A NEW METHOD FOR THE DETERMINATION OF DNASE II AND A COMPARISON WITH TWO OTHER ASSAY PROCEDURES*

T. R. KOSZALKA, S. HORNE, B. SCHLEGEL AND K. I. ALTMAN

Division of Experimental Radiology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. (U.S.A.)

(Received December 27th, 1958)

SUMMARY

A new method of assay for DNase II was developed based on the reaction of derivatives of desoxyribose with *p*-NPH. This new method was compared with two other existing procedures for the determination of enzyme activity, *i.e.*, the DPA and u.v. methods. All three procedures were found to be equally accurate. In contrast to the DPA and u.v. methods, the *p*-NPH procedure was found suitable for the assay of DNase II in subcellular particulates suspended in hypertonic sucrose. Examination of the assay conditions using the DPA method showed that optimal DNase II activity is obtained at pH 4.9 in the presence of 0.1 *M* sodium acetate buffer and 0.1 *M* NaCl. In the absence of NaCl, the maximal activity was somewhat lower and the pH optimum was extended from 4.9 to 4.8–5.6.

INTRODUCTION

Methods generally used for assay of the enzymic activity of DNase II have been based either (a) on the reaction of desoxyribose with diphenylamine, first used by DISCHE[1], or (b) on the absorption of light at 260 mμ by acid-soluble degradation products of DNA[2]. Since neither of these methods gave entirely satisfactory results in certain studies in this laboratory concerned with tissues homogenized in hypertonic sucrose, a new method of assay was developed based on the reaction of derivatives of desoxyribose with *p*-NPH which was suitable for use under these conditions. In this paper, this new method will be compared with the already existing procedures. In addition, the optimal conditions of pH, ionic strength and EDTA di sodium salt concentration for a partially purified enzyme preparation[3] used in all of these studies will be described.

EXPERIMENTAL

Reagents

(1) DNase II. A 5-fold purified preparation of DNase II was obtained by fractionation with $(NH_4)_2SO_4$ from aqueous extracts of fresh or frozen calf spleen[3].

DNase II = Desoxyribonuclease II; *p*-NPH = *p*-nitrophenylhydrazine; DNA = Desoxyribonucleic acid; DPA = Diphenylamine; EDTA = Ethylene diamine tetra-acetate; TCA = Trichloroacetic acid; u.v. = Ultraviolet.

References p. 202.

The sedimented fraction was taken up in H_2O and dialyzed against H_2O before use. (2) EDTA di sodium salt solution. A stock solution of 0.009 *M* EDTA di sodium salt was diluted 1:100 with water before use. (3) Stock solution of acetate buffer–EDTA di sodium salt mixture. Three vols. of 0.2 *M* sodium acetate buffer, pH 5.6, were mixed with one vol. of $9 \cdot 10^{-5}$ *M* EDTA di sodium salt. This mixture was stored at room temperature and dispensed by automatic pipette. (4) DNA. A 0.4 % aqueous solution of highly polymerized DNA from salmon sperm, purchased from the California Foundation for Biochemical Research, was used in all experiments. (5) Solution of acetate buffer containing EDTA di sodium salt and DNA. This mixture was prepared daily from 3 vols. of 0.2 *M* sodium acetate buffer, pH 5.6, 1 vol. of EDTA di sodium solution No. 2 and 1 vol. of 0.4 % DNA solution No. 4. (6) TCA. 2.88 *M* in aqueous solution. (7) DPA. A preparation, purchased from the Eastman Kodak Co. and used without further purification, was dissolved in a mixture of sulfuric acid and acetic acid to make a solution of the following final composition: 0.05 *M* DPA; 0.68 *M* sulfuric acid and 14.3 *M* acetic acid. (8) *p*-NPH. *p*-NPH purchased from the Eastman Kodak Co. was dissolved in ethanol as a 0.5 % solution was prepared daily for each series of determinations. (9) *n*-Butyl acetate. This solvent, obtained from the Eastman Kodak Co., was used without purification.

METHODS

The quantitative determination of DNase II by three different methods

1. *The incubation of DNase II with its substrate*: The same procedure for the incubation of enzyme and substrate was used for the three methods of DNase II assay. This incubation procedure which represents a modification of the procedure described by KOWLESSAR *et al.*[4] constituted the initial step in the determination of enzymic activity.

The complete enzymic assay system was composed as follows:

DNase II solution	0.5 ml
Acetate buffer, DNA, EDTA mixture No. 5	2.5 ml

In the control system 2.0 ml of acetate buffer (solution No. 3) was substituted for solution No. 5. This latter mixture, which was devoid of DNA, was incubated simultaneously with each complete assay system. Duplicates of each mixture were incubated for 3 h at 37°. After the reaction was stopped by adding 1.0 ml of 2.88 *M* TCA to all tubes, 0.5 ml of a 0.4 % DNA solution was added to the control system. After mixing, the suspensions were centrifuged for 10 min at 2,800 rev./min in a Model R-2 International Centrifuge. The resultant supernatant solutions were used for the final steps of the assay of DNase II. Henceforth, the supernatant obtained from the controls will be referred to as solution B whereas that from the complete system will be designated as solution A.

The aforementioned conditions of incubation were modified in the following manner for the study of DNase activity as a function of time. The complete incubation mixture (30–40 ml) was placed in the bath for 5 min and then maintained at 37° for 3 h. Aliquots of 3 ml were withdrawn at appropriate intervals and added to 1.0 ml of 2.88 *M* TCA. After mixing and centrifuging, the resultant supernatant solutions were analyzed by the DPA method.

References p. 202.

2. *Procedures used for the final steps of the enzyme assays. The DPA method.* To Folin-Wu tubes containing 1.0-ml aliquots of solutions A or B, was added 3.5 ml of DPA solution and the resultant mixture was placed in a boiling water bath for 20 min. After allowing 20 min for color development, the O.D. of the solution was determined in a Coleman Junior Spectrophotometer at 600 mμ. The enzymic activity is expressed either as the difference in O.D. (Δ O.D.) between solution A and solution B or as the O.D. of solution A.

The u.v.-absorption method. 0.5-ml aliquots of solutions A and B were diluted 20-fold with water. The O.D. of the diluted solutions was determined in a Beckman DU spectrophotometer at 260 mμ. DNase II activity is expressed as stated for the DPA method.

The p-*NPH method.* 3.0-ml aliquots of solutions A or B were diluted with equal vols. of water in tubes which, after being covered with a marble, were placed in a boiling water bath for 30 min. After cooling 2.0 ml of each solution were mixed with an equal vol. of 5 % TCA. 0.2 ml of the *p*-NPH solution was added and the mixture finally heated in a boiling water bath for 20 min. When cooled, the solution was extracted 3 times with 10 ml of butyl acetate and the organic phase discarded. If the organic phase still exhibited a yellow color after the third extraction, the treatment with butyl acetate was continued until the organic phase was colorless. To 3.0 ml of the remaining aqueous phase were added 2.0 ml of 0.1 *N* NaOH and immediately thereafter the O.D. at 560 mμ was determined in a Coleman Junior Spectrophotometer. Because of the instability of the color after the addition of alkali, no more than four samples were treated simultaneously with NaOH.

3. *Protein determinations.* Protein was determined by the method of LOWRY *et al.*[9].

RESULTS

A comparison of the three methods for the determination of DNase II

In the DPA and *p*-NPH methods, the enzymic activity is measured on the basis of a chromogenic product formed by the interaction of desoxyribose or its derivatives with the respective reagent. In the u.v. method the DNase II activity is measured in terms of an increase in the O.D. in the u.v. region due to the production of acid-soluble purine and pyrimidine-linked products of the enzymic hydrolysis of DNA.

A plot of DNase II activity in terms of concentration of DPA-reactive material formed *vs.* time of incubation is shown in Fig. 2. If the curves were extrapolated to the origin of the coordinate system, the slope representing the initial reaction velocity would be less steep than that representing the subsequent time course of the reaction. Thus, the initial reaction velocity suggests that a lag period exists similar to that reported for placental DNase II[5].

Plots of the DNase II activity *vs.* protein concentration of the test system have been drawn for each assay procedure (Fig. 1). A 3- to 4-fold change in concentration of the enzyme does not affect the linearity of the curve. Since the control measurements did not vary appreciably even at high enzyme concentrations, they may be omitted. The statistical analyses of the slopes of these curves reveal that the DPA and u.v. methods are equal in their analytical accuracy and sensitivity. The *p*-PNH method is twice as sensitive as the other methods but is accompanied by twice the

References p. 202.

error. Since the standard deviation from the mean in all three cases is small, the methods are equally accurate.

Optimal conditions for DNase II assay using the DPA method

A reinvestigation of the pH optimum of DNase II in the presence of monovalent cations was prompted by a recent report of KOERNER AND SINSHEIMER[6] indicating that Na^+ and K^+ provided maximal DNase II activity at pH 4.5. Using the DPA assay method, the optimal conditions of incubation were established with regard to three parameters, *i.e.*, ionic strength, pH and the effect of chelating agent with varying pH. The addition of Na^+ (as NaCl, final concentration 0.1 *M*) resulted in a moderate but significant activation of DNase II maximal at pH 4.9 (Fig. 3). It will be noted that the pH dependence curve of the sodium acetate test system devoid of NaCl was unchanged by the addition of EDTA di sodium salt. The pH optimum of this curve was 4.8–5.6.

DISCUSSION

All three methods described were satisfactory for the assay of DNase II in the 5-fold purified enzyme preparations used here as well as in crude extracts of various rat tissues[7].

For active preparations of DNase II in any state of purity, the incubation time may be decreased from 3 h to 1 h or less. The relatively long incubation time of 3 h used in the present experiment is necessary when the activity of DNase is low. Although the conditions for incubation were nearly optimal for DNA hydrolysis by relatively crude splenic DNase II preparations, the experimental conditions would probably require modification with respect to incubation time and Na^+ activation when assaying more highly purified enzyme preparations.

Although the u.v. assay method is adequate for most purposes, certain limitations as to its applicability should be pointed out. First, any substance absorbing light in the range of 260 mμ will interfere with the measurements; secondly, light scattering due to slight turbidity may be the cause of serious errors in the spectral reading. Finally, unlike the DPA and the *p*-NPH procedures, the u.v. method is characterized by high O.D. in solution B.

The DPA and *p*-NPH methods for the determination of DNase II have been found suitable for rapid serial analyses of DNase II and are specific for purine-linked desoxyribose. Since sucrose reacts with DPA by forming colored products, the DPA method is unsuitable for studies of DNase II in subcellular particulates. Such a limitation does not apply to the *p*-NPH method which can be used in the presence of sucrose. Also, as WEBB has shown[8], most substances present in biological fluids do not interfere with the determination of DNA by this procedure*. A disadvantage of the *p*-NPH method is that the color produced by the addition of alkali fades rapidly. WEBB[8] has reported that the color intensity decreases at the rate of 2 %/min for the first 5 min. However, the instability of the color is not a serious disadvantage of the method if the O.D. is determined after a constant time interval has elapsed.

The chemical reactions involved in the DPA and *p*-NPH methods have been

* The *p*-NPH assay procedure can probably be applied to the determination of DNase I activity by changing the conditions of enzyme incubation and composition of the assay system.

References p. 202.

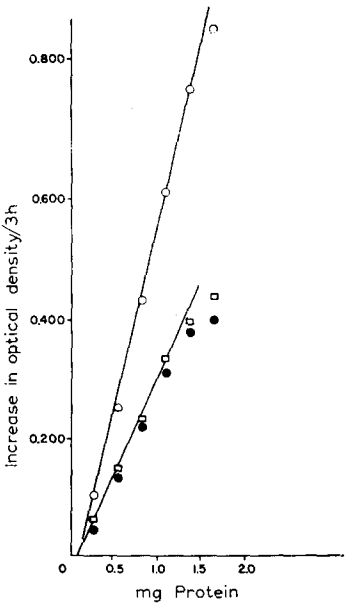

Fig. 1. Activity of purified splenic DNase II as a function of enzyme concn. The ordinate shows the increase in optical density as measured by the DPA (-●-), the *p*-NPH (-○-) or the u.v. (-□-) methods.

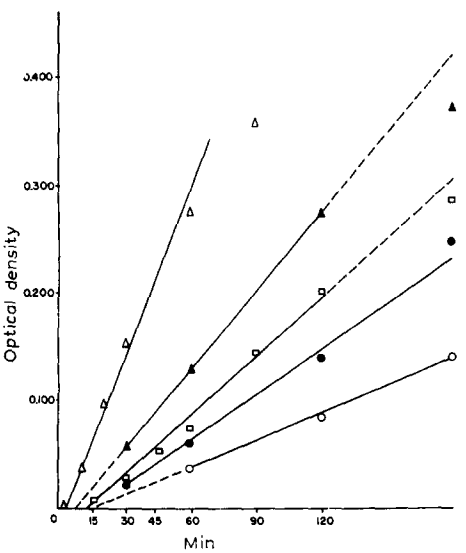

Fig. 2. Extent of enzymic hydrolysis of DNA as a function of time and enzyme concn. The reaction was carried out in the presence of $1.0 \cdot 10^{-1}$ *M* sodium acetate buffer pH 5.6, $1.5 \cdot 10^{-5}$ *M* EDTA di sodium salt, 0.067% DNA and varying amounts of enzyme prepared from spleen. The total protein concns. in the reaction mixtures are represented as follows: △, 0.94 mg/ml; ▲, 0.47 mg/ml; □, 0.37 mg/ml; ●, 0.28 mg/ml; and ○, 0.19 mg/ml. All determinations were carried out by the DPA method.

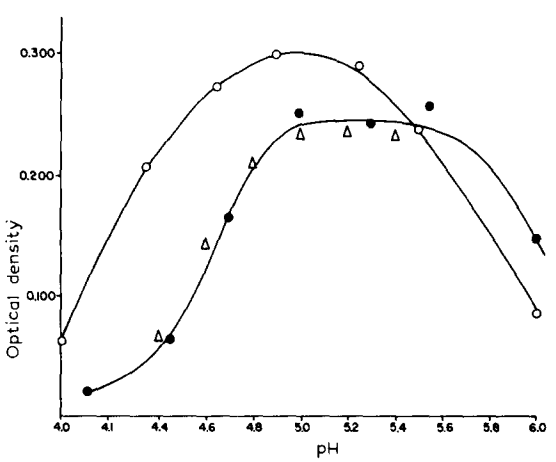

Fig. 3. Activity of Purified DNase as a function of pH. No additions (-△-), $1 \cdot 10^{-1}$ *M* NaCl (-○-), $1.5 \cdot 10^{-5}$ *M* EDTA di sodium salt (-●-). All determinations were carried out by the DPA method. Sodium acetate buffer ($1.0 \cdot 10^{-1}$ *M*) was present in all instances. All concns. refer to the final concns. in the complete system.

References p. 202.

found to yield identifiable end products with the expected chromogenic properties[7]. These end products proved to be derivatives of desoxyribose. In the case of the *p*-NPH method a single derivative of desoxyribose was detected, namely, the phenylhydrazone derivative. In the case of the DPA method, however, free desoxyribose, as well as a second unidentified diphenylamine reactive substance, was found in the incubation system. These findings will be published in the near future.

ACKNOWLEDGEMENT

This work was supported, in part, by a grant from the United States Atomic Energy Commission.

REFERENCES

1 Z. DISCHE, *Mikrochemie*, 8 (1930) 4.
2 W. C. SCHNEIDER AND H. G. HOGEBOOM, *J. Biol. Chem.*, 198 (1952) 155.
3 T. R. KOSZALKA, R. FALKENHEIM AND K. I. ALTMAN, *Biochim. Biophys. Acta*, 23 (1957) 647.
4 O. D. KOWLESSAR, K. I. ALTMAN AND L. H. HEMPELMANN, *Arch. Biochem. Biophys.*, 52 (1954) 362.
5 S. BRODY, *Acta Chem. Scand.*, 7 (1953) 721.
6 J. F. KOERNER AND R. L. SINSHEIMER, *J. Biol. Chem.*, 228 (1957) 1039.
7 T. R. KOSZALKA, unpublished.
8 J. M. WEBB AND H. B. LEVY, *J. Biol. Chem.*, 213 (1955) 107.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

REDUCTION OF CERTAIN AROMATIC ACIDS TO ALDEHYDES AND ALCOHOLS BY *POLYSTICTUS VERSICOLOR*

V. C. FARMER, MOIRA E. K. HENDERSON AND J. D. RUSSELL

Macaulay Institute for Soil Research, Aberdeen (Scotland)

(Received December 1st, 1958)

SUMMARY

1. *Polystictus versicolor* reduced the following acids to the corresponding aldehydes and alcohols: *m*-, *p*-methoxybenzoic, 3:4-dimethoxybenzoic, β-naphthoic. Only the alcohols were detected from *o*-methoxybenzoic and benzoic acids. *o*- and *p*-Hydroxybenzaldehydes were reduced to alcohols. The following acids were not reduced: 2:4-dimethoxybenzoic, phenylacetic and α-naphthoic.

2. The rate of conversion varied with the position and nature of ring substituents.

3. There was a small amount of demethoxylation of the following with production of the corresponding hydroxy acids: *o*-, *m*- and *p*-methoxybenzoic and 2:4-dimethoxybenzoic acids. The following were hydroxylates in the *para* position: cinnamic, benzoic, and phenylacetic acids, the last two in only small amounts. Some breakdown of the aromatic structure occurred during metabolism of cinnamic, β-naphthoic, *p*- and *o*-hydroxybenzoic acids.

4. The fungus produced an extracellular alcohol dehydrogenase.

References p. 211.