Methods of BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

VOLUME 9

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PREFACE TO THE SERIES

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need for material achievement to keep in sight of the advance of useful ideas.

The current volume is the first of a series which is designed to try to meet this need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological and, if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

DAVID GLICK

Minneapolis, Minnesota
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Assay of DEOXYRIBONUCLEASE ACTIVITY*

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I. INTRODUCTION

Although depolymerases for deoxyribonucleic acid (DNA) have been found widespread in nature, their function remains unclear. The enzymes have been found in all animal (2,3,129,133,147) and plant (98) (including bacteria (119,130,149) and viruses (31,72,113, 154,155)) cells in which they have been sought (70) except the human leucocyte and its precursors (73), and in many secretions, including serum (19,44,78,81,85,107,153), bacterial filtrates (55,114,144,145), pancreatic juice (1,25,32), bile (82), and snake venoms (10,26,140). The deoxyribonucleases (DNase) from different species can be differentiated immunologically by the preparation of specific antisera (104,144), and by their response to various intracellular inhibitors. The enzymes from different sources differ also in pH optima, but fall generally into two groups, one with a pH optimum between 4.2 and 5.5 (2,13,22,87,99,135,148), designated DNase II by Cunningham and Laskowski (22), and the other with a pH optimum in the vicinity of 7.5 (DNase I) (71). Most cells contain representatives of both types of enzyme (22,133). The alkaline enzyme appears to be destroyed in preparations treated with organic solvents such as acetone (22,133). The electrolyte requirements differ for the two groups also, a fact which is reflected in the assay method. In general the acid enzymes require the presence of low concentrations of electrolytes. Various electrolytes are effective activators—$Mn > Ba > Sr > Ca > Mg > Co > K > Rb > Cs > Na > Li$, and $Cl > Br > No_3 > I > SO_4$—and the optimum concentration is a function both of the electrolyte and pH (87). On the other hand, the alkaline enzyme is activated most effectively by Mg^{2+}, with few exceptions. *Cl. septicum* is said to produce an enzyme with pH optimum 7, not activated by Mg^{2+} (133), while various micrococci produce an enzyme with optimum activity at pH 8.6, which requires Ca^{2+} for activation

(149). Biologically occurring inhibitors have been reported for the alkaline enzyme by a number of investigators (8,9,17,20,23,49,50, 68,84,90,138,157). In the case of the bacterial cells it appears to be a ribonucleic acid (8,9). In the case of the animal cells, an inhibitor with a species specificity, which is stable in the frozen state and withstands heating to 60°C., has been identified as a protein (90). Another, which is very unstable and is nonspecific, differs from the preceding inhibitor in its distribution (49,50). An inhibitor of the acid DNase has been detected in human urine (67) and in horse leucocytes (133). Changes in the intracellular content of the enzymes and their inhibitors have been correlated with X-irradiation injury (30,84, 84a,111), with age of tissues (2,84,133), and with phage infection of bacteria (113). The mystery of the function of the enzymes in the metabolism of DNA is further complicated by the fact that both the enzymes and inhibitors appear to be cytoplasmic (mitochondrial) (124), where DNA is not found (although deoxyribose and mononucleotides of deoxyribose have been identified). A number of investigators (13,91,92,102) have localized the enzymes in the cell nucleus in mammalian cells. Their experience is contrary to the current consensus; it should be noted that the enzyme is readily soluble, and its extraction from an original intranuclear site or the contamination of nuclear preparations cannot be excluded. Thus, Van Lancker and Holtzer (142) found acid DNase in all fractions of mouse pancreas, including nuclei, mitochondria, microsomes, and supernatant.

The methods of assay may be classified according to their dependence on (*1*) increase in hydrogen ion concentration during the course of the hydrolysis of the phosphate bonds; (*2*) changes in the physicochemical characteristics of the substrate (viscosity, sedimentation constant, ultraviolet absorption, solubility, flow birefringence, dielectric constant, light scattering); (*3*) change in affinity of the substrate for methyl green and proteins; (*4*) detection of the products of depolymerization by (*a*) assay of dialyzates, (*b*) acid-soluble products, or (*c*) alcohol- and acetone-soluble products; and (*5*) changes in biological activity. Assay of inhibitors may be performed by most of these methods by duplicate assay of DNase activity, one with and the other without the added inhibitor. In the discussion of each method we shall note if assay for the inhibitor introduces any complications. This subject has been reviewed previously by Schmidt (122).

II. ASSAY METHODS BY MEASUREMENT OF INCREASE IN HYDROGEN ION CONCENTRATION

The hydrolysis of an internucleotide link—i.e. a sugar-phosphate link—results in release of a secondary phosphoric acid group and thus an additional dissociable hydrogen ion. Several methods have been proposed to follow this change.

1. Manometric

The method of Bargoni (5) depends upon the release of carbonic acid from a bicarbonate solution adjusted to pH 7.3–7.4. The released carbonic acid is determined in the Barcroft-Warburg apparatus.

The pH range in which this method is applicable is very limited. It is not applicable to the determination of acid DNase activity. The author notes that the enzyme (alkaline DNase) is inhibited by magnesium chloride, which is contrary to the experience with other methods. This observation suggests that the formation of insoluble carbonates causes spurious results. The presence of other buffers, as may occur in tissue homogenates, would presumably also interfere with the method. The presence of other hydrolytic enzymes such as phosphatases and proteases would also contribute to the formation of hydrogen ions by action on nucleotides produced by the DNase (123) and on substrates present in the homogenates, thereby vitiating the results.

Zittle (158,159) has described a manometric method similar to Bargoni's. He performed the reaction in the presence of dilute silver nitrate, added to inhibit adenosine deaminase.

2. Indicator Dyes

Cavalieri and Hatch (18) measured the change in optical density in *p*-nitrophenol phenolate buffer, 10^{-3} *M*, pH 7.1. This is near the pK for this buffer. The change in optical density is determined at 440 mμ. Since the pH is permitted to fall during this reaction, the rate of the reaction would not be expected to be constant. This effect is minimized by limiting the pH change to 0.37 pH unit, which, however, is not an insignificant fall in pH. As with the preceding method, the applicable pH range is limited to that for the assay of the alkaline DNase enzyme only, and then not at the optimum for it.

The authors consider that at pH 7 competition by secondary phosphate groups for the liberated hydrogen ion would be negligible because of the pK of 6 for such groups, resulting in 90% dissociation at pH 7. The authors note that substrate inhibition causes the curve of hydrogen ion release versus time not to be straight. They also observe that at high and low substrate concentration the reaction rate is not proportional to the enzyme concentration; i.e. an optimal substrate concentration is found. The authors ascribe this to "alterations in the macromolecular structure of DNA" as a function of its concentration in solution.

Khouvine and Grégoire (60) and Grégoire (45) described an indicator system similar to the preceding with phenol red instead of nitrophenol. They followed the decolorization of the indicator as a measure of release of H^+ ions. Allfrey and Mirsky (2) attempted to apply a similar method for the determination of acid DNase by using indicators in the acid range, such as brom-cresyl green, but without success. The method of Khouvine and Grégoire (60), modified by Allfrey and Mirsky (2), is performed as follows: the substrate consists of 1 ml. of 2 mg./ml. DNA + 1 ml. of 0.1*M* $MgSO_4$ and 0.4 ml. of phenol red solution (10 mg. of phenolsulfonphthalein, 0.28 ml. of 0.1*N* NaOH, 3 ml. of 95% ethanol, 40 ml. of 0.067*M* pH 7.55 phosphate buffer, made up to 100 ml. with H_2O). The mixture is adjusted to pH 7.55 with 0.01*N* NaOH and to a final volume of 3 ml. To this is added 1 ml. of the enzyme solution. The test is carried out in the spectrophotometer cuvettes in a Beckman photometer equipped with constant temperature sample holder at 25°C. The slope of the curve of the optical density at 558 mμ vs. time is the measure of DNase activity. The authors point out that possible errors arise from the competition of buffers present in the tissue homogenates used as enzyme source for the released H^+ and the adsorption of the indicator dye by constituents of the enzyme preparation.

3. Titrimetry

A. PH STAT

Schumaker, Richards, and Schachman (125) used a "pH stat" with unbuffered substrate containing 0.2*M* sodium chloride and 0.03*M* magnesium sulfate at pH 7.6. This apparatus maintained the pH at 7.6 automatically by the continuous addition of dilute alkali.

A plot of the volume of alkali added vs. time gave a slope proportional to the enzyme activity. Because of the competition for the released hydrogen ions from the secondary phosphate groups and other buffers, this method is applicable also only in the alkaline pH range. It has the advantage over the preceding methods of not permitting the pH to change during the course of the reaction, but otherwise shares their disadvantages. Thomas (141) used a similar apparatus in following enzymic hydrolysis.

B. INTERMITTENT TITRATION

Carter and Greenstein (15) used intermittent titration, as well as other methods. This is identical in principle to the pH stat method, but has the disadvantage of permitting pH to change during the reaction. Fischer *et al.* described a titrimetric method which they stated was satisfactory for pure enzyme preparations (34), but not for crude tissue extracts. Kunitz (71) used a similar method with intermittent titration at pH 7.5 (phenol red indicator). He found that the titration curve was not linear with time. Kunitz carried out the procedure as follows: 4 ml. of 0.5% DNA + 0.5 ml. of 0.3*M* $MgSO_4$ + 1 drop of 0.1% phenol red + a trace of 0.02*M* NaOH to pH 7.5 + 0.1 ml. of 0.01% crystalline DNase—incubate at 25°C., and titrate with 0.02*M* NaOH at intervals.

Methods which depend upon the determination of hydrogen ion release would appear to be limited to systems that contain only DNA, a pure enzyme, and electrolytes. The presence of other reagents, such as proteins, which might bind hydrogen ions and of other enzymes which might release hydrogen ions from the partial breakdown products of the DNA or from other substrates would interfere with this method. The method is also limited in the pH range to which it may be applied, and therefore is suitable for the assay only of the alkaline DNase. Automatic titration would appear to be the most favorable application of the method.

III. PHYSICOCHEMICAL METHODS

1. Viscosimetry

Viscosimetric methods have been popular since their introduction by P. de la Blanchardière (25) and in principle have remained the same as described by him. He observed that DNA solutions exhibited a marked drop in viscosity when exposed to certain enzyme systems. Capillary viscosimeters have been most commonly used (43,93).

As pointed out by Holoubek and Hupka (53), viscosimetric methods are influenced by the contribution to the fall in viscosity by the interaction of other enzymes and substrates in the homogenate. These authors also point out that highly polymerized DNA is not suitable for capillary viscosimetry because the rate of fall affects the orientation of the asymmetric DNA molecules, resulting in an apparent reduction in viscosity with increased rate of flow, and therefore an apparently greater reduction in viscosity than actually occurs: as the fall in viscosity occurs due to DNase action, the rate of flow, through the capillary increases, resulting in greater orientation of the remaining asymmetrical DNA molecules, and hence greater apparent enzymatic depolymerization than had actually occurred. This anomalous viscosity of solutions of DNA and other similar asymmetric molecules is well known, and it is recognized that the concentration dependence of viscosity is non-Newtonian. These authors furthermore point out that in the capillary viscosimeter the rates of flow are not uniform in the solution, being most rapid at the capillary wall and slowest at the center of the column. They therefore recommend the use of a rotating viscosimeter which keeps the rate of flow constant and very slow. The method appears to us to obviate very few of the objections to the capillary viscosimeter, however. The effect of the other substrates and enzymes, proteins, and electrolytes would not appear to be obviated at all, and that of orientation due to flow would be only somewhat reduced in significance. Indeed, in a subsequent paper, Holoubek (52) observed that the hyaluronic acid content of chicken sarcoma was so high as to give a homogenate with a viscosity greater than that of the DNA substrate. Clearly, the presence of hyaluronidase would have seriously impaired the validity of the method.

Greenstein and Jenrette (44) observed a marked reduction in viscosity of DNA solutions by electrolytes, urea, amino acids, and proteins. Greenstein (41) attempted to calculate a correction for the protein effect by using an albumin solution with nitrogen content equivalent to that of tissue homogenates. However, he found that the procedure was unreliable and gave results which were not proportional to the enzyme concentration. Laskowski and Seidel (93) have also recognized the limitations of the method and recommend it only for pure systems. The method was successfully applied by McCarty (103) to purified enzymes and to mixtures containing 0.2–0.8% serum.

A typical viscosimetric method is that of McCarty (104). The substrate consists of 4 ml. of 0.1% DNA in 0.005*M* magnesium sulfate and 0.025*M* veronal buffer, pH 7.5, to which is added 0.5 ml. of enzyme solution. The viscosity is determined in an Ostwald viscosimeter immersed in a 37°C. water bath. Repeated determinations are performed at timed intervals and the curve plotted. Siebert, Lang, and Corbet (132) used the same method, but plotted per cent reduction in viscosity in given time, thereby allegedly making the method independent of changes in the initial viscosity. This conclusion would appear not to be valid, since the rate of depolymerization is not directly proportional to the original substrate concentration. Indeed, high substrate concentration is inhibitory (79). In practice, the authors obviated this difficulty by working with a constant DNA solution and adjusting the enzyme concentration so as to make the activities similar. They also used the time required for a 50% reduction in viscosity, which allegedly permits comparison of very different enzyme activities. Haas (46) and Henstell and Freedman (48) also adopted this method. Again, the time required for 50% reduction in viscosity as a measure of enzyme concentration is valid only if the identical substrate preparation and concentration are used, since different lots of DNA differ in viscosity. Also, the enzyme solutions must affect the initial viscosity (i.e., nonenzymic effect) equally.

Sherry, Johnson, and Tillett (130) used a viscosimetric method in which 0.1 ml. of enzyme was added to 2.5 ml. of a substrate pH 7.4, with a relative viscosity of 3.5, consisting of 0.5–0.2% DNA (prepared according to the method of Mirsky and Pollister (105)) dissolved in 0.40*M* barbital buffer and 0.003*M* magnesium sulfate. One unit of enzyme activity was defined as that which caused a drop of 1 viscosity unit in 10 minutes at 37°C. The substrate used by these authors was heated at 56°C. for 2 hours to destroy deoxyribonuclease activity prior to use. It should be noted that this temperature may cause some denaturation of the substrate, with consequent loss of affinity for the enzyme (80). However, with a given substrate, the authors found that the slope of the viscosity curve was linear with enzyme concentration. The DNase of streptococcal origin was found to have a temperature optimum of 45°C. and a pH optimum of 8–8.5. Sherry *et al.* were able to study the pH optimum by adding increments of 3% acetic acid over the pH range 5.2–9.0.

Zamenhof and Chargaff (157) used a very similar method, with smaller volumes, in a microviscosimeter of the Ostwald type. Like McCarty (103), they stabilized the enzyme solution by the addition of gelatin, 0.01%. They operated at pH's in the vicinity of 5.5–6.5, indicating the applicability to the acid range. However, pH does have a significant effect on DNA viscosity, and at sufficiently low pH precipitation occurs. Also, as noted previously, the gelatin influences the viscosity.

Holoubek and Houpka (53) used a substrate consisting of 8 ml. of 0.1% DNA in distilled water. To this they added 2 ml. of enzyme solution which was prepared by homogenizing 3 g. of rat liver in 4 volumes of water in the Potter-Elvehjem grinder, allowing this to stand for 16 hours at 3°C., and centrifuging 30 minutes at 2500 r.p.m. The mixture was placed in a rotating viscosimeter at 24°C. At measured intervals, starting from zero time, the viscosity was determined, and the log of the viscosity vs. time was plotted for the first 5–6 minutes. These workers noted rapid reduction in the rate of the reaction with time, which they attributed to inhibition of the enzyme by the products of depolymerization, since the addition of a dialyzate from a DNA–enzyme mixture to a fresh DNA–enzyme mixture caused inhibition of the reaction. The reaction is therefore measured during the very early period, before inhibitory products accumulate significantly.

Laskowski (95) compared viscosimetric methods to the acid solubility method (see Section V.2). He found that the two methods did not give parallel results. He noted that reducing agents increase the activity of the enzyme as measured by viscosimetry, but inhibit the release of acid-soluble phosphorus compound. The same enzyme compared against different batches of DNA prepared by Hammarsten's method (47) gave striking differences between the activities as measured by the two methods. He attributed the effect of reducing agents to their action on the substrate, making it more susceptible to reduction of viscosity. Furthermore, at high concentration of cysteine and cyanide there was a fall in viscosity with time without the addition of enzyme, which suggested a chemical reaction which progressed slowly. Surprisingly, peroxide was noted to stabilize the enzyme during storage, and cystine enhanced the activity of the enzyme as measured by production of acid-soluble phosphorus, but had no effect on its activity as measured by viscosity.

Since reducing agents cause progressive, nonenzymic reduction

of the viscosity of other polymers such as hyaluronic acid, starch heparin, and chondroitin sulfuric acid (136), it may be that the apparent activation of DNase as measured viscosimetrically by Laskowski (95) reflects simultaneous slow nonenzymic denaturation of the DNA. Denaturation, in fact, inhibits DNase activity (80), which is consistent with the diminished DNase activity in the presence of reducing agents observed by Laskowski by the acid-soluble method. The slow reduction in viscosity by nonenzymic reagents imposes a further limitation on the applicability of viscosimetric methods.

Greenstein *et al.* (42) used a capillary (Bingham-Jackson) viscosimeter in which the external pressure was maintained constant at 16 cm. of water. The relative viscosity was plotted against time, and the enzyme activity measured from the slope of the curve. No buffer was used other than that present in the tissue extract itself, which maintained a pH of 6.4–6.8. Since this pH is not optimal for either the acid or the alkaline DNase, it is probable that both were active at suboptimal levels, at least when Mg^{2+} was present. Greenstein *et al.* observed that adjustment of the pH to 7.8–8.0 by the addition of $NaHCO_3$, without Mg^{2+} caused inhibition. This effect may be ascribed to the fact that at pH 7.8–8.0, acid DNase is inactive, while the alkaline DNase requires Mg^{2+} for activation rather than to a specific effect of bicarbonate ion.

Schumaker, Richards, and Schachman (125) also used a viscosity method, but employed for each determination several viscosimeters with different sheer gradients and extrapolated to zero sheer gradient. Theoretically this technique should overcome the effect of orientation due to rate of flow. However, since the rate of flow is not uniform in the cross section of the capillary, it is doubtful that this purpose is achieved.

As noted, various methods have been suggested for quantitative determination of the velocity of the enzymic action as measured viscosimetrically. One index is the time required to reduce the viscosity to one-half the initial level. Another uses the slope of the curve of viscosity plotted against time. A third (29) defines the unit of enzyme activity as that which causes a drop of 1 viscosity unit (defined by the flow time of water) in 10 minutes at 30°C. Warrack, Bidwell, and Oakley (145) used the flow-time function f_e defined by Swyer and Emmens (139):

$$v = 1000(f_s - f_e)/(f_s - f_0)$$

where f_0 is flow time for buffer, f_s is flow time for substrate, f_e is flow

time for substrate enzyme mixture. They found that this function, plotted against enzyme concentration, gives an S-shaped rather than a linear curve.

As with other methods, so with viscosimetry, the definitions of units of enzyme activity are as numerous as the investigators. No efforts have yet been made to standardize the unit.

2. Change in Ultraviolet Absorption

Kunitz (70) observed that during the course of depolymerization of DNA by DNase the optical density increased throughout its ultraviolet absorption spectral range, most markedly at 260 mμ, where an increase of nearly 30% occurred with complete depolymerization. By plotting the optical density at 260 mμ against time, the enzyme activity is determined. The substrate consists of 2 ml. of 2 mg./ml. DNA in water plus 10 ml. of 0.05*M* magnesium sulfate + 10 ml. of 1.0*M* acetate buffer, pH 5.0, plus water to 100 ml. To 3 ml. of this substrate, 1 ml. of water is added; this serves as the blank. To another 3 ml. sample of substrate, 1 ml. of the enzyme solution is added. The optical density at 260 mμ is read every 0.5–1 minute for 5 minutes in a spectrophotometer, preferably with a thermostatically controlled sample holder. Following a 1–2 minute lag, which is greater at lower enzyme concentrations, the curve is linear. Since Kunitz used the crystalline alkaline (bovine pancreatic) DNase, the pH he selected is far from optimal. The method may, however, be used at other pH's and electrolyte concentrations. Since it is limited to pure systems, it is not readily applicable to tests for inhibitors.

Since the increase in optical density is now known to be a function of cleavage of hydrogen bonds linking the purine of one DNA chain to the pyrimidine of the complementary chain (120,141), it is apparent that native (undenatured) DNA must be used as the substrate. Similar increase in UV absorption occurs with other means of denaturation, such as heat (77). The method is very sensitive, but is unsuitable in the presence of other ultraviolet-absorbing constituents, such as are commonly present in tissue homogenates. It is limited, therefore, to pure systems, such as the crystalline alkaline DNase system which Kunitz used. Thus, Kovacs (65) found that the method was not applicable to the determination of DNase in spinal fluid because "autolytic changes in the optical density of the cerebrospinal fluid which have been largest in the region of 2600–2650 A" interfered with the interpretation of the results.