that heparin requires a co-factor for full activity.

We are not yet able to relate antithrombin IV to the other antithrombins. It is possible that this activity represents another separate entity. It is also possible that more than one substance is involved, and that these substances are known by other manifestations in the complex interactions of the blood clotting mechanisms.

Summary. The antithrombin capacity of plasma can be differentiated into 4 main effects. Antithrombin I is the adsorption of thrombin on fibrin. Antithrombin II is the co-factor of heparin. Antithrombin III neutralizes thrombin activity even in the absence of heparin, while antithrombin IV destroys thrombin only while it is being formed from prothrombin. Some of the properties of antithrombins II, III and IV have been investigated, together with those of the factor which acts with heparin to inhibit the activation of prothrombin, and which is referred to as the '39 factor. The 4 activities have the common property of resistance to heating at 60°C for three minutes, but are destroyed by heating at 70°C. They are not adsorbed on $BaCO_3$. They are water soluble. None of these activities is found in a commercial crystalized bovine plasma albumin. Antithrombin III activity is the only one removed by ether treatment of plasma. In ammonium sulfate fractionation antithrombin II is found predominantly in the 50-70% fraction, while antithrombin III is found only in the 0-50% fraction. It is believed that these 2 factors are two distinct entities. The relation of antithrombin IV to these 2 is not yet clear. It is possible that the '39 factor and antithrombin II are identical.

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Monolayer Tissue Cultures I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells.*† (20830)

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Recent reports by Dulbecco(1,2) on the use of monolayer tissue cultures for the study of certain animal viruses has made possible the application, in studies of animal viruses, of quantitative technics similar to those used with bacterial viruses. Dulbecco has demonstrated production of plaques of degenerated cells by Western equine encephalitis virus in monolayer chicken-fibroblast cultures(1) and similarly in monolayer monkey kidney cultures, plaques have been produced by the Brunhilde strain of Type 1 poliomyelitis virus(2).‡

Methods for treatment of kidney tissue fragments with trypsin to reduce the time required for cell outgrowth in plasma clot cul-

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tures have been reported by Simms and Stillman(3) as well as by Robbins, Weller, and Enders(4). Dulbecco(2) has described a technic for dispersing monkey kidney cells with trypsin to obtain cell suspensions for preparation of monolayer cultures. It is the purpose of this report to describe certain modifications in the methods used by Dulbecco for preparing the trypsin-dispersed kidney cell suspension. These modifications include a) use of mechanical rather than manual agitation for dispersing the cells, b) use of a different nutrient medium, and c) quantitative adjustment of the dispersed cell suspension on the basis of total cell count or optical density. The procedures described are adaptable to the production of relatively large amounts of standardized dispersed cell suspension; the use of these suspensions for preparation of different types of monolayer cultures will be described.

Materials. All solutions used in this study were sterilized by Seitz filtration under positive pressure. *Phosphate buffered saline* (PBS), pH 7.5, was prepared according to the formula used by Dulbecco(2). *Trypsin solution* was prepared from Bacto-Trypsin 1:250 (Difco Corp., Detroit, Michigan) in a concentration of 0.25% by weight using PBS as diluent. The *horse serum* used in these studies was obtained from the blood of two horses and was heated at 56°C for 30 minutes before use. *Nutrient fluids—Synthetic mixture 199* of Morgan, Morton, and Parker (5) was used as the basic constituent of all media and differed from that described in the use of Hanks' balanced salt mixture in place of Earle's salt mixture. Since Hanks suggested separate addition of different quantities of sodium bicarbonate, this was used to permit adjustment of initial pH of the medium as desired for different purposes. Medium D, used for washing and suspending dispersed cells, contained the following ingredients per 100 ml of medium: synthetic mixture 199, 2.8% $NaHCO_3$, horse serum, 95, 3 and 2 ml, respectively. Penicillin and streptomycin were added to a concentration per ml of 100 units and 0.1 mg, respectively. The pH of this medium was 7.2-7.4 and upon incubation rose to 7.8-8.0.

Methods. Preparation of trypsin-dispersed cell suspensions. Kidneys were removed from healthy 5-7 lb rhesus or cynomolgus monkeys, exsanguinated under sodium pentobarbital anesthesia. The cortical area was dissected as free as possible from medulla, and minced with scissors into pieces about 4-5 mm in diameter. Minced cortical tissue was transferred to a 250 ml centrifuge tube, washed several times with PBS to remove red blood cells, and suspended in about 20 ml of trypsin solution per kidney. After 10 minutes at 37°C, the trypsin solution was discarded, replaced with about 20 ml of fresh trypsin solution per kidney and the suspension transferred to a Waring blendor the motor of which was controlled by means of a rheostat at a speed that produced agitation of the tissue without cutting or foaming. Mixing was allowed to proceed for 10 minutes. The supernatant fluid was saved after decanting through 3 layers of sterile cheesecloth to eliminate floating tissue particles and connective tissue fibers; the retained portion contained mainly clusters of cells, but also some single cells and cell debris. Fresh trypsin solution was added to the tissue remaining in the blendor and agitation repeated. After 8-10 such extractions, the tissue was near exhaustion as far as yielding additional cell clusters. Further extraction yielded increasing amounts of connective tissue fibers which were too fine to be retained by the cheesecloth filter and could not be separated easily from cellular components. The turbid trypsinized cell suspension was centrifugated at 1000 RPM for 5 minutes; the sediment was resuspended in approximately 20 volumes of medium D and centrifugated at 600 RPM for 2 minutes. Resuspension and washing was repeated 3 times at the slow speed. Final centrifugation of the pooled cell sediment was performed in graduated 15 ml centrifuge tubes. A yield of 1.0 to 1.5 ml of packed cellular material was usually obtained from the cortex of each kidney, the weight of which was approximately 4-5 g.

Standardization of cell suspensions. Packed cell sediment was diluted with medium D to make a 1:50 dilution based upon packed cell volume. An aliquot of this suspension was

further diluted to 1:200 for purposes of standardization. Since the work was done upon which this report is based, it has been found desirable to pass the 1:50 dilution through 3 layers of cheesecloth to remove aggregates of connective tissue fibers which appear to form as the result of packing of sediment during centrifugation. The *optical density* (O.D.) of the 1:200 cell suspension was determined in the following manner. Four ml of the cell suspension was acidified with 0.3 ml of 0.2 N HCl; acidification minimized variations in light absorption by phenol red in the medium. Optical density (neg. log of transmittance) was determined in a Coleman Junior Spectrophotometer using a wavelength of 590 mμ where light absorption by acidified phenol red was least. Four ml of similarly acidified medium was used as a blank. *Total cell count* was determined by enumeration of nuclei using an adaptation of the method suggested by Sanford and her associates(6). To 2.0 ml of the 1:200 cell suspension, in a graduated 15 ml centrifuge tube, was added 10 ml of 0.1 M citric acid. After one hour of incubation at 36°C, the tube was shaken vigorously and centrifugated at 1800 RPM for 15 minutes. The supernatant fluid was discarded and 1.0 ml of 0.1% crystal violet dissolved in 0.1 M citric acid added. The pellet was resuspended by mixing vigorously with a 1.0 ml pipette and the resulting suspension allowed to stand at room temperature for 5-10 minutes. At this time, 10 ml of 0.1 M citric acid was added, the tube thoroughly shaken and the contents centrifugated at 2000 RPM for 15 minutes. All but 1.0 ml of supernatant fluid was discarded, the volume brought to 2.0 ml with 0.5% Methocel§ (viscosity 4000 CPS) and the pellet containing nuclei resuspended in this solution. Nuclear counts were made without further dilution in a haemocytometer using the white blood cell counting squares; counts were expressed as the number of nuclei per ml of 1:200 cell suspension.‖

The relationship between O.D. and the nuclei count of the 1:200 dilutions of trypsin-dispersed cells, is illustrated in Fig. 1. Points are based on data derived from 16 successive batches of kidney, each utilizing tissue from 1 to 6 monkeys.

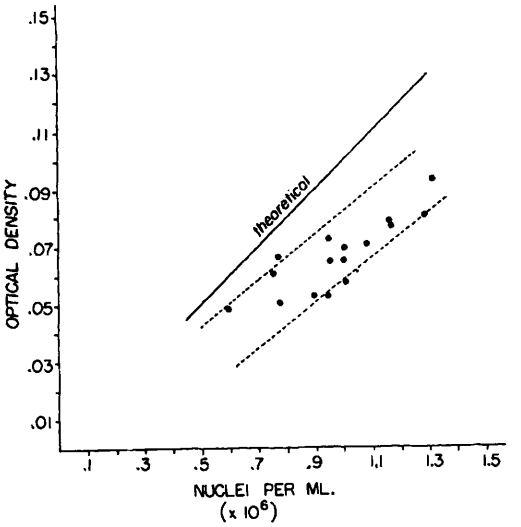

FIG. 1. Relation between optical density and nuclei/ml in 1:200 dilution of trypsin-dispersed cells.

On the assumption that there is a direct proportionality between O.D. and cell count, it is possible to envisage a straight line relationship if one were dealing with uniform suspensions of single cells. This theoretical relationship is illustrated graphically by the solid line in Fig. 1. Experimental points, plotted from data obtained with 16 preparations, all fall below the theoretical line. The most likely explanation for this observation is that cell suspensions are not composed of single cells but are made up of cell clusters of different sizes. The greater the number of cells in each cluster, the higher the total cell count in relation to the observed O.D. In addition, scattering of experimental points in Fig. 1 most likely is produced by variations in distribution of cell cluster sizes in different preparations. Data in Fig. 1 permit estimation of cell count by measuring the O.D.

§ A methylcellulose product which acts as a suspending agent, kindly furnished by Dow Chemical Company, Midland, Mich.

‖ The following formula was used to calculate number of nuclei/ml:

$$\frac{\text{Total nuclei in 4 white blood cell squares}}{4} \times 10000 = \text{nuclei/ml}$$

The 1:50 cell suspension stock is diluted with medium D to adjust to the desired number of cells per ml. Data in Fig. 1 indicate that 1:200 dilutions of trypsin-dispersed cells, adjusted on the basis of packed cell volume alone, contained between 600,000 and 1,320,000 cells per ml. It is possible to utilize the 1:200 dilution for preparation of cultures without additional standardization. However, standardization of cell content by the methods described permits utilization of more uniform cell populations as well as more economical use of cell suspension since it may contain sufficient cells to permit dilution to 1:300 or 1:400.

Maximum viability and reproducibility of results are obtained when dispersed cell suspensions contain 600,000 to 700,000 cells per ml. Such cell suspensions have been used to prepare several different kinds of cultures.

Preparation of cultures. Uniformity of suspension is maintained during distribution by the use of a magnetic stirring device together with a Cornwall automatic pipetting unit. *Petri dish (60 mm) cultures* are prepared with 4.0 ml of cell suspension containing 600,000-700,000 cells per ml, and incubated at 36°C in an atmosphere of 3% CO_2 as described by Dulbecco(2). Fluid is removed after 3 or 4 days of incubation and 4.0 ml of fresh medium D are added. After 6 or 7 days of incubation a confluent epithelial monolayer is developed.

To prepare *culture tubes*, 0.5 ml of suspension, containing approximately 300,000 cells, is added to screw-topped tubes (16 x 150 mm) with rubber inserts in the caps. These are incubated at a slight tilt from the horizontal at 36°C for 6 or 7 days without rolling; in this interval, a zone of confluent kidney epithelium covers the area of the tube in contact with cell suspension and pH of the medium has fallen to about 6.8. Between 800 and 1000 culture tubes can be prepared from cortical tissue of a single monkey by this method. Over 90% of tubes prepared in this manner are suitable for virus assay or other studies. For virus or antibody titrations medium D, containing horse serum, is not used. Instead, the medium employed is made up of 97.5 ml of mixture 199 to which is added 2.5 ml of 5% $NaHCO_3$. Cultures have also been prepared in *bottles* used normally for milk dilution tests. Each bottle receives 8.0 ml of standard cell suspension containing 600,000-700,000 cells per ml and is incubated without motion in a horizontal position for 6 or 7 days. After this interval, a confluent monolayer of kidney epithelium covers the entire flat glass surface (40 x 110 mm). Cultures can either be stoppered with rubber stoppers or with cotton plugs. In the latter case, incubation is carried out in an atmosphere of 3% CO_2. The application of this type of culture for the propagation of poliomyelitis viruses will be reported separately.

Viability of cell suspensions is retained at a usable level for at least 2 days beyond the day of preparation when kept at 4°C. For convenience, suspensions are stored as 1:50 dilutions and brought to the desired concentration with fresh medium D just before use.

Summary. An adaptation of the method of Dulbecco for preparation of trypsin-dispersed monkey kidney cell suspensions has been described. Procedures employed yield quantitatively standardized cell suspensions which can be used in the preparation of large numbers of replicate cultures.

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