in our article to carry out acute experiments on the effect of diuretics on kidney function (P. Govaerts, *C. R. de la Soc. Biol.*, 1928, v99, 647; *Arch. Internat. Pharmacodyn. et de Thérapie*, 1929, v36, 99). The technic was also employed by Brull (*C. R. de la Soc. de Biol.*, 1931, v107, 248, 249) for short-term experiments.

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Received June 27, 1957. P.S.E.B.M., 1957, v96.

Colorimetric Method for Estimating Number of Cells in Monolayer Cultures without Physiological Damage.* (23463)

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A colorimetric method for determining the number of cells in monolayer cultures of monkey kidney cells has been developed. The method depends on production of hydrogen ions by the cells and consists in measuring change in absorption of a standard physiological salt solution of phenol red after a 15-second exposure to the cultures. The procedure avoids the physiological damage usually resulting when cells are removed from the glass surface for counting and makes it possible to estimate reliably the number of cells in the particular culture which is being studied.

Studies on many thousands of cultures during the last 6 months have shown that the amount of phenol red acidified under these conditions is correlated with the number of cells in the culture over a wide range of cultivation conditions and of age. The method proved useful particularly as a method of selecting from among a large number of cultures those having the same cell number within ± 10%. The mechanism of the reaction is not completely understood, and it is therefore not possible to evaluate all of the factors which might give anomalous results. In this paper the details of the procedure and factors controlling the sensitivity of the method will be given. Some of the possible sources of error, as determined from these limited studies, will be considered.

* Aided by grant from The National Foundation for Infantile Paralysis.

Material and methods. Monolayer cultures of monkey kidney cells were grown out from freshly trypsinized rhesus monkey kidneys in either medium M + H (0.5% lactalbumin-hydrolysate, 2% calf serum, Hanks' salt solution) (1) or the completely synthetic, protein-free medium SM-2(2) in 2-3 oz Brockway prescription bottles. *Phenol red* was the commercial LaMotte soluble product used either as obtained or after recrystallization from dilute alcohol. *Cell counts* were done by a modification of the Parker(3) method for counting nuclei. The modifications consist in using small volumes of citric acid to obviate the need to concentrate the cells by centrifugation and in adding the crystal violet within at least 5 minutes after the citric acid in order to stop the continued swelling and bursting of the nuclei. The exact procedure is as follows: 1) The supernatant fluids from the cultures are decanted, the bottles drained, and the cell layer covered with 1% tannic acid for 1½-2 minutes. 2) The tannic acid is decanted, the bottles drained on adsorbent paper, and exactly 2 ml of 10% citric acid is added to the cell layer. 3) After about a minute in contact with the citric acid, the bottles are shaken vigorously for 10-15 seconds. The time required to remove the cells from the glass and the amount of shaking depends on the age of the cells and whether they have been grown in a serum-containing or protein-free medium. With both cell types the older cultures (more than 5 days) are removed easily in 15-30 seconds, whereas younger cul-

tures may take as much as 5 minutes with vigorous shaking. Three- to 5-day-old cultures grown in synthetic medium may take as much as 5 minutes with vigorous shaking. 4) The nuclei are stained by adding 0.5 ml of 0.1% crystal violet in 0.1 M citric acid (4). *Chemical determinations.* Total endogenous polysaccharide was determined with the anthrone reagent as modified by Fales(5). The monolayers were first collected by solubilization in 0.2 N NaOH for a minute. *Spectrophotometric determinations* were made using the Beckmann Model DU spectrophotometer. *Temperature* in all of these experiments was the room temperature of 24-25°C.

Preliminary studies. Physiological salt solution. These studies were done using a physiological salt solution similar to both Hanks and Earles, containing NaCl 8.0 g, KCl 0.4 g, $MgSO_4 \cdot 7\ H_2O$ 0.2, $CaCl_2$ 0.14, and Na_2HPO_4 0.426 g, per liter of demineralized distilled water. The combined solution has a variable pH which declines on storage, due to the slow supersaturation with CO_2. To be assured of the same pH, it is necessary to make up the medium as follows: The NaCl, KCl, and $MgSO_4$ are made at 10 times concentrated combined stock. The $CaCl_2$ is kept at a separate 10% solution. A quantitative 0.30 M solution of Na_2HPO_4 made from the commercial anhydrous form which is kept in a desiccator is made and distributed to test tubes which are tightly stoppered. Each time the physiological salt solution is constituted, a previously unopened tube of dibasic phosphate is used. The final solution combined from the stocks diluted with distilled demineralized water equilibrated at room temperature (24-25°C) has a pH of 8.05 ± .05 as measured by the Beckmann Model H-2 pH meter. *Phenol red* is a weak acid which dissociates in the pH range 6.8 to 8.2. The peak of maximum absorption in the visible range of the spectrum in the physiological salt solution at alkaline pH is at 560-562 mμ. On acidification, the absorption at this region declines, with the appearance of an absorption band between 400 and 460 mμ. The absorption at 484 mμ is invariant with pH. It has been found that phenol red obeys Beers' law within the range of concentrations which can

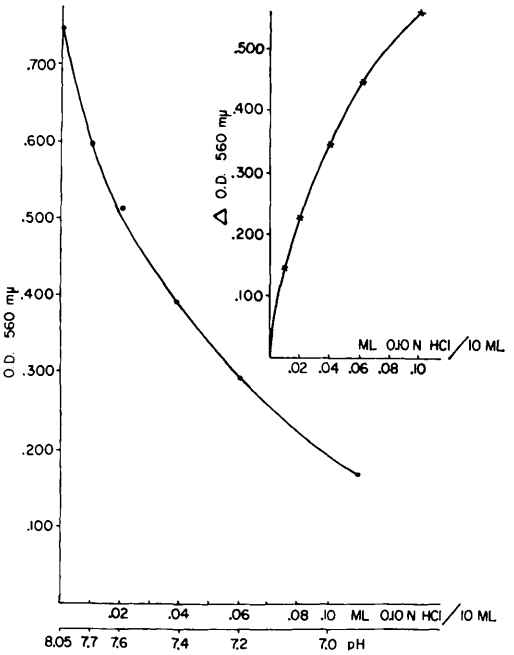

FIG. 1. Absorption of standard phenol red solution at 560 mμ between pH 8.0 and 7.0.

FIG. 1a. Change in absorption (delta o.d.) of standard phenol red solution as a function of amount of acid (Δ H^+) added to 10 ml of the salt solution.

be measured directly. It has also been found that when working at the level of 6-8 g per liter of NaCl, as in physiological salt solutions, the extinction is not affected by changes in the concentration of the common ions.

The change in adsorption at 560 mμ on addition of measured quantities of 0.10 N HCl is shown in Fig. 1. It is clear that the change in absorption (delta o.d.) for a given quantity of acid (delta H^+) is constantly declining in the pH range of 8-7. This relationship is seen more clearly in Fig. 1a where the delta o.d. has been plotted against delta H^+. These data mean that when phenol red is used to measure a change in H^+, the change in absorption must be calibrated by titration with a standard acid, in order to eliminate differences in absolute quantities of phenol red, the amount of true titratable phenol red in the preparation, and to a lesser extent the sensitivity of the spectrophotometer used to measure delta o.d. Since the absorption peak at 560 mμ is very sharp, the band width of the light incident on the sample will influence

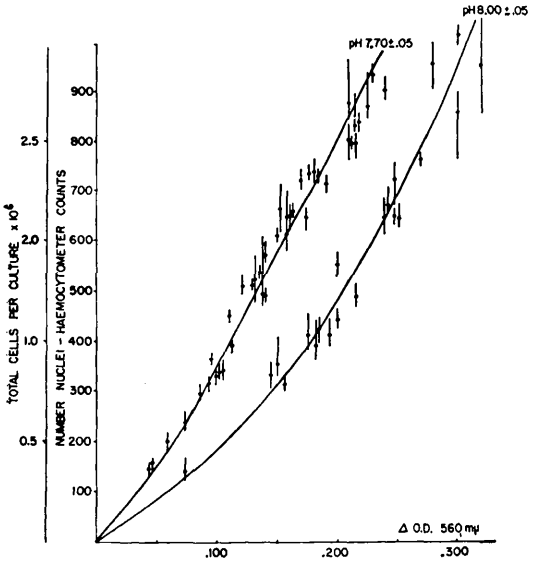

FIG. 2. Relationship between phenol red index and cell number in monolayer cultures of monkey kidney cells. Mean avg nuclei count plotted against change in absorption (Δ o.d.) at 560 mμ during a 15-sec. exposure of the cultures to standard phenol red solution containing 3×10^{-3} M Na_2HPO_4 at pH 8.05 or adjusted to pH 7.70 with standard acid. Total fluctuation in nuclei counts (3-4 samples) indicated by bars.

the magnitude of the delta o.d. with the same delta H^+.

Procedure to determine cell number. The method depends on determining amount of hydrogen ion produced by monkey kidney cells, by determining the change in absorption at 560 mμ of a standard phenol red solution. The procedure is as follows: The medium from the culture to be titrated is decanted and the bottles drained 10-15 seconds. The residual culture medium is removed by rinsing the bottles 2 times with 10-15 ml portions of the physiological salt solution and the bottles drained. The bottle is placed so that the standard phenol red solution can be pipetted directly onto the cell layer. Using a stop watch timed from the second that the solution touches the cell layer, 3.0 ml of the standard solution is added to the bottle. After 15 seconds, the fluid is decanted and absorption at 560 mμ is determined. The difference in absorption between the standard solution and the effluent solution (phenol red index) is determined.

The phenol red index was determined on more than a hundred monolayer cultures of monkey kidney cells and compared with cell number as determined by counting nuclei of the same cultures. The cultures had been grown out in 2 different media, a serum containing medium, and the completely synthetic medium SM-2, and were studied between the 3rd and 35th day of *in vitro* life. Several cultures which had degenerated because the medium had not been changed were intentionally included. The data for these calibration curves have been presented in Fig. 2. It is seen that the change in absorption is correlated within $\pm$ 10% with the number of cells (nuclei) in the cultures.

The pH of the effluent solution as determined by the Beckmann Model H-2 pH meter and the pH expected from the change in o.d. (Fig. 1) agreed within the limits of measurement with this meter ($\pm$ 0.1 pH unit). The change in absorption therefore can be ascribed primarily to production of hydrogen ions. The calibration curves show that the sensitivity of the method is greater with the pH of the standard solution at 8.0 than at 7.7. This agrees with the titration curve of the phenol red solution (Fig. 1a). The calibration curves also show that the delta o.d. is not a straight line function of cell number, but as would be expected from Fig. 1a, decreases with increasing cell number. Since the method is actually measuring delta H^+ as a result of cell number, it is useful to plot delta H^+ as a function of cell number. This may be done (Fig. 3) by determining the delta H^+ from the delta o.d. (Fig. 1a) for a given cell number (Fig. 2). It is seen that the amount of hydrogen ion produced is a linear function of cell number. This finding indicates that between pH 8.0 and pH 7.2, the range indicated by maximum delta o.d., the mechanisms involved in the production of hydrogen ion by the cells are independent of pH of the medium.

The phenol red index is now determined routinely on all cultures grown out from the trypsinization fluids in order to select cultures having the same cell number within a desired range for further study. The manipulations involved, including timing of the rinses,

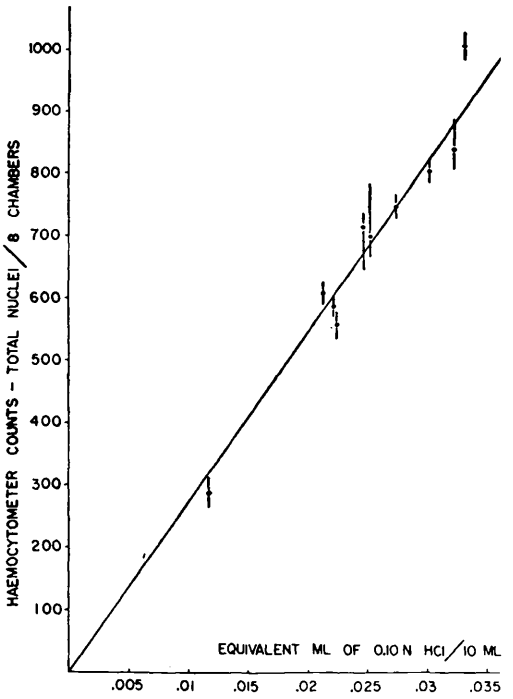

FIG. 3. Amount of H^+ produced as a function of number of cells in monolayer monkey kidney cultures. pH of the standard solution was varied between pH 7.4 and 8.0 ± .05.

amount of fluid used to rinse or pH of the rinse solution between 5.7 and 8.0 have not been found to affect the phenol red index. One obvious difficulty when large numbers of cultures are studied is the change in pH of the standard phenol red due to diffusion of CO_2 from the air. This may be obviated simply by covering the solution with mineral oil (Dracol) and using a dispensing flask with an outlet at the bottom attached to an automatic Cornwall pipetting outfit. The connection to the pipetting outfit should be of glass except for the minimum amount of gum rubber needed for manipulation. In experiments where a large number of bottles are being screened, time of exposure has been reduced to 10 seconds, which is within the time error of manipulations when using the automatic pipetting outfit. When screening a large number of cultures it was also useful to use a spectrophotometer not requiring special cuvettes. We have used both the Bausch and Lomb Spectronic 20, and the Beckmann Model C with a Corning glass 560 mμ filter and matched ordinary test tubes. With these instruments the accuracy is somewhat less than with the Beckmann model DU and is about ± 15%.

Standardization of phenol red solution. It has been shown that the amount of hydrogen ion produced by monolayer cultures of monkey kidney cells is a function of the number of cells in the culture. The amount of hydrogen ion may be determined, as was done above directly from the delta o.d. of the standard phenol red solutions, provided two criteria are met. 1) The standard solution must have the same pH, since the delta o.d. with delta H^+ varies with pH (Fig. 1). A method has already been given for obtaining a standard solution with a constant pH. 2) The standard solution must have a constant quantity of titratable phenol red. In the salt solution used, the buffering capacity of the medium is due to the Na_2HPO_4. Since phenol red obeys Beers' law, a 2-fold difference in concentration of phenol red gives a 2-fold increase in the delta o.d. for a given delta H^+. When using recrystallized phenol red solution, a constant quantity of phenol red may be obtained by determining the absorption at 560 mμ. Since most commercial preparations of phenol red are impure, and since in fact even recrystallized material changes on standing, the extinction at 560 mμ is not a sufficient criterion of the amount of titratable phenol red in the solution. One method for adding the same amount of titratable phenol red when using different commercial samples is as follows: From a 0.5% stock solution in water, 0.05 ml, 0.10 ml, and 0.150 ml are added to 100 ml aliquots of the salt solution. The o.d. at 560 mμ of these solutions is determined and the delta o.d. obtained on adding 0.01 ml of standard 0.1 N HCl per 10 ml for each of the solutions is determined. Delta o.d.'s are plotted versus milliliters of the phenol red added; the amount of stock solution giving a desired delta o.d. for the standard solution is extrapolated from the curve. For these studies, a delta o.d. of 0.150 ± .005 was selected. This represents a concentration of 5.0 mg per liter, with our best recrystallized phenol red preparation. Several commercial preparations have been standardized in this

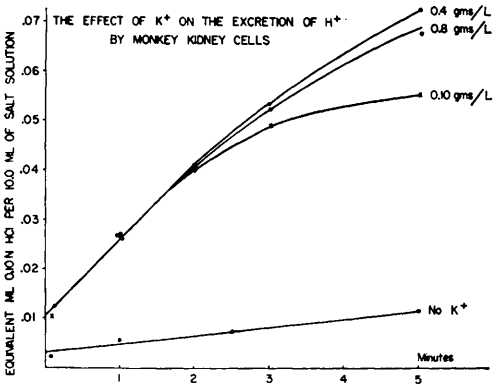

FIG. 4. Kinetics of H^+ production and requirement for K^+. Cultures had been selected from a series of 9-day-old "M" cultures to have the same phenol red index.

way and found to give the same phenol red index as the recrystallized material. The phenol red stock solutions are stable for several weeks if kept in the dark.

Kinetics of acidification of phenol red and effect of Na^+ and K^+. The amount of H^+ produced was determined as a function of time. It is seen (Fig. 4) that the rate is constant between 5 seconds and 2-3 minutes. Since this rate does not extrapolate back to zero, there is indication of a very rapid primary reaction. The K^+ has been found to be very critical for the H^+ production. As shown in Fig. 4, the production of H^+ is almost completely suppressed in the absence of K^+ in the salt solution. It is limited after 2 minutes at a concentration of 0.1 g/liter of K^+, even in the presence of high Na^+ concentration (8 g NaCl/liter).

In contrast, the effect of the Na^+ was not so great in this system. In Table I, changes in absorption and amount of acid produced in the salt solution without NaCl are given. The concentration of Na^+ could not be reduced below 0.134 g per liter, because of the presence of Na_2HPO_4 needed for the buffer. Since omission of NaCl significantly changes the spectrum of phenol red, it was necessary to retitrate the phenol red with standard acid in this solution. Table I shows that there was no significant effect on the primary reaction in the absence of NaCl; the secondary reaction was only slightly inhibited.

Invariance of the phenol red index with endogenous polysaccharide reserves. In the studies already presented, the production of hydrogen ions by monkey kidney cells was followed in a solution in the absence of any exogenous carbon. If energy is required for this reaction it is presumably made available by the endogenous polysaccharide reserves of the cells. Thus if the phenol red index was dependent on a critical level of these reserves it might give erroneous indication of cell number under different metabolic regimes. In an effort to check this the phenol red index was studied in cultures which had different levels of endogenous reserves.

The amount of polysaccharide (anthrone-positive material) in cultures of monkey kidney cells varies with age of the culture and with frequency of replenishment of the medium. The variations in the level of endogenous polysaccharide reserves in cultures between the 8th and 12th day after seeding and the effect of time of replenishment are shown in Table II. For this experiment the cultures had been matched for cell number on the 4th day after seeding by taking the phenol red index. At the time of the experiment the phenol red index was determined for each culture before the anthrone test. Several cultures of

TABLE I. Kinetics of H^+ Production in Salt Solution with Different Concentrations of NaCl.

0.134 g Na^+*			3.22 g Na^+		
Time	Δ o.d.	Equivalent ml 0.1 N HCl/10 ml of same low sodium salt sol.	Time	Δ o.d.	Equivalent ml 0.1 N HCl/10 ml of same salt
7 sec.	.115	.011	5 sec.	.155	.011
1 min.	.170	.019	1 min.	.265	.027
2 "	.210	.030	2 "	.415	.053
5 "	.300	.052	5 "	.470	.067

9-day-old cultures had been selected to have the same phenol red index.

* Contributed by the Na_2HPO_4 used for buffer. Salt solution had no added NaCl.

TABLE II. Changes in Endogenous Polysaccharide Reserves and Invariance of the Phenol Red Index.

Age of culture and replenishment schedule	Phenol red index (pH 8.0)	Equiv. cell No. $\times 10^6$ $\pm$ 10%	Cell No. $\times 10^6$ (avg haemocytometer count)	γ equivalents of glucose	
				Total per culture	Per 10^6 cells
8 days old—replenished at 6 days	.290	2.81		60	21
	.280		2.32		
	.272	2.5		49	21
	.273	2.5		51	20
	.280		2.40		
	.278	2.6		52	20
	.285	2.72		55	20
	.275	2.55		50	19
9 days old—replenished at 6 & 8 days	.320		3.31		
	.300	2.95		105	35
	.310	3.12		120	37
	.340	3.45		130	38
9 days old—replenished at 6 days	.265	2.40		32	13
	.270	2.50		28	11
	.270	2.50		30	12
	.260	2.35		27	11
	.280		2.52		
	.275	2.55		32	12
12 days old—replenished at 6, 8, 10 & 11 days	.275	2.50		180	72
	.285	2.72		205	75
	.280	2.60		192	74
	.282	2.60		187	72
	.277		2.55		

Cultures had been matched for cell number on the 4th day of outgrowth.

each series were also used for cell counts. The number of nuclei recovered agreed within $\pm$ 10% with the number expected from the phenol red index. On the other hand, on the basis of cell count the polysaccharide reserves varied between 11 γ and 70 γ per 10^6 cells. It may be concluded that the phenol red index is independent of endogenous polysaccharide reserves above a level of 10 γ per 10^6 cells.

The effect of severe starvation in a salt solution on the phenol red index has been studied. The phenol red index and the total nuclei were determined in a series of matched cultures which were changed from the M-H medium to Hanks salt solution containing no glucose. The number of cells expected from the phenol red index and the number of nuclei actually recovered after fixing with tannic acid and shaking with citric acid have been plotted at different times during the starvation. It is seen (Fig. 5) that when 7-day-old cultures are changed from the complete serum-containing medium to the salt solution without glucose, there is a rapid dissolution of the cells. This was reflected not only in decrease in the number of nuclei actually recovered but also in the uneven staining and broken membranes of those nuclei which were recovered. The count is only an approximate indication of the number of normal cells in the cultures. The count of cells expected from the phenol red index was significantly lower during the first 8 hours than the number of nuclei recovered. At 24 hours, however, the phenol red index was a reliable indication of the number of nuclei in the culture recoverable under these conditions. Rate of decline of the phenol red index during the first 8 hours is only about 2 times the rate of loss of recoverable nuclei. The observation suggests that mechanisms involved in excretion of hydrogen ions by the cells are maintained by the lowest metabolic rates compatible with the integrity of the cell.

Acid properties of glass. The excretion of H^+ shown to occur in salt solution presumably continues to some extent during growth. Since glass binds H^+ during rinses with strong acid, or even during autoclaving in water, it may be expected that the glass be-

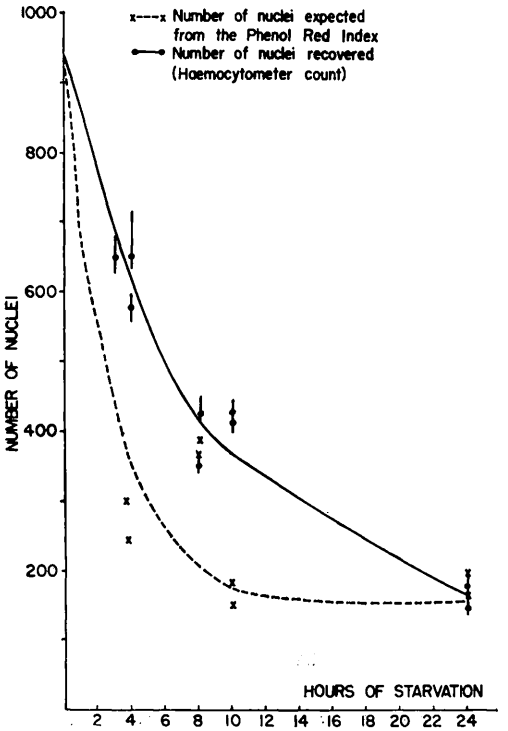

FIG. 5. Effect of starvation on phenol red index. Seven-day-old cultures transferred after rinsing in salt solution from the lactalbumin-hydrolysate-calf serum medium to Hanks salt solution without glucose. Number of nuclei indicated by value of the phenol red index determined at different times during starvation is compared to the number of nuclei recovered after treatment with citric acid.

comes acid during growth. That glass does retain H^+ is shown by the fact that the side of the glass on which the cells were not grown will acidify phenol red. Indeed, as will be discussed elsewhere, glass scraped clean of cells will still acidify phenol red. The glass used in these studies does not bind H^+ significantly before the cells have attained a population of 0.5-1.0 x 10^6, at which time the glass is nearly covered. If the phenol red solution is placed directly onto the cell layer, the contribution from the glass is negligible and has already been accounted for in the calibration curve. Glass, however, differs in the rate at which it binds H^+. It is probable that the calibration curve is valid only for cultures growing in the same type of glass.

Discussion. It has been found empirically that the amount of hydrogen ions excreted by monolayer cultures of monkey kidney cells is correlated with number of cells in the culture over a wide range of cultivation conditions and of age. The amount of hydrogen ions excreted seems to be independent of levels of endogenous polysaccharide reserves of the cells. Even under conditions of extreme physiological stress, *i.e.*, starvation in a salt solution without glucose, rate of hydrogen production decreased at only twice the rate of the decline in cell number. The results suggest that the excretion of hydrogen ions is a primary function of the cell which is maintained by the lowest metabolic rates compatible with the integrity of the cell.

The excretion of hydrogen ions has been studied extensively in perfused kidney(6), in stomach mucosa(7), and in yeast(8). Preliminary studies in this laboratory indicate that it is a characteristic function of other mammalian cells growing *in vitro*. The mechanism of hydrogen ion excretion is not understood and the studies presented here were designed only to evaluate the possible sources of error when it is used as a quantitative indication of cell number. Unfortunately, all the possible errors can not be evaluated until the mechanism has been clarified. It would seem, however, that mammalian cells growing *in vitro* may be used for quantitative studies on the mechanism of ion exchange.

Summary. A method has been presented for determining the number of cells in monolayer cultures of monkey kidney cells without physiologically damaging them. The method consists of determining the change in absorption of a phenol red solution after a 15-second exposure of the cultures under standard conditions. These conditions include the use of a standard solution defined with respect to pH, quantity of titratable phenol red, and buffering salt, and the use of the same type of glass in the different cultures. The change in absorption appears to be a simple consequence of the production of hydrogen ion by the cells. It is critically dependent on the presence of potassium but independent of an exogenous carbon source, and, independent of endogenous polysaccharide reserves over a wide range. The amount of hydrogen ion produced may be sufficiently independent of cell age

and metabolism to be a reliable method for the estimation of growth in cultures.

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Received July 3, 1957. P.S.E.B.M., 1957, v96.

Diabetes Mellitus and Serum Vitamin B_{12} Concentrations: 333 Patients. (23464)

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Since a role for vit. B_{12} in transmethylation and the transformation of carbohydrate to fat has been invoked(1), it is natural that deviations in the B_{12} metabolic pattern should be looked for in the most obvious defect of carbohydrate metabolism, human diabetes mellitus. It is conceded that patients with diabetes show an accelerated rate of development of cardiovascular degenerative disease, not directly correlated with age of onset, duration, or severity of the diabetes. Vascular disease of the retina, diabetic retinopathy and glomerulosclerosis with or without the Kimmelstiel-Wilson syndrome, are regarded as advanced manifestations of vascular degeneration. Observations correlated with these conditions raise the question whether the relationship is with severe vascular disease independent of diabetes or with diabetes itself.

Methods. Patients from 4 large diabetic clinics* were studied. Single serum samples were obtained from fasting patients and were assayed for their B_{12} content by the *Lactobacillus leichmanii* method(2,3,4). All sera were frozen and held at –20°C until assay and those specimens which were *not frozen* upon receipt were discarded. The diagnosis of diabetic retinitis in the patients studied was accepted only on the basis of an examination by an experienced ophthalmologist within a week of assay. The diagnosis of diabetes in all patients studied was established by history and the requirement of insulin for prolonged periods.

Results. The results are recorded in Tables I to IV. Normal groups for comparison were not obtainable from all institutions for it is not common for normal individuals in any number to be institutionalized. The statistical methods seemingly most appropriate to this study have been employed in the analysis of the data(5,6). Certain variables inherent in such a study as has been carried out, cannot be eliminated. The onset of diabetes mellitus is superimposed upon *pre-existent* physiology and pathology and diabetes in "pure" form in a patient free from abnormalities which, in the absence of diabetes would be regarded as falling within the limits of normal, is rare.

It is clear that where controls were available there was no consistent difference between diabetics and these "normals." Similarly, in the 3 studies where the subjects had been screened for retinopathy there was no apparent difference between patients with and

* The authors wish to express their indebtedness to Dr. Wallace W. Dyer of the Philadelphia General Hospital and Dr. Harry S. Lipscomb of Jefferson Davis Hospital, Houston, Texas, for their kindness in having supplied us with the sera from diabetic patients.