–9*).

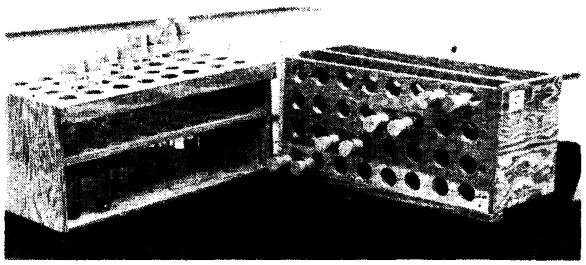

FIG. 1. Wooden racks holding tissue culture tubes in upright position or in slanted position.

Of a number of substances tested with a view toward replacement of chick embryo extract and horse serum, bovine plasma hydrolyzate[1] proved most satisfactory. Consequently, a medium incorporating this material was devised; it is designated Medium E. Medium E is rather simple, consisting of 75% Hank-Simms (Hank's 3 parts, Simms' 1 part) solution and 25% of the bovine plasma hydrolyzate. It is adjusted to pH 7.0–7.2 by the addition of sterile sodium bicarbonate solution. Recently it has been found that the Simms serum ultrafiltrate may be omitted entirely with equally good results.

The tissue cultures are prepared with fragments of monkey testicular tissue covered with 2 cc of the medium in ordinary bacteriological test tubes, 15 × 150 mm, similar to the techniques of Youngner, Ward, and Salk (*5*) and Ledinko, Riordan, and Melnick (*6*). The tubes are inserted into especially designed wooden racks, 11½ × 5½ × 5½ in., with holes for 4 rows of tubes, 8 tubes/row. When the rack is placed sidewise, a strip of wood about 1 cm thick across the upper back keeps the tubes in a slanted position at an angle of about 5° above horizontal level, and the medium just covers the tissue. When the tubes are moved from one place to another, the rack is carried in an upright position to prevent the fluid from touching the rubber stoppers (Fig. 1).

The cultures are incubated at 35° C for 5–7 days. No change of the medium is required during this period. The medium is then removed, and the virus, or other material desired to be cultured (in an amount of about 0.1 cc), is placed in contact with the cells for about 10 min, after which 2 cc of fresh medium is added. Incubation is continued and the cells are observed. With this procedure, both the fibroblasts and the virus grow well in Medium E, and, although the cells do not grow so richly and densely as those cultured in Medium A of Youngner, Ward, and Salk

[1] Obtained commercially as Travamin, a sterile 5% solution of bovine plasma hydrolyzate, w/v in water, from Baxter Laboratories, Inc., Morton Grove, Ill. Recently Melnick and Riordan (*8*) reported that lactalbumin enzymatic hydrolyzate is also satisfactory.