METHODS OF BIOCHEMICAL ANALYSIS

Volume IX

METHODS OF
BIOCHEMICAL ANALYSIS

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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.

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Minneapolis, Minnesota
January, 1954

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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):

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

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.

3. Precipitation of Remaining Substrate

A number of methods dependent upon the fact that polymerized DNA is insoluble in acid alcohol, or acetone have been described. These are primarily qualitative methods; the solubility principle has been more effectively applied in the converse, namely, the measurement of soluble products of depolymerization (see Section V.2).

Gilbert, Overend, and Webb (37) described a quantitative assay method based upon the measurement of the mass of substrate which remains insoluble following enzyme action. To 2 ml. of 2% DNA in water + 1 ml. of 0.1*M* magnesium sulfate + 1 ml. of 0.1*M* veronal buffer, pH 7.0, were added 0.5 ml. of enzyme solution + 0.5 ml. of water or a solution to be tested for inhibitory activity. This was incubated for 16 hours at 37°C., then cooled to 0°. To a 4 ml. aliquot, 0.1 ml. of 5*N* HCl was added, and the precipitate was centrifuged, washed with ethanol and ether, dried *in vacuo*, and weighed. The loss of weight, as compared to a control which contained no enzyme, indicated the DNase activity. Of course, if the enzyme solution contains other acid-precipitable constituents, such as proteins—and particularly if these also change in amount during the course of the digestion, owing, for example, to the presence of proteolytic enzymes—the method becomes invalid as a test for DNase activity. This method, like the UV absorption method, is therefore applicable to pure systems and fraught with risks in crude systems.

Weckman and Catlin (149) used a semiquantitative test. This depended upon the determination of the length of incubation time necessary to prevent the formation of a fibrous precipitate when a drop of the DNA–enzyme mixture was allowed to fall into 90% acetone. A very similar procedure had been developed by McCarty (104). McCarty incubated 0.5 ml. of 0.1% DNA in 0.005 *M* magnesium sulfate in 0.025*M* veronal buffer, pH 7.5, with 0.5 ml. of enzyme solution for 30 minutes at 37°C., then added 1 ml. of ethanol, and examined for the presence of a floating fibrous precipitate to indicate undigested DNA. Carter and Greenstein (15) incubated a solution consisting of 1 ml. of 0.5% DNA and 1 ml. of tissue extract, at 37°C. for 1–3 hours, added 0.2 ml. of 0.1*N* HCl, and then determined the turbidity. With a highly polymerized substrate, however, the precipitate is quite cohesive and cannot be readily dispersed to permit turbidimetry.

Oakley and Warrack (108) used a method similar to that described by Burnet (14) for hyaluronidase. This depends on the formation of a blue "blob" (a cohesive gelatinous mass) when a mixture of DNA solution and congo red is dropped into 1% HCl in 70% alcohol. They incubated 0.5 ml. of a 0.13% solution of highly polymerized DNA in 0.025*M* veronal buffer pH 7.5, containing 0.003*M* Mg^{2+} with 1.5 ml. of the enzyme solution for 1 hour and determined the highest dilution of enzyme which would prevent the "blob' formation. They found that lower concentrations of DNA were unsuitable because of difficulty in reading the formation of the "blob." They also found that the test was complicated by the presence of proteins which precipitated on addition to the acid–alcohol.

A simple semiquantitative method for determining DNase activity in bacteria was described by Jeffries, Holtman, and Guse (56). An agar medium containing 2 mg./ml. DNA is used for culturing the organisms. After a suitable period of incubation the plate is flooded with 1*N* hydrochloric acid. Precipitation of the DNA produces a cloudy medium, with a clear zone surrounding colonies which produce DNase. The width of the clear zone is a rough measure of DNase production. The method was modified by DiSalvo (28), by the addition of 0.8 mg. of calcium chloride per ml. This is based on the observation by Weckman and Catlin (149) that the DNase of micrococci is activated by calcium ion, with a pH optimum of 8.6. However, since this pH is inhibitory to bacterial growth, the reaction does not occur at optimum pH in the agar plate method.

All the methods based upon the formation of precipitates by the fraction of the DNA which remains insoluble in acid or organic solvents following action on the enzyme, suffer from the disadvantages of error introduced by other precipitable constituents, particularly if these change in amount during the reaction, and by the interference by gelatin, neopeptone, and probably other proteins with the precipitation of DNA by strong acid (71). This interference, as well as the precipitation of other constituents such as mucopolysaccharides and proteins, may vary with different acids, any of which may be used, including hydrochloric acid, sulfuric acid, perchloric acid, trichloroacetic acid, etc.

4. Change in Sedimentation Constant

Rabatin, Friedland, and Frajola (118) have described the assay of DNase activity by use of the ultracentrifuge. These authors centri-

fuged, at intervals, aliquots of a solution consisting of 5 ml. of 1.1% deoxyribonucleoprotein dissolved in 1*M* sodium chloride + 0.5 ml. of 0.1*M* magnesium sulfate + 0.5 ml. of a 0.012% crystalline DNase solution (Worthington). The reaction of the enzyme is stopped by the addition of 1 ml. of 0.82*M* sodium citrate to the enzyme substrate mixture prior to centrifuging. They observed that prior to enzyme action, a single sharp peak is obtained. With enzyme activity, this peak becomes reduced and a second slower peak begins to appear. The enzyme activity is measured by determining the reduction in area under the curve of the fast sedimenting component. As with other methods, an initial lag phase is observed. The simultaneous determination of fall in viscosity demonstrates that the reduction in viscosity is complete before the fast-sedimenting component begins to disappear.

Rabatin *et al.* selected deoxyribonucleoprotein as the substrate rather than DNA because they contended that the nucleoprotein is a more "physiological" substrate and gives more clear-cut changes in the ultracentrifugal pattern than does pure DNA. It should be pointed out that 1*M* sodium chloride dissociates the nucleohistone and is markedly inhibitory for DNase activity (79).

F. Flow Birefringence, Dielectric Constant, Light Scattering

Depression of flow birefringence, change in dielectric constant, and the reduction of molecular weight as measured by light scattering may all be followed to determine the depolymerization of DNA by DNase (71). These are laborious methods, applicable only in pure systems, interfered with even by electrolytes, and therefore very little used for assay of DNase activity. These methods have found their principal applications in the study of the structure of the DNA molecule. Thus, Hammarsten (47) noted changes in streaming birefringence (as well as osmotic pressure and viscosity) during enzymic attack. The method was used also by Thomas (141) in combination with light-scattering methods, while Jungner (57,58) studied DNase action on DNA by dielectric determinations.

Frajola, Rabatin, and Smith (35) described a quantitative assay method based on flow birefringence. In their system, the intensity of the polarized light transmitted through the solution, which is a function of the concentration of the birefringent (undepolymerized DNA), is measured at intervals. The rotor is permitted to run only

intermittently because of heating. The test solution contains 0.04% polymerized DNA, 0.044*M* magnesium sulfate, and 0.039 μg. of DNase (Worthington) per ml. It is observed that the addition of magnesium sulfate causes an immediate 37.5% reduction in birefringence. If 0.33*M* sodium chloride is also added, the birefringence falls 62.5% (cf. effect on lowering viscosity). Thereafter the rate of fall in birefringence proceeds as a first order reaction. This concentration of sodium chloride (0.33*M*) caused approximately 75% inhibition. Frajola *et al.* found that it was also possible to use deoxyribonucleoprotein in 1*M* sodium chloride as substrate, but this required approximately 100 times as much enzyme to permit detection of the activity because of inhibiton by the salt.

IV. CHANGES IN AFFINITY FOR DYES AND OTHER REAGENTS

1. Methyl Green

In a series of experiments beginning in 1946, Kurnick (74) demonstrated that the affinity of DNA for methyl green was dependent upon the native structure of the DNA (75,76). Changes in the configuration of the DNA molecule by chemical, physical, or enzymic means result in loss of affinity for methyl green.

Kurnick and Foster (83) have shown that the methyl green–DNA complex has a different visible absorption spectrum from that of free methyl green and that the color of the complex is stable at pH 7.5, while that of free methyl green fades to almost colorless with the formation of the carbinol. These characteristics permit the colorimetric assay of DNase activity by measuring the progressive reduction of bound methyl green (77). The method is applicable over a wide pH range and permits the testing of the influence of various electrolytes and salt concentrations on enzyme activity At high salt concentration some dissociation of the methyl green–DNA complex occurs, but since the enzyme does not appear to distinguish between free DNA and DNA bound to methyl green, the reaction remains linear and proportional to the enzyme concentration.

Because of the different pH and electrolyte requirements, two different substrates are used. For assay of the alkaline enzyme (78).

the substrate consists of 15 ml. of a 2 mg./ml. DNA in water solution + 15 ml. of 0.1*M* magnesium sulfate (or chloride) + 100 ml. of 0.05*M* trimethylolaminomethane buffer (38) + 15 ml. of 0.04% methyl green in 0.02*M* pH 4.2 acetate buffer (which has been extracted exhaustively with chloroform) and made up to 200 ml. with water. Merthiolate,* 0.2 ml. of 10 mg./ml. solution may be added as a preservative. This substrate is allowed to fade at 37°C. for approximately 18 hours and then stored at 0–2°C. For the determination, 15 ml. of the substrate is brought to 37°C., and enzyme solution (usually a tissue homogenate in water) is added to a final volume of 19 ml. At intervals, 3 ml. aliquots are removed and added to 0.5 ml. of 0.33*M* sodium citrate, mixed, and allowed to stand overnight at room temperature in subdued light; the optical density is read at 640 mμ.

For the assay of the acid enzyme (88) the substrate mixture consists of 100 ml. of 2 mg./ml. aqueous methyl green + 200 ml. of 0.05*N* acetate buffer, pH 4.6 (or other pH if desired) + 100 ml. of 0.6*N* magnesium chloride + 700 ml. of distilled water + 1 ml. of 1% aqueous sodium ethylmercurithiosalicylate. To 12 ml. of this substrate mixture at 37°C. 1 ml. of the enzyme is added. At intervals 2 ml. aliquots are pipetted into 2 ml. of a mixture of one part 0.33*M* sodium citrate and 3 parts 0.05*M* Tris buffer, pH 7.5 This mixture stops the reaction by adjusting the pH to 7.5, at which the acid enzyme is not active, and the addition of sodium citrate inhibits the alkaline enzyme. The final concentration of electrolyte after the addition of the citrate is inhibitory to both enzymes. After standing overnight in subdued light, the optical density is read at 640 mμ.

At very low pH, where DNA–protein complexes tend to precipitate, the reaction may be carried out by setting up an individual tube for each assay: 1.9 ml. of substrate is added to 0.1 ml. of the homogenate to be tested. At appropriate intervals, 2 ml. portions of the Tris–citrate mixture are added to the tube. At the final pH of 7.5, the complex redissolves.

The unit of enzyme activity for the methyl green methods is the same and is defined as that which produces a reduction of 1 optical density unit per minute. Mixtures of enzyme solution and inhibitor solution may be tested. The presence of particulates does not interfere with the reaction, since these may be removed by centrifugation if they do not dissolve during the course of the experiment. The

*Merthiolate is the trademark of Eli Lilly & Co., for Thimerosal, made by it.

theoretical possibility of the adsorption of methyl green–DNA complex has not in practice proved to be a problem, and the adsorption of free methyl green would have no effect on the reaction.

The methyl green method is simple, permits simultaneous assay of many samples, is sensitive, requires relatively little DNA, and may be performed with crude and even heterogeneous systems. It is, therefore, very well suited for the analysis of tissue enzymes and inhibitors.

2. Other Dyes

It is probable that other dyes may also show changes in affinity to DNA as its structure is modified by DNase. The appearance of altered nuclear material stained with hematoxylin suggests that the absorption spectrum, and perhaps the amount of dye bound, in the DNA–hematoxylin complex differ with different states of the DNA molecule (73). However, this problem has not been investigated.

3. Proteins

Donahue, Houck, and Coffey (29) described a turbidimetric method based on the observation that DNA forms a stable colloid with serum albumin in the presence of gelatin. The presence of other reagents which might produce turbidity would, of course, interfere. The method for diluted serum is as follows: to 0.5 ml. of a 0.12% solution of DNA in 0.05*M* veronal buffer, pH 7.2, an equal volume of serum diluted 1:10 with 0.04*M* magnesium chloride is added. The mixture is incubated for 30 minutes at 37°C., following which 5 ml. of 0.1*M* acetate buffer, pH 3.7, containing 0.3 mg. of serum albumin and 1 mg. of Knox gelatin per ml. is added. After incubation for 5 minutes at 37°C. the optical density at 450 mμ is measured. The enzyme activity is defined as the mg. of substrate depolymerized per 0.05 ml. of serum in 30 minutes at 37°C. A series of standards containing known quantities of DNA provide the standard curve. In a further study Houck (54) slightly modified this method. He used a substrate containing 0.8–1.2 mg./ml. DNA (Herring Sperm DNA from the California Foundation, DNA from Nutritional Biochemical Corp., or Schwarz Company DNA; these are all moderately to extensively degraded DNA), in 0.1*M* acetate buffer, pH 6.3 with 0.1*M* magnesium chloride, final pH 5.9 (not optimal for either tissue

enzyme). To 0.5 ml. of this substrate, 0.5 ml. enzyme solution is added, and the mixture incubated for 30 minutes at 26°C., whereupon 5 ml. of 0.3 mg./ml. fraction V (human serum albumin) in 0.1*M* acetate buffer, pH 3.7, containing 1 mg./ml. of Knox gelatin is added. After incubation for 5 minutes at 37°C., the turbidity of the colloid is read at 450 mμ. The author comments that the turbidity is greatly influenced by ionic strength, being maximal near 0.1 ionic strength, and only one-third as high at 0.4 ionic strength for magnesium and calcium, and two-thirds as high for sodium and potassium. He notes that boiling the DNA solution for 5 minutes has no effect upon the reaction, but prolonged exposure to alkali reduces the enzyme activity. The extensive denaturation of the DNA produced by boiling diminishes greatly the affinity of DNase for it (79,80). Denatured DNA is not applicable for most methods, and its use in this turbidimetric method must also interfere with standardization of the method. Thus, Houck (54) noted that various DNA preparations and different albumin preparations gave different turbidities. Houck recognized that the presence of protein in the enzyme preparation would also affect the result, but stated that this is not a problem in the dilution of serum (2:25) used. However, varying the amount of serum used did vary the turbidity. The author also noted that the reaction is not linear with time or enzyme concentration, but that the enzyme activity is linear with DNA concentration. The slope of the curve is different for different DNA's. The author does not offer an explanation for the linearity of the reaction rate with substrate concentration. However, since he increased the magnesium ion concentration proportionately to the DNA concentration, his observation may be a function of approaching electrolyte concentration optimum. He also noted that heparin, 20 mg./ml. (which does not interfere with the methyl green method in anticoagulant concentration (77)), inhibited about 30% as do other polyelectrolytes (51), and that polyelectrolytes react with albumin, thereby interfering with the test reaction. Houck found the pH optimum for pancreatic DNase to be 5.9 and the optimum temperature to be 26°C., whereas for serum he found two optima, at pH 5.0 and 7.0 with a temperature optimum of 31°C. These optima are at variance with the observations of others who have found the optimal pH for both the pancreatic and serum enzymes at pH 7.5 (78).

A somewhat similar method based upon changes in protamine

binding by DNA following DNase attack was described by Mazia (102).

V. ASSAY OF PRODUCTS OF DEPOLYMERIZATION

1. Dialysis

Carter and Greenstein (15) determined nitrogen, phosphorus (cf. 16), purine (ultraviolet absorption), and deoxyribose (diphenylamine reaction of Dische (27); other color reactions for the sugar may also be used) content of the dialyzate of a mixture of DNA and tissue homogenate. This method is based upon the observation that polymerized DNA will not pass through a cellophane membrane. The assay may be interfered with by the binding of partial breakdown products, which would otherwise be dialyzable, to other constituents in the homogenate, and by the action of other enzymes such as nucleotidases, phosphatases, and deaminases on the otherwise nondialyzable early products of DNase digestion. Wiberg (151) also contends that the dialyzability of the products of digestion is influenced by electrolytes, in agreement with Chargaff and Shapiro (18a). The method is complicated by the fact that the result is influenced simultaneously by the rate of enzyme action and by the rate of diffusion of the dialyzable products.

2. Acid and Organic Solvent Soluble Products

The determination of the formation of acid-soluble products by the action of DNase on DNA is the converse of the determination of the remaining acid-insoluble substrate previously described. The determination of the acid-soluble products may be performed by any of the methods applied by Carter and Greenstein to dialyzates as described in Section V.1. Carter and Greenstein (15) incubated tissue extract with an equal volume of 0.5% DNA, then added 5 ml. of 5% trichloroacetic acid, centrifuged, and analyzed the supernatant for purine.

In a modification of the method of Laskowski (94,95), in which acid-soluble phosphorus is measured, Gilbert, Overend, and Webb (37) incubated 2 ml. of 1% DNA with 1 ml. of 0.1*M* magnesium sulfate and 1 ml. of 0.01% DNase (Worthington) plus 1 ml. of water or

inhibitor at 37°C. (no mention is made in this paper of a buffer, but in the previously described assay by the weighing of acid-precipitable DNA, the authors used 0.1*M* veronal buffer, pH 7.0). At the end of incubation 0.1 ml. of 5*N* HCl was added, the material centrifuged, and the phosphorus content of the supernatant determined.

Probably the simplest assay method using the principle of acid-soluble products is that described by Allfrey and Mirsky (2). A substrate containing 2 mg. of DNA (Worthington) in 1 ml. of water or 0.05*M* magnesium sulfate + 1 ml. of the appropriate 0.2*M* buffer is incubated with 1 ml. of enzyme solution at 35°C., following which 1 ml. of TCA is added at 0°C.; the mixture is then filtered or centrifuged, and the supernatant analyzed for deoxyribose by the method of Dische. The unit is defined as that which releases 1 μg. of acid soluble phosphorus per hour.

A slight modification of the assay of acid-soluble products by the diphenylamine reaction just described was introduced by Koszalka *et al.* (64). These authors introduced the color development with *p*-nitrophenylhydrazine in lieu of the diphenylamine reaction because of the interference of hypertonic sucrose with the latter. The substrate consisted of 3 volumes 0.2*M* sodium acetate buffer, pH 5.6, 1 volume of $9 \times 10^{-5}M$ disodium ethylenediaminetetraacetic acid solution, and 1 volume of 0.4% DNA (California Foundation for Biochemical Research; this material is prepared with a heating step and is therefore considerably degraded—we have found that its methyl green binding capacity (73) is much less than that of the Mirsky and Pollister (Worthington) preparation and of preparations made by the methods of Kay, Simmons, and Dounce (59) and of Simmons (134); and Rowen (121) found that the molecular length as measured by flow birefringence is approximately two-fifths that of the other preparations). To 2.5 ml. of this substrate 0.5 ml. of enzyme solution was added. After incubation for 3 hours at 37°C., 1 ml. of 2.88*M* trichloroacetic acid was added, and the precipitate removed by centrifugation. The control consisted of 2 ml. of the acetate buffer–Versene solution + the enzyme, simularly incubated, to which were added the trichloroacetic acid and then 0.5 ml. of 0.4% DNA solution. This method, as noted for the method of Schneider and Hogeboom (124) (see below), runs the risk of underestimating the enzyme activity by subtracting enzyme action on

DNA contained in the enzyme preparation. The contribution of soluble products by this DNA should not be deducted, since the enzyme would not be expected to differentiate between DNA included in the enzyme preparation and that in the added substrate. The significance of the addition of Versene is discussed in the section on inhibitors in connection with the effect of electrolytes (Section XII.1). The *p*-nitrophenylhydrazine color was developed by adding to 3 ml. of the supernatant of both the test and the control an equal volume of water, heating in a boiling water bath for 30 minutes, cooling, and mixing 2 ml. of this solution with an equal volume of 5% trichloroacetic acid, adding 0.2 ml. of fresh 0.5% *p*-nitrophenylhydrazine in ethanol, and heating in a boiling water bath for 20 minutes. The solution was cooled and extracted three times with 10 ml. of butyl acetate, and the organic base discarded. To 3 ml. of the aqueous phase was added 2 ml. of 0.1*N* sodium hydroxide, and immediately thereafter the optical density at 560 mμ was determined. The color is unstable and must be read immediately. The results were compared to color development with the diphenylamine reaction and with ultraviolet absorption. The contribution of trichloroacetic acid to UV absorption at 260 mμ is referred to below (p. 23). Koszalko *et al.* observed about 30% activation of the acid enzyme by the addition of 0.1*M* sodium chloride, which also shifted the pH optimum from a broad shoulder near 5.3 to 4.9. Versenate, $1.5 \times 10^{-5}M$, had no such effect. The lack of effect of this low concentration of Versenate is consistent with our observations on the action of Versenate as an electrolyte in the activation of acid DNase (87). The activation by sodium chloride is consistent with the observation of Koerner and Sinsheimer (61), as well as with our own (87). Both latter observers, however, found the pH optimum in the presence of electrolyte to be lower than the 4.9 reported by Koszalka *et al.* This results from the fact that pH optimum is a function of the electrolyte concentration, which is optimal at approximately 1.5 times the sodium chloride concentration used by Koszalka *et al.* The method of Koszalka *et al.* appears to have only the advantage of suitability in the presence of high sucrose concentration, which they used in the fractionation of subcellular particles. The sucrose produces color reactions with dipyenylamine, thereby making this method of deoxyribose assay inapplicable, unless the subcellular particles are first separated from the sucrose solution by centrifugation. The

method has the disadvantages of requiring that unreacted nitrophenylhydrazine be extracted and of the great instability of the color reaction. It would appear to have no advantages over phosphorus analysis on the supernatant, with which the sucrose would not interfere.

Kunitz (71) also used an acid-soluble method. To 9 ml. of 0.5% DNA + 1 ml. 0.3*M* magnesium sulfate, adjusted to pH 7.6 with 0.02*N* sodium hydroxide, he added 0.1 ml. of 0.01% crystalline DNase. At intervals, 0.5 ml. samples of this mixture were added to 4 ml. of ice cold 0.25*N* sulfuric acid, the mixture centrifuged, and the optical density of the supernatant at 260 mμ determined. Kunitz observed that gelatin and neopeptone, both recommended for stabilizing the enzyme (103), interfered with the acid precipitation of DNA and that the optical density of the products of digestion is greater than that of the original DNA because of the hydrogen bond cleavage. The latter, however, does not interfere with the assay method.

Because sulfuric acid does not remove naturally occurring constituents which absorb in the UV, the method just described is limited to pure systems, as is the ultraviolet absorption method of Kunitz previously described. Kurnick (78) has modified the method to permit somewhat more general application. One milliliter of the enzyme solution is added to 6 ml. of substrate, which is made up as follows: 3.75 ml. of 0.0143% DNA-phosphorus; 10 ml. of 0.067*M*, pH 7.5, Sorenson's phosphate buffer; 7.5 ml. of 0.1*M* magnesium sulfate; and water to 50 ml. At intervals, 1 ml. of this mixture is added to 3 ml. of ice cold 2% perchloric acid, centrifuged in the cold, and the supernatant compared against water at 260 mμ. The use of perchloric acid causes the precipitation of proteins which are responsible for most of the interfering absorption at 260 mμ. Phosphate buffer is used since both the veronal and tris buffers absorb at 260 mμ.

Schneider and Hogeboom (124) adopted the procedure with slight modification. They incubated 1 ml. of 0.01*M* $MgSO_4$ in 0.02*M* sodium succinate buffer, pH 4.5, containing 0.2 mg. of DNA and tissue homogenate, for 30 minutes at 37.5°C., then added 0.2 ml. of 12% perchloric acid, and determined the optical density of the supernatant at 260 mμ. They used a blank which was incubated as above, except that the DNA was added after the perchloric acid. In addition to the other sources of error inherent in the acid-soluble methods, this

blank would cause the subtraction of acid-soluble products formed by depolymerization of the DNA present in the tissue homogenate. Since the reaction is not influenced by small changes in substrate concentration, the use of such a blank would result in underestimation of the DNase activity.

Sherry, Johnson, and Tillett (130) determined phosphorus and nitrogen concentration in 8% trichloroacetic acid filtrates of the enzyme-substrate mixtures described in their method for viscosimetry. They found the liberation of acid-soluble phosphorus and nitrogen to proceed more slowly than the drop in viscosity and to continue after the viscosity had been reduced to its lowest limit. These authors also used the increase in absorption at 260 mμ of the TCA filtrates. This method is subject to considerable error because of the marked absorption of TCA at this wavelength, so that slight variations in the concentration of TCA due to precipitation with protein or technical errors would materially affect the result.

The assay of DNase activity by the measurement of acid-soluble products formed is interfered with, as mentioned previously, by the presence of proteins which may alter the solubility of the products of depolymerization. Thus, whereas serum or egg albumin enhanced the activity of crystalline DNase as measured by the methyl green method, they caused apparent inhibition when the perchloric acid-soluble method was used (78). This was thought to be due to the precipitation of complexes of proteins with the split DNA products.

Of course, organic solvents may be used to precipitate the DNA, and the supernatants analyzed by any of the procedures described for dialyzates and acid-soluble components.

The methods based on the assay of products soluble in acid or organic solvents permit testing the effects of pH, electrolyte concentration, and inhibitors (within the limitations imposed by interference of tissue proteins in the inhibitor preparations with precipitation of the DNA and products of depolymerization).

VI. BIOLOGICAL ACTIVITY

Zamenhof *et al.* (156) have followed the loss of transforming action by extracts of bacteria following exposure to DNase. This is obviously a laborious and time-consuming method of assaying DNase activity. It is, however, a very sensitive measure of changes in the

DNA molecule. The loss of transforming activity is linear with time. This is in contrast with the loss of viscosity which Zamenhof *et al.* (156) followed simultaneously. The viscosity shows a lag in its response and then falls only slightly while the transforming activity falls to zero. Zamenhof *et al.* suggest that the difference in the rates of change of viscosity and of transforming activity is due to slight denaturation without significant change in molecular asymmetry early in the depolymerase activity. As we have discussed elsewhere, it is likely that a few scattered cleavages in one or the other of the strands, not opposite each other so as to cleave the helical molecule, would result in no change in molecular asymmetry, but would result in change in transforming activity as well as such other parameters as methyl green binding and hydrogen ion release.

Zamenhof *et al.* performed the analysis as follows: to 1 ml. of solution of purified transforming agent, containing 530 μg. of DNA per ml. of citrate buffer, 0.4 ml. of enzyme solution was added. The enzyme solution was crystalline deoxyribonuclease dissolved in aqueous solution containing gelatin (0.035%) and $MnCl_2$ (0.012*M*) diluted in gelatin–$MnCl_2$ solution to an enzyme concentration of 3×10^{-4} μg. per ml. The enzyme-substrate solution was immediately placed at 30°C., and at intervals 0.5 ml. aliquots were removed and immediately precipitated by two volumes of absolute alcohol. The resulting fibers were washed five times in 5 ml. portions of 75% alcohol and redissolved in 0.5 ml. portions of the citrate buffer. The solutions were immediately tested for their transforming activity. The logarithm of the per cent transforming activity $\times 10^{-3}$ was plotted against time in minutes.

VII. HISTOCHEMICAL METHODS

Daoust (24) has described a method in which 0.1% DNA is incorporated into a 2.5% solution of gelatin, which is painted on a glass slide and fixed in 10% formaldehyde. A freshly frozen tissue slice is placed in contact with the gelatin film, and, after incubation, the film is stained in 0.2% toluidine blue for 10 minutes. Clear zones indicate the regions of DNase activity. The reaction, as described, is carried out in the dry state, with no control of pH or electrolytes. We have not found this method satisfactory for the localization of DNase.

Aronson, Hempelmann, and Okada (4) have modified the acid phosphatase method of Gomori (39) using DNA as a substrate in the presence of added acid phosphatase, which is required to liberate phosphate from the products of DNase action (123). Tissue washed with 5% cold sucrose is frozen with dry ice, cut at 15 μ in a cryostat, fixed at -10°C. for 5 minutes in acetone–formalin–water (50:10:40) and then for 3 minutes in 50% acetone, rinsed in water at room temperature for 5–15 minutes, and incubated in a substrate consisting of 10 ml. of 0.05*M* sodium acetate buffer, pH 5.0, 0.3 ml. of 0.1*M* lead acetate, 0.5 ml. of 0.4% DNA (California Foundation for Biochemical Research, a partially heat-denatured DNA), and 0.5 ml. of 2 mg./ml. acid phosphatase (Nutritional Biochemical Corporation). After 6–8 hours at 37°C., the slide is rinsed in water, immersed 2 minutes in 0.2% ammonium sulfide, counterstained with fast green, dehydrated, and mounted. The method suffers from the numerous disadvantages of the Gomori acid phosphatase method and is probably unreliable both in specificity and localization.

Marshall (97) has described the use of fluorescein-labeled anti-DNase antibodies for the histological localization of DNase. This method is applicable only when a DNase preparation free of other tissue antigens is available. At present, the method has been limited to the alkaline DNase of beef (pancreas). Daoust (24) has pointed out too that the method does not distinguish active from inactive enzyme, since it depends only upon the protein nature of the enzyme.

VIII. PREPARATION OF SUBSTRATE

Although crude DNA preparations, prepared by procedures which include heating, may be used in some of the methods, particularly those which depend upon acid solubility of the reaction products, the appropriate substrate for determining DNase activity is one which is highly polymerized and undenatured. The methods of Mirsky and Pollister (105), Simmons (134), Kay, Simmons, and Dounce (59), Schwander and Signer (126), Hammarsten (47), and Marko (96) are all suitable for the preparation of DNA (more accurately referred to as sodium deoxyribonucleate). Most of these procedures depend upon the extraction of the nucleoprotein with strong electrolyte solutions followed by shaking with chloroform, as

described by Sevag (128), or with detergents, to denature and precipitate proteins. Most commercial DNA preparations are prepared with heat and are therefore not undenatured. Denatured DNA shows much less affinity for DNase than does the native molecule (79,80).

IX. PREPARATION OF ENZYME

Unrefined tissue extracts or tissue fluids obviously present the simplest preparations for the enzyme assay. In many of the assay methods such preparations can be successfully used. For some, however, the enzyme must be purified.

With certain limitations, discussed in connection with the methods, crude heterogeneous tissue homogenates may be used in the acid solubility and methyl green methods. For assay of alkaline DNase, either water or saline homogenates, and the supernatant of either, may be successfully used, since the enzyme is completely extracted by grinding in the Potter-Elvehjem homogenizer or a Waring Blendor. Fresh or frozen tissues may be used. Acetone powder or tissue preparations made with organic solvents, such as the Behrens (7) technique for isolating nuclei, contain only the acid enzyme (2), the alkaline enzyme being destroyed in this type of preparation (22).

Acid DNase is extractable with distilled water, and either heterogeneous homogenates or the supernatants from such homogenates are suitable for its assay, depending upon the limitations imposed by the assay method. Saline extraction is incomplete for this enzyme, presumably because of inadequate rupture and extraction from the mitochondria (109,110,142,146). This enzyme is also extractable from acetone powders or fractions prepared with organic solvents (2,22).

Some of the methods are not adaptable to impure systems, and even the acid-soluble method may suffer interference from proteins in solution. Partial purification of alkaline DNase from pancreas has been described by Fischer, Botger, and Lehmann-Echternacht (33), Laskowski (94), McCarty (103), and Baumgarten *et al.* (6). These authors used acid extracts of fresh beef pancreas and precipitation with ammonium sulfate. Kunitz (70) succeeded in crystallizing the enzyme from such preparations. Following the fractionation

with ammonium sulfate, he used selective denaturation of proteins at 37°C. in alcohol, followed by fractionation with ammonium sulfate at pH 4.0 and crystallization in dilute ammonium sulfate at pH 2.8. The enzyme he obtained was a soluble protein of the albumin type with a molecular weight of about 60,000 and an isoelectric point near 5.0. This enzyme preparation, which is now commercially available, is very well suited for assays which require pure systems. Privat de Garilhe *et al.* (117) have purified the bacterial enzyme.

The purification of the acid enzyme has also been accomplished by a number of investigators, but the enzyme has not yet been crystallized. Early attempts were described by Laskowski *et al.* (95a), Siebert *et al.* (133), Maver and Greco (100,101), and Webb (147). Koszalka, Falkenheim, and Altman (63) achieved 200-fold increase in specific activity by the extraction of fresh or frozen calf spleen with sulfuric acid followed by ammonium sulfate precipitation and chromatographic separation on an IRC-50 (XE-64) resin column. Koerner and Sinsheimer (61), Fredericq and Oth (36), and Shimomura and Laskowski (131) have used similar procedures for the partial purification of acid DNase. Polson (115) has described the enzyme purification by multimembrane electrodecantation.

Manipulations to purify the enzyme result in considerable loss of enzyme, usually in the vicinity of 80%. They are therefore not applicable to quantitative assays of tissue enzyme activity. Of course, they have the advantage of making possible more precise description of the enzyme and its mode of action. In the pure enzyme and substrate system, delicate biophysical methods which are not applicable to impure—let alone heterogeneous—systems may be applied, and interference with the enzyme-substrate reaction from other constituents of the impure system can be eliminated.

X. STABILITY OF ENZYME

Crude tissue extracts or homogenates retain their enzyme activity indefinitely when kept frozen. They are also reasonably stable in the refrigerator, although this is not recommended for longer than 24 hours. Pure enzyme solutions are reasonably stable when concentrated, but are unstable when dilute. Spontaneous denaturation occurs readily (69,70). In preparing solutions of

crystalline DNase, shaking and violent mixing must be avoided to prevent the denaturation of the enzyme, which may result in the formation of insoluble strands. The crystalline enzyme should therefore be kept in stock either in the dry state or in concentrated solution (preferably frozen). Dilute solutions may be stabilized by the addition of hydrogen peroxide (47), gelatin, neopeptone, or various proteins and amino acids (103). These materials, as well as denatured proteins, appear to increase the activity of the enzyme. However, their interference with many of the assay methods, particularly those for which a purified enzyme is required, makes it desirable to avoid the addition of such stabilizing reagents.

XI. SOME BIOLOGICAL ASPECTS OF MAMMALIAN DEOXYRIBONUCLEASE

The suggestion that only acid DNase occurs in mammalian tissues other than the pancreas (2) appears to have been a consequence of the use of organic reagents in preparation of the tissues for analysis. Cunningham and Laskowski (22) and Siebert *et al.* (133) have demonstrated that both enzymes occur in the tissues, but that the acid enzyme is much more abundant in most. The enzyme activities vary considerably from tissue to tissue and from species to species. Body fluids within an individual and from different species also differ considerably in their enzyme content (44,82). In the radiosensitive tissues, the acid DNase activity is greatly increased by irradiation (30,40,84,84a,89,111,112,127) and also rises with age (84,89). The activity in the radioresistant organs is more stable both after irradiation and with advancing age (84,89). On the other hand, in embryonic tissues, including the placenta, the DNase activity appears to fall with advancing maturity (133). The serum of premature infants is found to be higher in alkaline DNase activity than that of term infants, a condition which has been attributed to hepatic immaturity (107), since the liver clears the portal plasma of alkaline DNase (82). It has been reported that human serum alkaline DNase is lower in tumor-bearing patients than in normal (153), but Kurnick has observed no such difference (78). That the activity does change in various disease states is, however, indicated in our observations of increased activity in hepatic disease and lupus erythematosus (85) and in pancreatic disease (66). Brody (11) and Brody and

Balis (12) have reported on changes in DNase activity in malignant tissues. The direction of change upon tumor development was not uniform for different tissues. The activity of the acid enzyme has been reported to rise in lymphoid organs following steroid administration (21).

XII. INHIBITORS

The determination of inhibition or activation of deoxyribonuclease activity is to some extent a function of the method used for assay. Thus, as we have already indicated, Laskowski observed that reducing agents appeared to activate the enzyme when the assay was performed by viscosimetry, but to inhibit when the assay was dependent upon the release of acid-soluble products (95). Likewise, whereas the methyl green method of measurement indicates that proteins activate the crystalline alkaline deoxyribonuclease, the acid-soluble product method indicates inhibition (77), presumably because of the binding of the products by protein and precipitation as a complex. Treatments which denature or depolymerize DNA, such as heat or X-irradiation, inhibit the enzyme activity as measured by methods which depend upon early enzymic changes in the substrate, such as methyl green affinity, viscosity, birefringence of flow, etc. (79). However, such procedures do not inhibit the enzyme as measured by methods dependent upon events which occur only late in the enzymic reaction, after the substrate has already been extensively depolymerized and denatured, such as the acid solubility product method. Indeed, initial denaturation and/or depolymerization by nonenzymic means might actually appear to accelerate the enzymic reaction as measured by such methods. The influence of the assay method on the result must, therefore, be carefully evaluated in the determination of inhibition or activation.

Inhibitors of deoxyribonuclease activity fall into several groups: (*1*) electrolytes, (*2*) antibodies, and (*3*) reagents other than antibodies which react with the DNA to prevent enzyme attack.

1. Electrolytes

Both alkaline and acid deoxyribonucleases are inhibited at high electrolyte concentration, but they require electrolytes for activation (62,77,87,106,129). In the case of the mammalian acid DNase,

the optimal electrolyte concentration is a function of the pH (87). As indicated in Section I, different electrolytes produce varying degrees of activation. Both the cations and the anions play significant roles (87). Since electrolyte concentrations greater than optimal are inhibitory (and this occurs at much lower ionic strength than for the alkaline enzyme and is more evident for the divalent than for the monovalent cations), it had been thought that the acid enzyme did not require electrolytes (2,147). The use of $MgSO_4$ for activation of the alkaline enzyme had led to its inclusion in substrates for the acid enzyme. Sulfate has an inhibitory effect and shifts the optimum electrolyte concentration to a very low level. This led to the conclusion that Mg ion was inhibitory. Therefore, the addition of Versene to bind cations has been recommended in some procedures for assay of the acid DNase. We have observed that Versene in low concentration may produce an activating effect, presumably acting as an electrolyte itself. We recommend the use of an optimum or near optimum electrolyte concentration, as described in the methyl green method, and do not recommend the addition of Versene.

The optimum electrolyte concentration and pH for the mammalian acid DNase are interrelated functions. With increasing pH, the optimum electrolyte concentration falls. In the pH range 5.2–5.9 (2,147), the required electrolyte concentration is very low and the inhibitory effect of $MgSO_4$ is therefore apparent even at minimal concentration, whereas at lower pH, the sulfate activates the DNase (although not optimally). Since final electrolyte concentration is important, the use of concentrated buffers (131) has also lead to erroneous conclusions about the effects of specific ions on the enzyme.

The alkaline mammalian DNase is activated by electrolytes, particularly by magnesium. It is therefore readily inhibited by the addition of anions which bind the activating cation. Citrate, arsenate, fluoride, and oxalate are examples of ions which have been used for inactivation in this manner. Copper, selenite, arsenite, and borate are also strongly inhibitory (122).

2. Biological Inhibitors

A number of investigators have studied the production of antisera against various DNases (104,137,144). These antisera may be assayed by their inhibition of the specific enzyme antigen.