methylation at the 3 position is an effective means of inactivating norepinephrine. It will be of interest to investigate the enzymatic mechanisms involved in this unusual methylation process and to attempt to alter it *in vitro* and *in vivo*.

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A New Chemical Method for Measuring Cell Populations in Tissue Cultures.* (23946)

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Among more popular criteria for measuring cell populations in tissue cultures are cell counts, respiration rate, "hematocrit," isotope incorporation, nutrient uptake, desoxyribonucleic acid (DNA) content, and protein content (1-7,9-13). In this report we wish to present a new chemical method for measuring size of tissue cultures, based upon determination of "total purines and pyrimidines" (TPP). TPP is determined simply by extracting washed cultures with hot acid salt solution, and measuring the absorbance of the extract at 268 mμ. While TPP values correlate well with cell counts, mainly we have used DNA as a "standard of reference" for measuring populations. The case for DNA as a measure of cell numbers was summarized by Healy, Fisher, and Parker(6). To evaluate TPP as a measure of cell populations we studied young, old, starved and well-fed cultures, and determined the TPP, DNA and protein in each. As would be expected, TPP and protein both varied somewhat and similarly with respect to DNA. The data indicate that TPP is at least as good as protein for quantitation of cell populations. The virtues of the TPP method are simplicity, reproducibility, stability of absorbance, and a wide range of operation. Ribonucleic acid (RNA) determination is a logical extension of the TPP method, which we hope to employ. If the cells are first extracted with cold trichloracetic or perchloric acid, the TPP of the residue will consist of only RNA and DNA. RNA then will be equal to the difference between TPP and DNA, both being determined in the same alkaline extract as described below.

Methods. Cell cultures. HeLa S-3, was obtained from Tuskegee Institute. The McCoy strain of human cells was kindly supplied by Prof. C. M. Pomerat. Strain L, NCTC929-209-7 was obtained through the kindness of Dr. Wilton R. Earle, Nat. Cancer Inst. Stock cultures were fed and divided twice weekly. Human cells were cultured in a synthetic medium very similar to Eagle's, with human serum non-dialyzable fraction equivalent to 10% serum, supplemented with 1 g of Difco Bacto Tryptose/liter. Strain L was cultured in the same synthetic medium with 10% horse serum instead of human serum. Incubation was at 37°C. Replicate cultures were planted by Earle's apparatus (4). Strain L and McCoy were suspended by mechanical agitation; HeLa required trypsin treatment. Cultures were planted in 18 x 150 mm test tubes graduated for 5 ml volume, with a conically tapered bottom and an indentation 7 mm deep and 15 mm from mouth

*We are grateful to Henry Kokko for technical assistance and to Jack Arzoomanian for the drawings.

of tube.† During incubation the tubes were horizontal. They were placed in special racks with the indentation over a rail which prevented rolling. Also, the indentation kept the media away from the silicone rubber stoppers. Each culture contained 2.5 ml of medium. For the chemical determinations, cultures must be rinsed carefully with physiological salt solution. In our experience equally good results may be obtained with 0.9% sodium chloride, adjusted to pH 4.5 with phosphoric acid, and Earle's salt solution, pH 7, without sodium bicarbonate. For cultures of 1 to 5 x 10^6 cells two 5 to 10 ml rinses should be sufficient. Centrifuge at 2000 rpm.

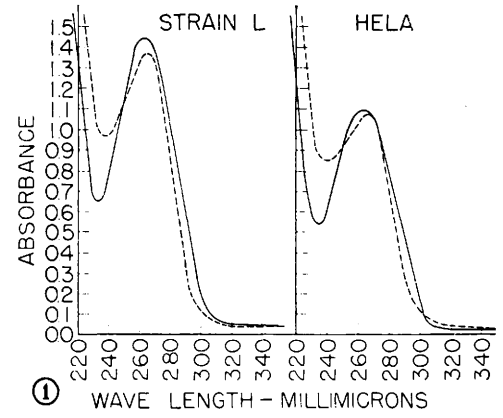
TPP procedure. To each washed culture add 5 ml of 0.1 N sulfuric acid containing 10% sodium chloride. Cover tube with glass cap. Arrange tube almost horizontally so that acid salt solution covers the cells. Heat in live steam 1 hr, cool, adjust to 5 ml volume, centrifuge, and read absorbance of the solution at 268 mμ. From the equation given for DNA by McIntire and Sproull(8) calculate TPP as DNA equivalents. Recently we have modified the procedure as follows: In each washed culture remove the cells from the glass with 2.5 ml of 0.1 N sodium hydroxide. Add 2.5 ml of 0.3 N sulfuric acid containing 20% sodium chloride, mix well, cover with a glass cap, heat in boiling water 15 minutes, cool, mix well, centrifuge and read the absorbance. A volume adjustment will not be necessary if the boiling water is slightly below the level of solution inside the tubes. *TPP, DNA and protein in the same culture. Reagents and apparatus*—0.3 N sulfuric acid containing 20% sodium chloride, 0.4 N sodium hydroxide and other reagents and apparatus required for DNA by the method of McIntire and Sproull(8) and for protein by the method of Oyama and Eagle(10). *Procedure.* Follow directions for determination of DNA through the heating in 0.4 N sodium hydroxide and diluting to 0.04 N. Take aliquots and proceed with the DNA determination. For TPP determination take a 0.2 ml aliquot of the .04 N sodium hydroxide extract, add 0.2 ml of 0.3 N sulfuric acid with 20% sodium chloride. Mix gently, heat in boiling water 15 to 30 minutes, cool, adjust to 0.4 ml volume,‡ and centrifuge. Read the absorbance of the acid salt solution in a quartz micro cell at 268 and 330 mμ. If the samples are allowed to stand overnight before concentration, the 330 mμ reading (haze correction) is usually negligible. For protein determination follow the directions of Oyama and Eagle for preparing Lowry's solution C and for standardizing the Folin-Ciocalteau (F-C) reagent. Take an aliquot of .04 N sodium hydroxide culture extract, or protein standard prepared just as the cell extract, and add water to make 1 ml. Mix with 5 ml of solution C and 0.5 ml of F-C reagent and follow through according to Oyama and Eagle. Two protein standards were employed. A purified protein from HeLa cells was the primary standard, human serum albumin was a secondary standard. Results are given in terms of HeLa protein nitrogen(PN).

Results. Significance of TPP determination. Fig. 1 shows absorption curves for TPP solutions prepared by hot acid saline extraction of HeLa and Strain L cells. Solid lines show absorption in the acid salt solution; broken lines show absorption after the acid solutions had been made approximately .05 N to sodium hydroxide. These curves are very similar to the curves for purified DNA treated similarly(8). By the same arguments advanced for the specificity of the DNA method, there is no detectable protein interference, and the TPP value appears to be a measure of purines and pyrimidines and very little else. This value of course includes DNA, RNA, building blocks for these molecules, and coenzymes. DNA and RNA make up approximately 75 to 80% of the total.

Figure 2 shows that the TPP value is a linear function of cell number when samples of different size are taken from a homogeneous population. For these data aliquots of different size were taken from a stirred suspen-

† This culture tube and a smaller one of the same type may be obtained from Bellco Glass Inc.

‡ These manipulations were done in 8 x 70 mm test tubes calibrated to 0.4 ml by Paul O. Moeller, Universal Instrument Corp., Chicago. For measuring the 0.2 ml aliquots we used micropipets made by Micro-Chemical Specialties Co.



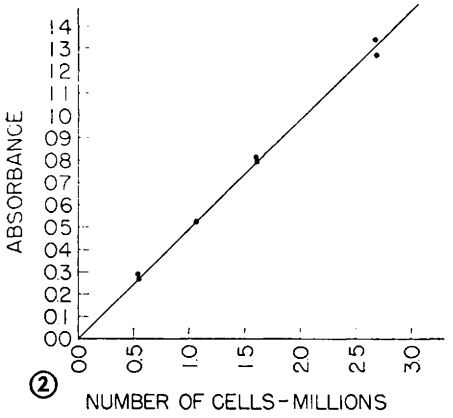


FIG. 1. Absorption spectra of TPP extracts of HeLa and Strain L.

FIG. 2. Relationship of cell number to absorbance of TPP extract of Strain L.

sion of Strain L cells. The cells were centrifuged, washed, and extracted with hot acid salt solution. Each point represents an individual determination, *not an average.*

Relationship of TPP, and PN to DNA in cultures. If TPP and PN are to be used as criteria for measuring tissue cell populations, one must know how constant are these constituents per cell under different conditions of growth. To investigate this question we chose to use the DNA content rather than cell count as a standard of reference, because the constancy of the average DNA per cell in a large population is widely accepted. This means that we will be talking about TPP/DNA and PN/DNA ratios, rather than TPP and PN per cell. To bring out the widest range of ratios that might be encountered in growing cells we analyzed young cultures where the cells were growing rapidly, older cultures that were crowded and in spent medium, and older cultures that had been fed 24 hours before termination.

The data of Table I show that both TPP/DNA and PN/DNA vary with the culture conditions. A 2-way analysis of variance indicates that the *differences among the 3 groups of cultures of each cell strain* are highly significant. Variance among the 3 groups of each strain was analyzed against variance among replicate cultures within each group. The groups were significantly different at the 0.995 level while there was no significant difference among replicates at the 0.95 level. PN and TPP varied similarly with respect to DNA, but the variations are not great in view of the differences in conditions, and are much less than might be predicted from the work of Davidson and Leslie(3). As would be expected, both PN and TPP were low in the older cultures in which the medium had not been renewed. For many experiments with these cell strains, either TPP or PN would be a suitable measure of cell population, and TPP is at least as good a criterion as PN.

The TPP value may be expressed in several ways. For routine comparisons absorbance units per culture, or per ml of a standard volume of extract, is satisfactory. It may also be expressed in terms of equivalents of DNA, RNA or any purine or pyrimidine which has an absorption maximum near 268 mμ. We chose to use DNA equivalents, because we had on hand a purified preparation of calf thymus DNA.

The center 3 columns of Table I show the DNA, TPP, and PN group average values and the standard deviation of the assays within each group of cultures. In general the standard deviation for PN is greater than for TPP and DNA. The significance of this was not tested statistically.

As a laboratory method TPP has the following advantages over protein determination: 1. TPP absorbance is very stable; it is potentially less capricious than the transitory color of the protein method which requires rigid control of the rate of mixing the reagents. 2. In the TPP method there is no variable blank to be subtracted and no stand-

TABLE I. TPP, DNA and PN in Cultures of Tissue Cells.

Culture, age (days)	No. of cultures	TPP,* μg/culture		DNA, μg/culture		PN,† μg/culture		TPP/DNA		PN/DNA	
Strain L		μ‡	S.D.§	μ	S.D.	μ	S.D.	μ ± S.E.‖		μ ± S.E.	
3	6	44.2	.88	16.5	1.16	45.2	3.18	2.69	.087	2.75	.127
6	6	107	2.81	46.7	2.6	106	5.38	2.22	.041	2.27	.178
6F¶	6	113	2.77	42.9	3.04	153	10.1	2.63	.032	3.62	.210
HeLa											
4	6	75.2	1.46	19.8	.72	86.3	1.46	3.79	.061	4.36	.21
7	6	64.1	.93	23.9	.70	78.7	2.94	2.64	.069	3.32	.14
7F	6	87.2	2.02	26.8	1.3	100	1.48	3.26	.008	3.75	.18
McCoy											
3	6	111	3.7	53.9	2.7	95	6.9	2.1	.054	1.93	.060
5	9	170	5.1	79.6	2.9	119	5.2	2.13	.039	1.61	.026
5F	8	240	3.2	99.5	2.7	157	4.6	2.42	.033	1.72	.022

* TPP is expressed in DNA equivalents. 10 μg of DNA/ml in acid salt solution has an absorbance of 0.260 at 268 mμ (1 cm light path).

† The reference protein was a purified preparation from HeLa cells.

‡ Avg values.

§ Stand. dev., calculated from difference between duplicate determinations for each culture, on the assumption that all cultures of a group were samples of the same population.

‖ Stand. error. Ratios were calculated for each culture individually. These values were used for calculation of mean ratio and stand. error of the mean.

¶ Letter F indicates that cultures were fed 24 hr before they were terminated.

ard is required with each experiment. 3. The TPP method has the much wider range of operation. Readings can be made very accurately, and there is a linear relationship between TPP and cell number between absorbances of 0.1 and 2.0.

Summary. This paper presents a new method for measuring populations of tissue cell cultures. The basis of this method is a simple extraction and the measurement of total purines and pyrimidines (TPP) by their absorbance at 268 mμ. A comparison of TPP/DNA to PN/DNA in 3 cell strains growing under different conditions shows that TPP is at least as good as PN for measuring cell populations.

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