a daily maintenance Vit. C dose of 2 mg per day. This response was manifested by appearance of large amounts of urinary p-hydroxy phenylpyruvic acid, the same compound which appears in the urine of uninjured guinea pigs fed tyrosine when ascorbic acid is removed from their diet. Metabolic abnormality reached its peak on the sixth post burn day, then declined to almost normal levels by the 20th post burn day. One hundred mg per day of Vit. C abolished the abnormality after the burn. These results agree with previous observations of altered Vit. C metabolism and an increased need for this vitamin after burns, as judged by the abnormal wound healing within the first 2 weeks after a burn.

1. Levenson, S. M., Pulaski, E. J., Upjohn, H. L., Chap. 1 in *Physiologic Principles of Surgery*, ed. by Zimmerman, L. M., Levine, R., W. B. Saunders Co., Philadelphia, 1957.

2. Levenson, S. M., Upjohn, H. L., Preston, J. A., Steer, A., *Ann. Surg.*, 1957, v146, 357.

3. Garrod, A. E., *Inborn Errors of Metabolism*, Oxford Univ. Press, London, 1909.

4. Sealock, R. R., Silberstein, H. E., *Science*, 1939, v90, 517.

5. Levine, S. Z., Marples, E., Gordon, H. H., *ibid.*, 1939, v90, 620.

6. Sealock, R. R., Silberstein, H. E., *J. Biol. Chem.*, 1940, v135, 251.

7. Bourne, G. H., *Lancet*, 1942, v243, 661.

8. McCarthy, M., *J. Lab. Clin. Med.*, 1945, v30, 1027.

9. Painter, H. A., Zilva, S. S., *Biochem. J.*, 1947, v41, 511.

10. Knox, W. E., Goswami, M. N. D., *J. Biol. Chem.*, 1960, v235, 2662.

11. Zannoni, V. G., La Du, B. N., *ibid.*, 1960, v235, 2667.

12. McElroy, O. E., Anderson, P. R., Gray, I., *Arch. Biochem. Biophys.*, 1958, v75, 69.

13. Anderson, P. R., McElroy, O. E., *ibid.*, 1959, v82, 256.

Received October 14, 1960. P.S.E.B.M., 1961, v106.

A Method for Measurement of Urinary Deoxyribonuclease Based on the Reaction of Indole with Deoxyribonucleotides.* (26306)

LEON L. MILLER, HARRY L. SEGAL AND PATRICIA A. HARRINGTON
Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The need for a more precise sensitive method to determine the activity of urinary deoxyribonucleases I and II (DNase I and DNase II) has led us to develop such a method based upon the indole reaction originally described by Dische(1) and used by Ceriotti for estimation of deoxyribonucleic acid(2). The failure to obtain good reproducibility even with zero time blanks, as noted with other urinary DNase methods(3,4), has been eliminated by insuring the quantitative precipitation of unreacted DNA. This has been accomplished by adding a protein such as casein or bovine serum albumin to the enzyme reaction mixture.

The use of purified, freshly distilled chloroform in Ceriotti's procedure has been circumvented by substituting commercially available analytical grade benzene as extracting solvent.

Insight into the mechanism of the indole reaction has been gained by comparing reactivity of purine and pyrimidine deoxyribotides and the corresponding deoxyribosides as well as that of certain possible hydrolysis products of DNA.

The method developed primarily for measurement of urinary DNase activity has been found equally applicable to measurement of DNase in serum and tissue homogenates. However, only a general method for determination of DNase I will be discussed here.

Experimental. I. *Reagents.* A. *General reagents for DNase I and II*: (a) A 0.2 g% solution of sodium deoxyribonucleate (DNA). DNA was prepared in this lab-

* Supported by U. S. Public Health Service grant.

oratory by the method of Kay, Simmons, and Dounce(5). (b) 2.88 M trichloroacetic acid (TCA). (c) 0.04 g% indole in distilled water(2). (d) Concentrated (12 N) HCl. (e) Benzene, thiophene free (C. P. Mallinckrodt has been found satisfactory without further purification).

B. *Reagents for DNase I*: (a) A buffer solution, pH 7.5, made by mixing 75 ml of 0.2 M tris (hydroxymethyl) aminomethane with 100 ml 0.1 M HCl, adjusting the pH to 7.5, and diluting to 300 ml with distilled water; this solution is then mixed with 100 ml 0.1 M magnesium chloride. (b) A 1.0 g% solution of Vitamin test casein (General Biochemicals) was prepared by mixing 1 g casein with 20 ml of water and adding 1 N NaOH (usually 11 to 12 ml) until the casein dissolved completely. This solution was then neutralized (pH 7.0) by adding 11 to 12 ml of 1 N acetic acid dropwise with continuous stirring. The casein solution was then diluted to 100 ml with the above pH 7.5 buffer.

II. *Methods for collection and storage of urine*: Urine was collected as a clean, single, freshly voided morning specimen (average interval, 8 hours). Under these conditions, the use of toluene as an antiseptic did not yield significantly different results and its use was eliminated. A 25 ml portion of the total measured volume was placed in a 30 cm segment of dialysis tubing (cellophane, 28 mm diameter, 0.18 mm wall thickness) weighted with a glass marble. Dialysis was continued for 24 hours. If the urine was not dialyzed before analysis, so much extraneous pink pigment was produced in the reaction with indole that 12 to 15 extractions with benzene were necessary. All urines were dialyzed immediately after collection. For dialysis, running cold tap water was used because no significant difference between tap water and distilled water dialysis was observed. After dialysis, the urine was immediately frozen and stored until assayed. Routinely, analyses (for both DNase I and II) of a given thawed specimen were run the same day. The stability of DNase I activity after freezing and thawing was tested on 8 urines; there was no significant change in activity after one thawing; after subsequent refreezing and thawing, as much as 50% of the activity was lost.

III. *General DNase assay method*: A mixture of 1.0 ml of the appropriate buffer, 1.0 ml of the specimen,† 1.0 ml of DNA solution, and 1.0 ml of a specific protein solution such as casein was incubated at 37.5°C for varying lengths of time as noted below. One ml of 2.88 M TCA was added to terminate the incubation and the resulting suspensions were filtered through Whatman No. 40 filter paper (7 cm diameter). Appropriate controls were set up in which reagents were mixed at zero time or in which the reaction was terminated at 1½ hours (urinary DNase I and serum DNase I respectively). All determinations except the zero time controls were carried out in duplicate. Two ml aliquots of the filtrate were then mixed with 1.0 ml of indole solution and 1.0 ml of HCl, heated in a boiling water bath for 10 minutes, cooled to room temperature with the aid of a cold water bath, and extracted repeatedly (usually 2 or 3 times) with 6 to 8 ml aliquots of benzene (used in place of the chloroform of the Ceriotti method(2)) until the benzene layer was colorless. Extraction with benzene was necessary to remove any pink-colored material which otherwise interferes with the final colorimetric measurement. The benzene was removed each time with the aid of suction. After extraction, the somewhat hazy yellow aqueous layers were clarified by centrifuging (International type SB, No. 1) at 2000 rpm for 10 minutes, and read at 490 mμ with the Beckman spectrophotometer against a blank solution containing 2 volumes of the appropriate buffer, one volume of TCA, and one-half volume of water. Against this blank solution the reaction zero time blanks with urine had optical density values generally less than 0.050.

By comparison with the optical density value obtained for a 10 μg and a 20 μg deoxy-

† In the assay method for human urinary DNase I, 1.0 ml of dialyzed diluted urine was incubated for one hour with 1.0 ml of 1% casein. Since undiluted urine was too active as such, human urine was diluted 1:20 with distilled water.

TABLE I. Reactivity of Deoxynucleotides and Related Substances with the Indole Method.*

Substances reactive with indole			Substances slightly reactive with indole			Substances non-reactive with indole	
Substance		O.D.	Substance		O.D.	Substance	O.D.
Deoxyadenylic acid, diammonium salt	(8.05)	.882	Deoxyadenosine	(.15)	.024	Cytidylic acid	.004
			Deoxyguanosine	(.13)	.020	Cytosine	.004
Deoxyguanylic acid, calcium salt dihydrate	(7.85)	.740	Thymidine	(.09)	.015	Deoxycytidine · HCl	.006
			Deoxyuridine	(.07)	.012	Guanine	.003
Thymidylic acid, calcium salt dihydrate	(1.91)	.193	2-Deoxy-D-ribose	(.12)	.036	Glucoseamine · HCl	.002
			Fructose-6-phosphate, barium salt	(.14)	.014	Adenosine	0
						D-(−)-ribose	.003
						Adenine · SO_4	.004
Deoxycytidylic acid	(0.49)	.064	Hexose diphosphate, calcium salt	(.11)	.010	Glucose-1-phosphate, dipotassium salt	.001
			Ribose-5-Phosphate, barium salt	(.09)	.010	Glucose-6-phosphate, barium salt	.002
						Barium phosphoglyceric acid	0

* Optical densities of 40 μg standards are given. (Values in parentheses are computed micromolar extinctions.)

adenylic acid (dAMP) standard‡ treated identically and run with each set of unknown specimens, the activity of the specimen in question was calculated and the results expressed in units defined as follows: One *unit* of *DNase* activity is equivalent to that amount of TCA-soluble nucleotides liberated during the selected incubation time and reacting with indole to the same extent as 1.0 μg of dAMP. In the case of urine, activity may be expressed in units per hour of urine collection.

IV. *Reactivity of deoxynucleotides and related substances in the indole reaction*: A stock solution of 500 μg per ml of 10% TCA was made of the following (all obtained from California Corp. for Biochemical Research): (1) Deoxyadenylic acid, diammonium salt (dAMP). (2) Deoxyguanylic acid, calcium salt dihydrate (dGMP). (3) Thymidylic acid, calcium salt dihydrate (dTMP). (4) Deoxycytidylic acid (dCMP). Solutions of 5, 10, 20, and 40 μg of each of the above per ml of 10% TCA were made from the stock solutions. Two ml aliquots of these dilutions were then treated with indole according to the procedure described above.

In addition, a variety of substances (Table I) related to the nucleotides and nucleosides as possible hydrolytic products or as substances of similar chemical structure were tested for their reactivity with indole. Standard solutions of 10, 20, and 40 μg per ml of 10% TCA were made and treated with indole as described above.

Studies were carried out with dAMP to determine the effect of time of heating in the boiling water bath on optical density at 490 mμ and to ascertain to what extent the color reaction was dependent upon time of addition of indole. These experiments were as follows: (a) Duplicate 20 μg dAMP and dGMP standards were treated with indole and HCl as above and heated in the boiling water bath for different time intervals from 2 to 40 minutes. Duplicate 20 μg dTMP and dCMP standards were treated in the same way, except that they were heated in the boiling water bath for 10, 20, 30, and 40 minutes. (b) Duplicate 10 and 20 μg dAMP standards were heated with HCl alone for the same time intervals as above. At the end of these times, indole was added and they were heated for 10 minutes longer. (c) Duplicate 10 μg dAMP standards were incubated at 37.5°C with HCl for time intervals of 2 to 30 minutes. At the end of these times indole was added and the mixtures were then heated for 10 minutes in the boiling water bath.

‡ For this purpose a stock solution of 500 μg of dAMP per ml of 10% TCA was made and stored in the refrigerator where it remains stable for months. This solution was diluted with a solution of essentially the same composition as the reaction mixture minus DNA to give a final concentration of 10 μg and 20μg of dAMP per 4 ml of the final indole reaction mixture.

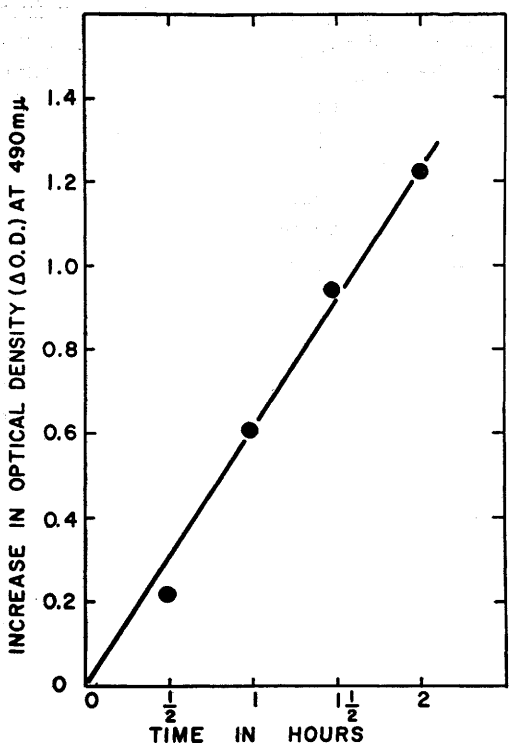


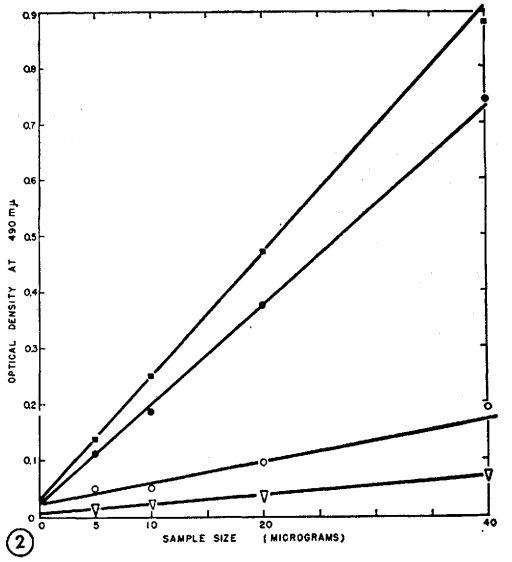
FIG. 1. DNase I activity (pH 7.5)—Human urine (1:20).

To compare the indole reaction with other methods for measuring urinary DNase activity, aliquots of the same TCA filtrates were examined by the diphenylamine method of Dische(6) and by the method of Schneider and Hogeboom(4).

Results and discussion. The reaction of indole with deoxyribonucleic acid first described by Dische(1) and used by Ceriotti (2) as the basis of a method for measuring DNA has been studied and affords a sensitive basis for quantitative measurement of the enzyme's urinary DNase I and DNase II. Fig. 1 shows the typical linear increase in TCA soluble hydrolytic products resulting from the action of human urinary DNase I on DNA as estimated by the indole reaction.

Early in the development of the indole reaction to measure urinary DNase activity, it was noted that the TCA filtrates were faintly hazy even in the case of the zero time controls, and that they gave poor duplicates and generally high values in the indole reaction. To precipitate what appeared to be colloidal DNA or apurinic acid, leaving only relatively low molecular weight fragments soluble in TCA, bovine serum albumin was added to the zero time controls and to incubation mixtures immediately before stopping the reaction with TCA. This gave crystal clear filtrates and consistently low zero time controls. After several other experiments with more readily available proteins, casein was chosen for use with the DNase I assay.

Fig. 2 reveals that in 10 minutes the purine



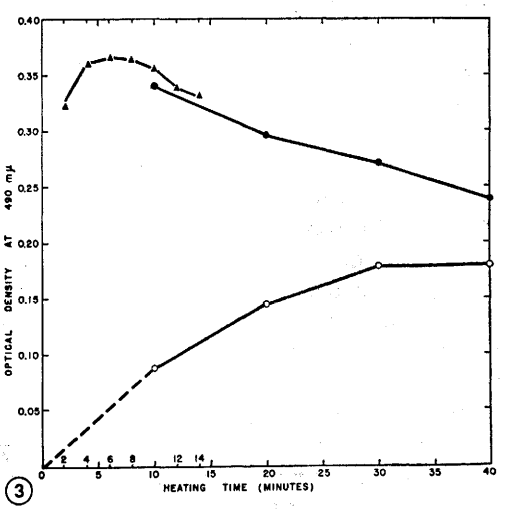


FIG. 2. ■—■ dAMP; ●—● dGMP; ○—○ dTMP; △—△ dCMP.

FIG. 3. ▲—▲ dAMP (20 μg/ml); ●—● separate determination of dAMP (20 μg/ml); ○—○ dTMP (20 μg/ml).

nucleotides react with indole to an extent more than 4 times as great as the pyrimidine nucleotides. When the absorption spectrum of the yellow solutions from the indole reaction with each of the nucleotides was plotted, identical curves were obtained with a prominent maximum at 490 to 495 mμ. These curves were similar to that described by Ceriotti(2) for DNA. Fig. 3 illustrates that maximum reaction of the dAMP standards heated with indole and HCl was reached in 4 to 10 minutes, and that further heating resulted in loss of activity. Fig. 3 also shows that with dTMP the extent of the indole reaction increases up to 30 minutes, then levels off. Although not shown in Fig. 3, dGMP and dCMP behaved qualitatively and quantitatively like dAMP and dTMP respectively.

Thus, where purine nucleotides have reached a maximum reaction within 10 minutes time, pyrimidine nucleotides do not yield a maximally intense color before 30 to 40 minutes. Since the color produced from purine nucleotides is destroyed to an appreciable extent by heating beyond 10 minutes time, the possible increase in sensitivity that one might anticipate from more protracted heating of the pyrimidine nucleotides is counterbalanced. This, in effect, explains why the 10 minute heating period selected by Ceriotti is optimal for DNA.

Heating dAMP with HCl alone from 2 to 40 minutes at 100°C completely destroys its reactivity with indole since further heating for 10 minutes after addition of indole yielded no characteristic color absorbing at 490 mμ. However, incubation of dAMP at 37.5°C with concentrated HCl alone, and then, after addition of indole, heating in the boiling water bath in the usual manner resulted in as much color development as was obtained in the samples treated in the routine manner.

The reactivity of the deoxynucleotides is in sharp contrast to the insignificant activity of the corresponding deoxynucleosides and to the minimal activity of 2-deoxyribose itself. Quantitatively, on a molar basis, the reactivity of 2-deoxyribose is less than 2% that of the purine nucleotides. The virtual complete lack of reactivity of the purine or pyrimidine bases and of the purine and pyrimidine nucleosides strongly suggests that the reactive substance derived from deoxyribonucleic acid is deoxyribose-5-phosphate or some precursor readily giving rise to deoxyribose-5-phosphate in the presence of HCl. The clear demonstration of the reactivity of an authentic specimen of deoxyribose-5-phosphate with indole in presence of HCl is necessary before this presumptive conclusion is proved.

In comparing the results obtained from the indole method with those obtained from the other methods tested, it was found that a given amount of DNA hydrolytic products gave a substantially greater optical density in the indole reaction than was observed with either of the other two methods. In an experiment using a rat spleen homogenate as a DNase source, optical density values obtained on the same aliquot of TCA filtrate from a given DNase reaction, with the indole reaction, Schneider and Hogeboom's technic and Dische's diphenylamine procedure, were 0.529, 0.386, and 0.130 respectively.

The reaction of indole with the deoxyribonucleotides and the failure of the corresponding nucleosides or free deoxyribose to react afford a basis for a simple sensitive quantitative test for measurement of deoxyribonucleotides in presence of the corresponding phosphate free compounds.

Summary. A method is described for measuring urinary deoxyribonuclease activity. The method is based on the reaction of indole in presence of HCl with the products of DNA cleavage. The reaction has been found specific for deoxyribonucleotides and occurs to an insignificant extent with deoxyribonucleosides, free purines or pyrimidines, deoxyribose, and a variety of sugar phosphates other than deoxyribose-5-phosphate.

The authors wish to thank Mrs. Leon L. (Elizabeth P.) Miller, Miss Gilda Guarasci, Mrs. Elizabeth Arrowsmith for their technical assistance, Dr. Thomas Koszalka for his advice, and Mr. Donald Gordon for providing us with normal serum.

1. Dische, Z., *Biochem. Z.*, 1929, v204, 431.
2. Ceriotti, G., *J. Biol. Chem.*, 1952, v198, 297.

3. Kowlessar, O., Altman, K., Hempelmann, L., *Arch. Biochem. Biophys.*, 1954, v52, 362.

4. Schneider, W., Hogeboom, H., *J. Biol. Chem.*, 1952, v198, 155.

5. Kay, E., Simmons, N., Dounce, A., *J. Am. Chem. Soc.*, 1952, v74, 1724.

6. Dische, Z., *Mikrochemie*, 1930, v8, 4.

Received October 17, 1960. P.S.E.B.M., 1961, v106.

Cardiac Temperature Gradients During Profound Hypothermia with Extracorporeal Perfusion.* (26307)

BERNARD FISHER AND E. J. FEDOR

Department of Surgery, Laboratory of Surgical Research, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

Whether or not cardiac temperature gradients during induction of hypothermia and rewarming are related to onset of ventricular fibrillation remains equivocal. That such differences in temperature do occur has been reported by Brooks(1), but no experimental data have been presented. He further added that " . . . such studies have not been carried far enough to justify statements other than that marked gradients are present when the heart is susceptible to fibrillation or is difficult to defibrillate." Prior to this Hegnauer (2) speculated that such gradients would be expected and might be responsible for ectopic activity in the ventricles. Because of the paucity of information relevant to cardiac temperature variations and fibrillation when either immersion or direct blood cooling was used to produce hypothermia, such a study was undertaken using the latter method in conjunction with extracorporeal perfusion.

Materials and methods. Fourteen mongrel dogs of both sexes, weighing 12 to 20 kg, were anesthetized with pentobarbital sodium, 25 mg/kg, attached to a Medtronic automatic respirator and connected to an extracorporeal system (consisting of a T. M.-2 Sigmamotor perfusion pump, a 13″ convoluted disc Kay-Cross oxygenator, and a gas type heat exchanger)(3) which permitted control of cooling and rewarming. The unit was primed with heparinized whole blood. An inflow catheter was inserted *via* a femoral artery into the aorta at the level of the renal arteries, and outflow catheters were placed in the vena cavae through femoral and jugular veins. Prior to cooling a thoracotomy was performed and 5 or 7 copper-constantan thermocouples† were implanted in various sites of the heart. Following closure of the thorax a similar thermocouple was passed orally into the mid-esophagus. The precise position of all thermocouples was confirmed by sacrifice of animals at conclusion of each experiment. Temperatures were automatically registered every 30 seconds on a Leeds-Northrup Speedomax Type G recorder. Variations of temperature as slight as 0.1°C could be detected. Cardiac activity was continuously monitored by a Sanborn Visoscope and recorded intermittently on a Sanborn Polyviso. The perfusion rate during cooling and rewarming was maintained at 50 cc/kg/min. Cooling was continued until the esophageal temperature registered 10°C, at which time rewarming was instituted. Perfusion was discontinued when esophageal temperature was between 25 and 30°C.

Results. Cardiac temperature differentials were noted in every animal studied. Four examples, each from a separate animal, representing the usual variations observed are shown in Fig. 1. Although temperatures were simultaneously obtained from sites other than those illustrated, for clarity of presentation they were omitted when they were similar or close to the ones that were used. While temperatures recorded from vari-

* Supported by U.S.P.H.S. grant, Dept. of the Army Contract and Southwestern Pennsylvania Heart Assn.

† Type NN-30-DT-Cu-Constantan-30 gauge BNS, Nylon Insulation—Thermo Electric Co., Inc., Saddle Brook, N. J.