8. Soret, M. G., and Sanders, M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 526.

9. Holtfreter, J., *J. Exp. Zool.*, 1943, v93, 251.

10. Wood, E. M., Yasutake, W. T., and Lehman, W. L., *J. Infect. Dis.*, 1956, v97, 262.

11. Duryee, W. R., and Doherty, J. K., *Ann. N. Y. Acad. Sc.*, 1954, v58, 1210.

Received April 4, 1957. P.S.E.B.M., 1957, v95.

A Simple Method for Determination of Desoxypentose Nucleic Acid in Tissue Cultures. (23251)

FLOYD C. MCINTIRE AND MURIEL F. SPROULL (Introduced by L. W. Roth)
Abbott Laboratories, N. Chicago, Ill.

The numerous methods now employed for quantitative determination of desoxypentose nucleic acid (DNA) suffer from either a laborious purification procedure or the capriciousness of colorimetric assays for desoxypentose(1,2,5-8,10-14,16). The desirability of quantitation by means of ultraviolet absorption has been recognized(8,10,14), but these methods are either too laborious or their specificity has been questioned(7).

A new method is presented here which consists of a very simple, *but highly specific,* purification of DNA and quantitation by means of the ultraviolet absorption. The basis of the method is the fact that DNA, in concentrations as low as 5 μg per ml, can be quantitatively precipitated from .04 N sodium hydroxide solution by salmine. Interference by ribonucleic acid is completely eliminated by alkaline hydrolysis(12,15) before the DNA precipitation. The purines and pyrimidines are recovered from the salmine precipitate by extraction with an acid salt solution. DNA recovery is nearly 100%. The method as presented is designed for a lower limit of 2 μg of DNA. By manipulation with smaller volumes a lower limit of 0.2 to 0.5 μg should be feasible.

Experimental. Reagents are 0.4 N sodium hydroxide; 0.1 N sulfuric acid containing 10% sodium chloride (C.P. grade); 1% solution of salmine (General Biochemicals Inc.) *Procedure.* 1. Separate cells from culture medium and wash generously with physiological salt solution. For cultures firmly adhering to glass, centrifugation is not necessary and two 10 ml washings are more than adequate for cultures of 1 to 5 x 10^6 cells. 2. Take up the cells in a small volume of 0.4 N sodium hydroxide; heat in boiling water 1 hour, with a glass cap over the neck of the culture tube to minimize evaporation; cool and dilute the sodium hydroxide to 0.04 N. The volume of 0.4 N sodium hydroxide required in the cell extraction depends upon the number of cells in the culture. If too much alkali is used, dilution to 0.04 N will give a solution too dilute for quantitative precipitation of the DNA. If too little alkali is used RNA will not be completely hydrolyzed and a high value for DNA will be obtained. The best solution to this problem is to have an extra replicate culture for "range-finding." Extract the extra replicate with 5 ml of the acid salt solution in boiling water for 30 minutes, cool, centrifuge and read the absorbance at 268 and 330 mμ. Use the following guide for the alkali extraction:

Absorbance, 268 mμ–330 mμ	ml of 0.4 N NaOH	Dilution after heating
.050 to .100	.05	.5 ml
.100 .200	.1	1
.200 .400	.2	2
.400 .800	.4	4

Cultures of 2 to 3 x 10^6 strain L cells will require 0.4 ml of 0.4 N sodium hydroxide for the extraction. 3. To 0.4 ml of diluted cell extract, in a 0.4 ml graduated test tube, add 0.02 ml of salmine solution.* Mix thoroughly

* Volumes less than 1 ml were measured with micropipets made by Microchemical Specialties Co. The 0.4 ml test tubes were 8 mm O.D., 70 mm long, graduated by Paul O. Moeller, Universal Instrument Corp., Chicago.

but gently (avoid foaming) and chill on ice for 1½ hours. Centrifuge for 10 minutes at 2000 rpm and 0 to 4°C; draw off the supernatant and wash carefully with 0.4 ml of ice cold water. Drain the tube in an inverted position for a few minutes and then shake out the last drop of water. 4. To the tube containing the precipitate add slightly less than 0.4 ml of acid salt solution, cover with a glass cap, heat in boiling water 30 minutes, cool, centrifuge briefly, dilute carefully to 0.4 ml with distilled water, mix well and centrifuge to pack down the slight precipitate. 5. Measure absorbance of the acid saline extract at 268 and 330 mμ. (A Beckman, Model DU spectrophotometer, equipped with a Lowry and Bessey micro cell(9), is recommended.) The reading at 330 mμ is a measure of a very slight haze which may be present. In most cases the 330 mμ reading is so small that it could be ignored. The difference between the 2 readings is proportional to amount of DNA in the original sample (Fig. 3). The quantity of DNA per culture may be expressed in any one of several convenient ways. As a standard of reference we used a preparation of calf thymus DNA purified(3,4) to an analysis of 14.9% nitrogen and 8.53% phosphorus. This DNA preparation, at a concentration of 10 μg per ml, dissolved by heating in the acid salt solution, had an absorbance of 0.260 at 268 mμ (1 cm light path). The amount of DNA per culture may be calculated in terms of the reference standard as follows: A = Absorbance reading, V = Total vol. of 0.04 N NaOH extract.

$$\mu g \text{ DNA/culture} = V \times \frac{A\ 268\ m\mu - A\ 330\ m\mu}{0.026}$$

Specificity of method. Fig. 1 shows the absorption spectra of the acid saline extracts of the salmine precipitates of purified DNA, HeLa extract and strain L extracts. (HeLa cells were obtained through the courtesy of Dr. William F. Scherer, Univ. of Minnesota; strain L, NCTC929-209-7, was obtained through the courtesy of Dr. Wilton R. Earle, Nat. Cancer Inst.) The spectrum of a 0.5% solution of salmine is included to show that this protein contributes nothing to the absorption of the extracts above 250 mμ. A high

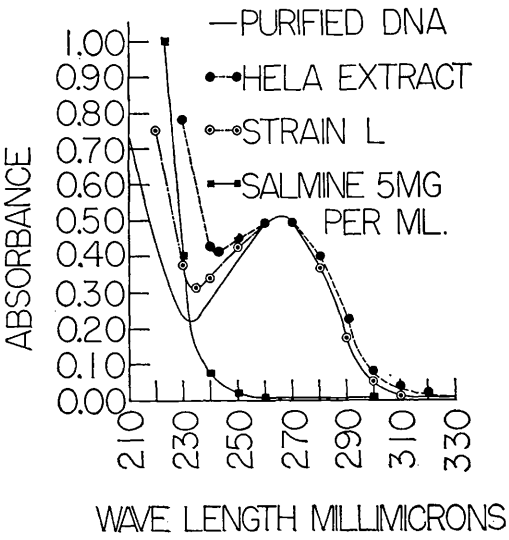

FIG. 1. Ultraviolet absorption spectra of acid saline extracts of salmine precipitates ("DNA extracts") from purified DNA and from alkaline extracts of HeLa and strain L cells.

degree of specificity for the method is indicated by the fact that the HeLa and strain L curves follow very closely the curve for purified DNA from 250 mμ on up to 330 mμ. As a further test of specificity the absorption spectra of these same extracts were obtained under alkaline conditions (~0.1 N sodium hydroxide). The tyrosine absorption peak of proteins, which appears at 275 and 280 mμ under acid conditions, is shifted to 290 mμ and intensified under alkaline conditions. If there were enough protein in the "DNA" extracts to contribute significanlty to the absorption at 268 mμ under acid conditions, this protein interference should show up as a hump at 290 mμ in the absorption curve under alkaline conditions. Also, the absorption at 290 mμ should be greater in alkali than in acid. That such is not the case is shown in Fig. 2. Both HeLa and strain L "DNA" extracts show smooth absorption curves above 270 mμ in alkali, and both alkaline curves are slightly less intense than the corresponding acid curves. Below 250 mμ there is a much greater difference between acid and alkaline absorption curves of conalbumin. In this range also there are relatively slight differences between the acid and alkaline absorption curves of HeLa and L "DNA" extracts. It is therefore clear that protein

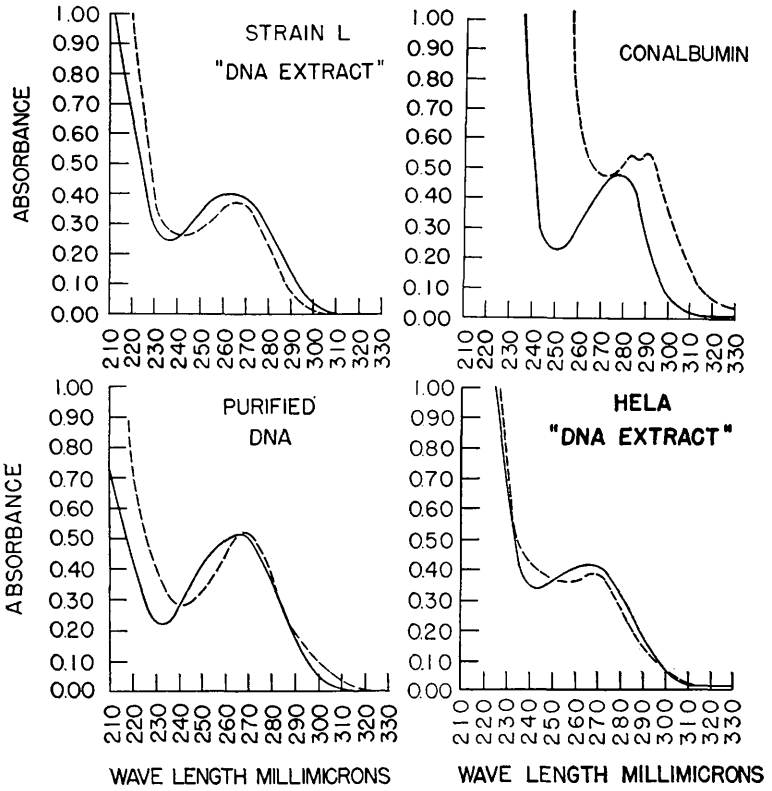

FIG. 2. Comparison of absorption spectra in acid (0.1 N H_2SO_4), solid lines, to those in alkaline solution (0.1 to 0.2 N NaOH), broken lines.

interference in this DNA determination is negligible. Conalbumin was chosen as a representative protein in these studies because it contains only moderate amounts of tyrosine and tryptophane. Insulin, which contains more tyrosine than conalbumin shows much greater differences in absorption between acid and alkaline solutions. Further evidence for the specificity of the salmine precipitation for DNA was obtained from the sugar color reactions. The orcinol test indicated that salmine precipitates from HeLa and strain L extracts contained negligible pentose. The diphenylamine reaction indicated that the salmine precipitates of HeLa and strain L extracts contained as much desoxypentose as the salmine precipitate of purified DNA, in relation to purine and pyrimidine content. The recovery data shown in Table I attest to the specificity of the salmine precipitation of DNA. There was no coprecipitation of either the mono nucleotide, cytidylic acid, or the alkaline hydrolytic fragments of RNA.

Reproducibility of the method and recovery of DNA. That the reproducibility of the method is very good is indicated by the data of Table I. The recovery of DNA is excellent alone or in the presence of alkali hydrolyzed RNA, alkaline cell extracts, or cytidylic acid. Of the 38 recovery figures reported, the mean value is 97.85 ± 0.87% (standard error). Fig. 3 shows the relationship at 268 mμ between absorbance of the acid saline extract of the salmine precipitate, and amount of DNA before precipitation. Each point on the graph represents the average of the 4 absorbance readings from which the corresponding recovery figures in Table I were calculated.

Summary. A new method for quantitative determination of desoxypentose nucleic acid has been presented. The innovation of this method is precipitation of DNA by salmine

TABLE I. Recovery of DNA in Presence of Thymidylate, Alkali Hydrolyzed RNA and Alkaline Cell Extracts.*

Constituents of 0.04 N NaOH solution	DNA added (μg)	DNA found (μg)†			
NaOH alone	5.44	5.16 (94)	5.44 (100)		
5.76 μg RNA‡	5.44	5.4 (99)	5.12 (93)		
20 μg calcium thymidylate	5.44	5.4 (99)	5.24 (96)		
40 μg " "	none	.31	.32		
11.5 μg RNA	"	.1	.1		
	21.8	21.6 (99)	21.8 (100)	21.6 (99)	21.8 (100)
	10.9	10.8 (99)	10.8 (99)	10.8 (99)	10.7 (98)
	4.36	4.1 (94)	4.1 (94)	4.18 (96)	4.2 (96)
	2.18	1.98 (91)	1.98 (91)	2.16 (99)	2.11 (97)
Strain L extract	none	3.48	3.58	3.6	3.66
	10.9 §	14.93 (104)	15.31 (108)	14.78 (103)	14.48 (100)
	2.18§	5.88 (106)	5.85 (104)	5.76 (100)	5.59 (92)
HeLa extract		3.84	4.0	3.97	3.97
	10.9 §	14.24 (94)	14.94 (101)	14.04 (93)	13.54 (88)
	2.18§	6.07 (98)	6.41 (113)	5.83 (87)	6.16 (102)

* Each "DNA found" value represents an individual determination, *not an avg value.*
† Figures in parentheses give % recovery.
‡ Hydrolyzed in 0.4 N NaOH, 60 min., 100°, then diluted to 0.04 N NaOH.
§ For calculation of DNA recovery from HeLa and strain L extracts, avg values of 3.58 μg DNA for strain L and 3.94 μg DNA for HeLa were used.

and dissolution of purines and pyrimidines from the precipitate by means of hot acid salt solution. The quantitation depends upon the absorption of the acid saline extract at 268 mμ. The method is very specific for DNA. Precision and DNA recovery are both good.

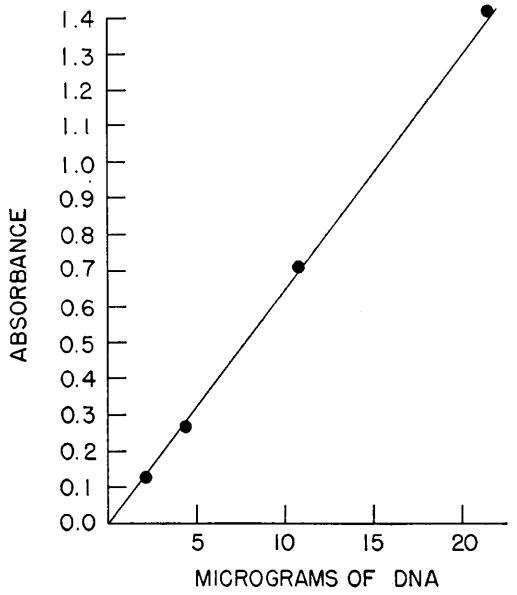

FIG. 3. Absorbance of acid saline extract of salmine precipitate as related to amt of DNA before precipitation.

The authors are grateful to Henry Kokko for technical assistance, to E. F. Shelberg and associates for microanalyses on DNA preparations, and to Jack Arzoomanian for the line drawings.

1. Bonting, S. L., and Jones, M., *Arch. Biochem. Biophys.*, 1957, v67, 340.
2. Ceriotti, G., *J. Biol. Chem.*, 1952, v198, 297.
3. Chargoff, E., *The Nucleic Acids,* v1, p327, Edited by E. Chargoff and J. N. Davidson, Academic Press, N. Y., 1955.
4. Cohen, S. S., *J. Biol. Chem.*, 1947, v168, 511.
5. Davidson, J. N., Leslie, I., and Waymouth, C., *Biochem. J.*, 1949, v44, 5.
6. Healy, G. M., Fischer, D. C., and Parker, R. C., *Can. J. Biochem. Physiol.*, 1954, v32, 319.
7. Loeb, H. G., and Dickinson, E., *Arch. Biochem. and Biophys.*, 1955, v57, 515.
8. Logan, J. E., Mannell, W. A., and Rossiter, R. J., *Biochem. J.*, 1952, v51, 470.
9. Lowry, O. H., and Bessey, A. O., *J. Biol. Chem.*, 1946, v163, 633.
10. Ogur, M., and Rosen, G., *Arch. Biochem.*, 1950, v25, 262.
11. Patterson, E. K., and Dakerman, M. E., *Arch. Biochem. Biophys.*, 1952, v36, 97.
12. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1945, v161, 83.
13. Schneider, W. C., *ibid.*, 1945, v161, 293; 1946, v164, 747.

14. Scott, J. F., Fraccastoro, A. P., and Taft, E. B., *J. Histochem. and Cytochem.*, 1956, v4, 1.

15. Steudel, H., and Peiser, E., *Z. Physiol. Chem.*, 1922, v120, 292.

16. Webb, J. M., and Levy, H. B., *J. Biol. Chem.*, 1955, v213, 107.

Received April 9, 1957. P.S.E.B.M., 1957, v95.

Relationship Between Sleep, Biotransformation Rates, and Plasma Levels of Pentobarbital and Secobarbital in Animals. (23252)

J. D. TAYLOR, ELEANOR M. SWENSON, JEANNE C. DAVIN AND R. K. RICHARDS
Department of Pharmacology, Abbott Laboratories, North Chicago, Ill.

Pentobarbital (Nembutal) (5-ethyl-5-(1-methylbutyl)-barbiturate sodium) and secobarbital (Seconal) (5-allyl-5-(1-methylbutyl)-barbiturate sodium) are very similar in structure and both are classed as "short-acting" in duration of action(1). Pentobarbital has been widely studied in animals with regard to its fate and metabolism, but there is little comparable data on secobarbital. Man seems to be the only species studied(2,3) in this manner.

It was our purpose to compare these 2 barbiturates with regard to sleeping time, body or plasma levels at the time of waking and rate of destruction in mice, rabbits, and dogs given single doses (equivalent by weight) of pentobarbital and secobarbital.

Materials and methods. The molecular weights of pentobarbital and secobarbital are within 5% of each other. This slight difference would not be evident in experiments that have biological errors which are considerably larger than 5%, so equivalent doses in weight per kilo were used throughout this work. Groups of female, albino mice, weighing between 18-22 g were injected intraperitoneally with either barbiturate as the sodium salt made to a 2% solution. Sleep was recorded as the interval between the time mice remained on their back to the time they woke and rolled over. For experiments measuring both body level and sleeping time of drug, each mouse was killed by impact, on waking, the whole animal homogenized in a Waring blendor and an aliquot analyzed for barbiturate. To determine rates of destruction, separate experiments were run with groups of mice analyzed at definite time intervals after injection regardless of whether they were asleep or awake. A group of female Sprague-Dawley rats weighing between 230-330 g was injected intraperitoneally with each barbiturate and sleeping time (righting time) recorded. One week later those animals that had received pentobarbital were given secobarbital and those that had received secobarbital were given pentobarbital. This cross-over arrangement equalized the effect of time and tolerance and the relatively large animal to animal variation was minimized. This design was used in some other experiments also (see below). Only sleeping times were studied on rats. Male rabbits weighing between 2-4.8 kg were injected intravenously with a 5% solution of the sodium salt of each barbiturate. Sleeping times were recorded from the time of injection to the time they righted themselves from a side position. Blood samples were taken by heart puncture at 1 and 2 hours and at the time of awaking. They were centrifuged, and an aliquot of the plasma analyzed for the barbiturate. Preliminary experiments showed that the plasma and tissues had reached equilibrium before one hour. One experiment was set up on a cross-over design as described above with a 5-day interval between experiments. Female mongrel dogs weighing between 5 and 12 kg were injected intravenously with 3% solutions. Blood samples were drawn from leg veins at 1 and 4 hours and at waking time and the plasma analyzed. The experiment was set up in a cross-over design as described above for rats with one week intervals between experiments. Pentobarbital and secobarbital were determined by the method of