528. *Deoxypentose Nucleic Acids. Part I. The Enzymic Degradation of Sodium Deoxyribonucleate by Deoxyribonuclease.*

By W. G. Overend and M. Webb.

Deoxyribonuclease has been isolated from ox-pancreas according to McCarty's procedure (*J. Gen. Physiol.*, 1946, **29**, 123), and a number of its properties re-determined. As a first step the enzyme depolymerises deoxyribonucleic acid to smaller units which are then hydrolysed to acid-soluble polynucleotides. In contrast to the results of Fischer *et al.* (*J. pr. Chem.*, 1941, **158**, 79) it has been shown that the diffusible degradation products exhibit a wide range of molecular size. The enzyme hydrolyses preferentially the pyrimidine nucleotide groups of deoxyribonucleic acid and leaves a core which contains an increased ratio of purines to pyrimidines and resists further action of the enzyme.

Although the importance of deoxyribonucleic acids in the structure of living cells has been recognised for many years, the study of the properties of these macromolecules has been hampered to some extent by the lack of suitable methods for their isolation in the native (undegraded) form. The stimulation of interest in the properties, composition, and function of this class of compound which followed the introduction of such methods by Mirsky and Pollister (*Proc. Nat. Acad. Sci.*, 1942, **28**, 344) has led to renewed investigations (cf. Gulland *et al.*, *J.*, 1947, 1129, 1131, 1141; Chargaff *et al.*, *J. Biol. Chem.*, 1948, **176**, 715; 1949, **177**, 405; 1949, **178**, 531), which have conclusively shown that the structural formulæ originally proposed (Levene and Tipson, *Science*, 1935, **81**, 98; *J. Biol. Chem.*, 1935, **109**, 623) for deoxyribonucleic acid were over-simplified. Unfortunately, little information concerning the structure of deoxyribonucleic acids is as yet forthcoming from degradative experiments with chemical reagents, especially acidic reagents, owing to the extreme lability of nucleic acids of this type. In consequence attention has been directed to a study of the enzymic degradation of deoxyribonucleic acids. Moreover a survey of the literature reveals that, although many results of enzymic hydrolysis of deoxyribonucleic acid have been reported, little agreement exists between the results and theories put forward by the various authors (see Allen, *Ann. Rev. Biochem.*, 1941, **10**, 221; Loring, *ibid.*, 1944, **13**, 295; Gulland, *ibid.*, 1945, **14**, 175). Therefore, as part of a wider investigation of methods of degrading deoxyribonucleic acids, it was considered advisable initially to re-investigate the properties and mode of action of deoxyribonuclease. The results of this investigation are recorded in this communication (cf. Overend and Webb, *Research*, 1949, **2**, 99).

Preparation and Properties of the Enzyme.—The deoxyribonuclease was isolated from ox-pancreas in highly active form by essentially the method of McCarty (*J. Gen. Physiol.*, 1946, **29**, 123). [Since this work was completed, Kunitz (*Science*, 1948, **108**, 19) following McCarty's procedure has announced the crystallisation of deoxyribonuclease, but an investigation of the mode of action of this preparation was not reported.]

McCarty (*loc. cit.*) and other workers (Fischer *et al.*, *J. pr. Chem.*, 1941, **158**, 79; Greenstein *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12**, 64) have shown that magnesium ions are essential for the activity of deoxyribonuclease. McCarty found 0·003M-magnesium ion to be the optimal concentration for the activation of systems containing varying amounts of enzyme in the presence of a constant amount of nucleic acid. In similar conditions with our enzyme preparation the critical concentration of magnesium ion was *ca.* 0·001M. and little hydrolysis of the substrate occurred if the concentration was less than 0·0005M. In agreement with McCarty who found deoxyribonuclease to have optimum activity between pH 6·8 and 8·2, the present preparation had optimum activity between pH 7 and 8. Moreover our preparation, although considerably less stable than the corresponding ribonuclease (Kunitz, *J. Gen. Physiol.*, 1940, **24**, 15), appeared more stable than McCarty's preparation. The latter lost 90% of its activity after 15 minutes at 55° and at various pH values, and after one hour at this temperature no residual activity could be demonstrated. Our enzyme did not undergo rapid inactivation until heated above 80°. Under the given conditions and concentration of substrate (see Experimental section) little degradation of the nucleic acid occurred when the enzyme concentration was less than 10^{-5}%, whereas maximum degradation occurred when it was greater than 10^{-4}%. A detailed investigation of the action of inhibitors on deoxyribonuclease will be reported elsewhere. From the results obtained it seems that substances which inhibit the enzyme activity may be divided into two groups, namely, those which exert their action by removing the activating magnesium ions either by precipitation or by co-ordination, and those which directly react with the functional groups of the enzyme protein. Among substances of the first group we have

confirmed the inhibitory action of fluorides, citrates, and borates. Among those of the second group, thioglycollic acid markedly decreased the activity of deoxyribonuclease. Sulphydryl inhibitors such as iodoacetic acid and iodoacetamide had no effect on the enzyme.

The action of deoxyribonuclease on deoxyribonucleic acid results in an increase in titratable acid groups without the liberation of inorganic phosphorus. Fischer *et al.* (*loc. cit.*) claim that this increase in acidity corresponds to one acid equivalent (titrated to pH 9) for each four atoms of phosphorus initially present and presumes the formation of tetranucleotides. When his experiments were repeated with our enzyme preparation, the acid groups liberated by the enzyme action corresponded to only 0·503 acid equivalent per four atoms of phosphorus. Since intermediate values of 0·78 and 0·81 acid equivalent per four atoms of phosphorus have been obtained by Carter and Greenstein (*J. Nat. Cancer Inst.*, 1946, **7**, 29) and by Zittle (*J. Franklin Inst.*, 1947, **243**, 334; *Arch. Biochem.*, 1947, **13**, 191) respectively, it is apparent that such measurements contribute little to interpretations of the mode of action of deoxyribonuclease.

FIG. 1. FIG. 2.

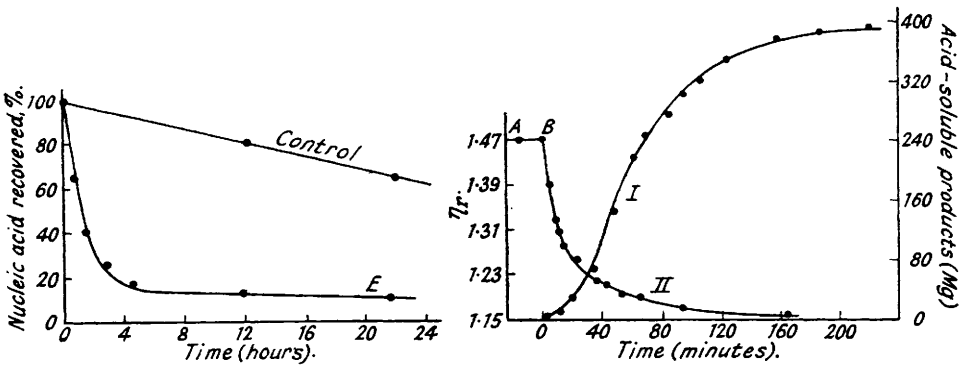

FIG. 1.—*Variation of deoxyribonuclease activity with time. Extent of the hydrolysis of thymus sodium deoxyribonucleate alone (control) and in the presence of deoxyribonuclease (curve E) at 37°.*

FIG. 2.—*Action of deoxyribonuclease on thymus deoxyribonucleic acid.*
Curve I. Formation of acid-soluble material. Curve II. Change in relative viscosity.
A—B shows that there is no change in viscosity before addition of enzyme.

Mode of Action of the Enzyme.—The variation of enzyme activity with time is shown in Fig. 1, which indicates that with 0·001% of enzyme and 1% of substrate the formation of acid-soluble material was complete within 5 hours. Fig. 2 shows the results obtained when more frequent determinations were carried out. [Control experiments indicated that, although prolonged incubation of the nucleic acid at pH 8 resulted in some non-enzymic hydrolysis (see Fig. 1), little (*ca.* 5%) formation of acid-soluble material, due to this cause, occurred during the first five hours.] It is seen that the formation of acid-soluble material does not occur to any marked extent in the first thirty minutes. It was observed, however, that during this lag period there was a rapid fall in viscosity of the substrate. This was essentially complete before the liberation of acid-soluble material became marked and obviously indicated a disaggregation of the deoxyribonucleic acid molecule. The changes in relative viscosity observed with the nucleic acids isolated from calf-thymus gland and herring sperm were considerably greater than the changes observed in solution of commercial (partly degraded) thymus deoxyribonucleic acid, but the solutions had approximately the same final relative viscosity. A full account of these results will be published later. From measurements of the influence of certain ions on the relative viscosities of solutions of the nucleic acid preparations used, it was concluded that the initial change in viscosity of enzyme–substrate systems (see Fig. 2) is caused entirely by the action of the enzyme and not by the non-specific action of ions, as has been suggested by Greenstein *et al.* (*loc. cit.*). Furthermore, no change occurred in the viscosity of a solution of the sodium deoxyribonucleate when 0·1M-magnesium sulphate was added, but a rapid decrease in the relative viscosity of the system followed the addition of a solution of the enzyme (0·002%) free from metallic ions. Moreover the disaggregation of the nucleic acid, as measured by the decrease in relative viscosity of the enzyme–substrate solution, was inhibited by the addition of sodium arsenate (see Fig. 3), which also inhibited the liberation of acid-soluble material (cf. Fischer *et al.*, *loc. cit.*). Since the viscosity measurements were carried out in the presence of 0·02M-magnesium

sulphate it appears probable that at this salt concentration the viscosity changes observed were not caused by disaggregation of the type recently suggested by Jungner *et al.* (*Nature*, 1949, **163**, 849). Thus it is concluded that the action of deoxyribonuclease on deoxyribonucleic acid results initially in the depolymerisation of the molecule to smaller units, which are subsequently hydrolysed to acid-soluble polynucleotides (cf. Greenstein and Jenrette, *Cold Spring Harbor Symp. Quant. Biol.*, 1941, **9**, 236).

Examination of "Acid-soluble" Polynucleotides.—Klein (*Z. physiol. Chem.*, 1933, **218**, 164) considered that the products of the enzymic (thymonuclease) degradation of deoxyribonucleic acid were simple mononucleotides, whereas Fischer *et al.* (*loc. cit.*) concluded that they were tetranucleotides, a conclusion accepted by Zittle (*loc. cit.*). Recent studies (cf. Gulland *et al.*, *loc. cit.*; Chargaff *et al.*, *loc. cit.*), however, conclusively show that the hypothesis of the "structural tetranucleotide" assumed by earlier workers is no longer completely acceptable. Consequently a large-scale enzymic hydrolysis of deoxyribonucleic acid was carried out and the products were examined.

FIG. 3.

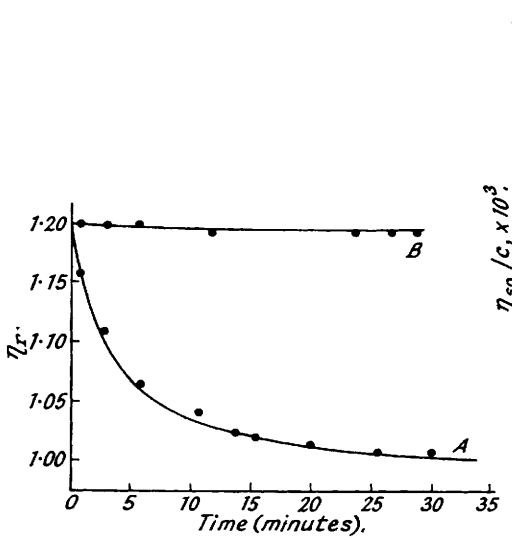

FIG. 4.

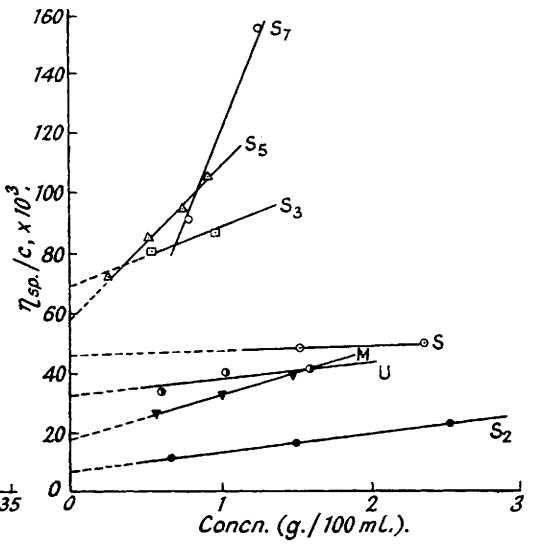

FIG. 3.—*Inhibition by sodium arsenate of the depolymerisation of thymus sodium deoxyribonucleate as measured by the decrease in relative viscosity (η_r) of the enzyme–substrate solution. Substrate:* 1% *(w/v) sodium deoxyribonucleate* (2 *ml.*), 0·2M-*veronal buffer* (1 *ml.*), *and* 0·1M-*magnesium sulphate* (1 *ml.*) *with the addition of* 0·001% *(w/v) solution of deoxyribonuclease* (1 *ml.*) *(curve A), or a mixture of* 0·002% *(w/v) solution of deoxyribonuclease* (0·5 *ml.*) *and* 0·1N-*sodium arsenate* (0·5 *ml.*) *(curve B).*

FIG. 4.—*Variation of $\eta_{sp.}/c$ with concentration (c) shown by the products resulting from the action of deoxyribonuclease on deoxyribonucleic acid.*

(a) *Dialysis.* A preliminary dialysis served to separate the material into dialysable and non-dialysable components (fractions S and R respectively). Graded dialysis of S yielded a number of fractions (S_1—S_6) and a non-dialysable residue (S_7). The fact that a non-dialysable fraction (S_7) was obtained from a mixture which initially was separated by dialysis is attributed to the fact that the fractional dialysis was against repeated changes of distilled water which effectively removed metallic ions from the system. It is known that polynucleotides dialyse to a greater extent in the presence of ions (Lehmann-Echternacht, *Z. physiol. Chem.*, 1941, **269**, 169, 187, 201). Fischer *et al.* (*loc. cit.*) state that the products of the enzymic hydrolysis of deoxyribonucleic acid are completely precipitated from solution by acidic solutions of molybdate ions whereas monoribonucleotides, deoxyriboguanylic acid, and muscle adenylic acid are not precipitated. In contrast to these observations certain of the fractions (*e.g.*, S_2—S_4) which dialysed most readily and presumably were of relatively low molecular weight were not precipitated completely by acid–molybdate reagent. The soluble material could be precipitated by the subsequent addition of uranyl acetate in trichloroacetic acid, and by this means S_2 was separated into two portions (M and U respectively). Consequently the fractions separated by dialysis were far from homogeneous.

(b) *Controlled precipitation with ethanol.* The fraction S was further separated into five

fractions (O_1—O_5) by controlled precipitation with ethanol. It is thought that fractionation by this procedure results in the loss of much of the polynucleotide material of relatively high molecular weight, by co-precipitation with inorganic fractions. In consequence fractions O_2—O_5, which were free from inorganic salts, probably represent the lower polynucleotide degradation products.

(c) *Viscosities of the fractions.* The specific viscosities of aqueous solutions of the fractions were determined in Ostwald viscometers at 37·5°. Results (Fig. 4) confirm that the products of the enzymic hydrolysis are not homogeneous and differ widely among themselves with respect to molecular size. The absence of suitable polynucleotide reference compounds, together with the complex nature of the relationship between molecular size and viscosity of polyvalent acids of high molecular weight (Kern, *Z. physikal. Chem., Abs. A.*, 1938, **181**, 283 ; Owens, Lotzkar, Schultz, and Maclay, *J. Amer. Chem. Soc.*, 1946, **68**, 1628), do not justify any attempt to obtain a measure of the relative molecular weights of the various fragments from these results. However certain general conclusions may be drawn. The relative positions and slopes of the curves ($\eta_{sp.}/c$ plotted against c) for the fractions S_2, S_3, S_5, and S_7 confirm that they are of increasing molecular weight. It is also of interest that $\eta_{sp.}/c$ of these fractions decrease with

FIG. 5.

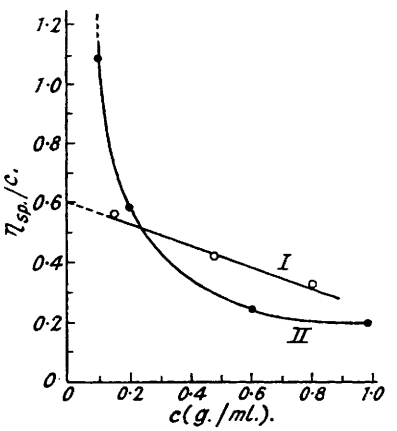

FIG. 6.

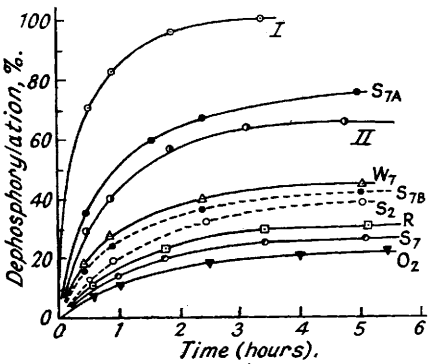

FIG. 5.—*Variation of $\eta_{sp.}/c$ with concentration (c) of deoxyribonucleic acids in water (I) and in 6% sodium chloride solution (II).*

FIG. 6.—*Liberation of inorganic phosphate by the action of prostate phosphatase on adenylic acid (I), adenosine–cytosine dinucleotide (II), and the products (O_2, S_7, R, S_2, S_{7b}, W_7, S_{7a}) resulting from the action of deoxyribonuclease on deoxyribonucleic acid.*

dilution as is the case with neutral polymers, whereas with the intact nucleic acid molecule $\eta_{sp.}/c$ increases with dilution (Fig. 5). This anomolous behaviour is shown by other strong polyelectrolytes (Fouss, *J. Polymer Sci.*, 1948, **3**, 603). With deoxyribonucleic acid the abnormal behaviour cannot be attributed solely to the electroviscous effect, since the relationship between $\eta_{sp.}/c$ and c is even more complex in the presence of an excess of salt, than in water alone (see Fig. 5).

(d) *Diffusion.* Results obtained with the apparatus described by Gage (*Trans. Faraday Soc.*, 1948, **44**, 253) indicate, in a general manner, the increasing molecular size of the dialysis fractions S_2, S_3, and S_4, and confirm the previous suggestion that the O-fractions contain the lower polynucleotides. [Anomalous values obtained with fractions S_{7B} (see later) and O_4 can only be attributed to the many sources of error associated with the measurements.] These results differ from those reported by Fischer *et al.* (*loc. cit.*) who measured absolute molecular weights. However it is considered that with polyelectrolytes of this type the diffusion method is unsuitable for accurate determination of molecular weight.

(e) *Action of prostate phosphatase* (cf. Schmidt, Cubilees, and Thannhauser, *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12**, 161, for analogous work on the products from the action of ribonuclease on ribonucleic acid). The work of Schmidt *et al.* (*loc. cit.*) shows that the amount of the total phosphorus liberated from a polynucleotide as inorganic phosphate by acid phosphatase is, within limits, a measure of molecular size. Fig. 6 indicates results obtained when using the fractions described and also two reference com-

pounds, namely adenylic acid and adenosine–cytosine dinucleotide (a commercial sample supplied by B.D.H. Ltd.). While the measurements were being made, no inorganic phosphate was liberated from deoxyribonucleic acid. From Fig. 6 it is seen that most of the polynucleotide fragments have a mean molecular weight considerably greater than that of the dinucleotide. The relative positions of the curves agree with the results obtained by viscosity measurements.

(f) *Further action of deoxyribonuclease.* Recent evidence (Chargaff and Zamenhof, *J. Biol. Chem.*, 1949, **178**, 531) indicates the existence of an enzyme-resistant core in deoxyribonucleic acid which does not dialyse. Our non-dialysable fraction (S_7) was converted by deoxyribonuclease into a readily dialysable fraction S_{7A} (27·5%, if experimental loses are neglected) and a non-dialysable fraction S_{7B}. In agreement with Zamenhof and Chargaff (*loc. cit.*) the non-dialysable fraction (S_{7B}) was completely resistant to the further action of deoxyribonuclease. From Fig. 6 it is clear that the fractions S_{7A} and S_{7B} differ considerably in molecular size. The fact that S_{7A} was dephosphorylated to the extent of 75% by the prostate phosphatase, whereas an authentic mononucleotide and dinucleotide were dephosphorylated to the extent of 100% and 60% respectively, suggested that S_{7A} contained some mononucleotides. This was confirmed by dephosphorylating fraction S_{7A} with prostate phosphatase and separating the products on a paper chromatogram. The positions of the products on the chromatogram were determined by the method of Hotchkiss (*J. Biol. Chem.*, 1948, **175**, 315). [Control experiments showed that nucleotides, *i.e.*, adenylic acid, adenosine–cytosine dinucleotide, or S_{7A}, did not move on the chromatogram, under the conditions used.] Two products were recognised with absorption max. at 266—267 and 258 mμ. (R_F values, 0·42 and 0·67) respectively. A comparison of these absorption maxima with the values previously recorded for the purines and pyrimidines and their corresponding deoxyribosides (Heyroth and Loofbourow, *J. Amer. Chem. Soc.*, 1931, **53**, 3441; Stimson and Reuter, *ibid.*, 1945, **67**, 847, 2191; Hotchkiss, *loc. cit.*) suggested the presence of thymine deoxyriboside, but did not completely identify the nucleosides.

Since chromatography of larger quantities of the initial enzyme digest failed to give a sharp separation of the components, the corresponding products from three chromatograms were combined and then hydrolysed with sulphuric acid at 175° for 2·5 hours in order to liberate the free bases. After neutralisation neither of the hydrolysates gave a precipitate with silver nitrate, but after appropriate treatment the solution of the base separated from the component with absorption max. at 258 mμ. gave a positive reaction for thymine [Hunter's method (*Biochem. J.*, 1936, **30**, 745), as adapted to microquantities by Woodhouse (*Biochem. J.*, 1949, **44**, 185)]. Since no purines were precipitated as silver complexes at pH 6 (cf. Gulland *et al.*, *loc. cit.*) it would appear that the second nucleoside (absorption max. 266—267 mμ.) is cytosine deoxyriboside. Confirmation of this should be possible by the recent method of Cohn (*Science*, 1949, **109**, 377) in which relatively large quantities of purines, pyrimidines, and nucleosides may be separated by ion-exchange resins. This is being investigated.

The presence of mononucleotides among the products of the enzymic degradation was only demonstrated by the above method in fraction S_{7A}; the components of fractions S_2, W_7 (see p. 2753), and O_5 after treatment with prostate phosphatase failed to move from the initial spot on the paper chromatogram.

(g) *Purine : pyrimidine ratios.* Fractions S_2, S_3, and S_{7B} were hydrolysed with sulphuric acid for 2 hours at 175° and the constituent purines and pyrimidines separated by precipitation as silver complexes (Gulland *et al.*, *loc. cit.*). Nitrogen analyses (micro-Kjeldahl) gave the following values for the ratio of purine-N : pyrimidine-N : S_2 0·44, S_3 0·46, S_{7B} 2·37. These values indicate an increased ratio of pyrimidines to purines in the readily dialysable products (S_2 and S_3), whereas the non-dialysable fraction (S_{7B}) which resists further action of the enzyme contains an increased ratio of purines to pyrimidines.

Discussion.—This work shows that in contrast to the conclusions of Fischer *et al.* (*loc. cit.*) the polynucleotides resulting from the enzymic degradation of deoxyribonucleic acid are not homogeneous tetranucleotides, but exhibit a wide range of molecular size. They appear to range from about tri- or tetra-nucleotides to a relatively large fragment which fails to dialyse in the absence of salts. Although mononucleotides are not normally encountered among the products of the enzymic degradation (cf. Klein, *loc. cit.*), subsequent action of the enzyme on the large fragment liberates some pyrimidine mononucleotides. This observation, coupled with the fact that the smaller, readily diffusible products of the enzyme action contain an increased ratio of pyrimidines to purines compared with the native nucleic acid, indicates that deoxyribonuclease preferentially hydrolyses the pyrimidine nucleotide groupings of the deoxyribonucleic acid molecule.

From the foregoing account it follows that the action of deoxyribonuclease on deoxyribonucleic acid is closely analogous to that of ribonuclease on ribonucleic acid. The latter action is known from micro-dilalometric measurements to involve an initial depolymerisation of the nucleic acid into smaller units (Chantrene, Linderstrøm-Lang, and Vanderdriesche, *Nature*, 1947, **159**, 877) which are then hydrolysed to lower polynucleotides of varying molecular size (Schmidt, Cubilees, Schwartz, and Thannhauser, *J. Biol. Chem.*, 1947, **170**, 759). Furthermore it is apparent from the work of Schmidt *et al.* (*loc. cit.*) and of Loring, Carpenter, and Roll (*ibid.*, 1947, **169**, 601) that ribonuclease preferentially hydrolyses the pyrimidine nucleotide groups of the ribonucleic acid molecule and leaves a non-diffusible core, which contains an increased ratio of purines to pyrimidines and is resistant to further action of the enzyme.

The fact that both ribonuclease and deoxyribonuclease preferentially hydrolyse the pyrimidine nucleotide groupings of the corresponding nucleic acid may, as discussed elsewhere (Overend and Webb, *Research*, 1950, **3**, 238), have some bearing on the specificity of these enzymes.

Experimental.

Preparation of Deoxyribonuclease.—Deoxyribonuclease was isolated from ox-pancreas (20 lbs.) according to McCarty's method (*J. Gen. Physiol.*, 1946, **29**, 123). The final precipitate obtained after repeated fractional precipitation with ammonium sulphate was collected with suction on hardened filter paper (Whatman No. 54) after 24 hours, redissolved in distilled water (15 c.c.), and dialysed at 1° against frequent changes of 0·002N-sulphuric acid until free from salts. The remaining solution was dried in the frozen state to give a white powder (5 g.) which dissolved readily in water. The enzyme preparation contained no ammonium ions, was free from adenine and guanine deaminase, and was devoid of ribonuclease and nucleotidase or phosphatase activity. It had weak proteolytic activity when examined in 1% (w/v) solution against a casein substrate, but exhibited no hydrolytic activity against Witte peptone. In the Tiselius apparatus, one major electrophoretic component was observed together with a small amount of a second component which had approximately the same mobility.

Some Properties of the Enzyme.—The properties of the enzyme were determined, using both a commercial preparation of thymus sodium deoxyribonucleate (B.D.H. Ltd.) and highly polymerised preparations of deoxyribonucleic acid isolated from calf thymus gland and herring roe according to the method of Mirsky and Pollister (*Proc. Nat. Acad. Sci.*, 1942, **28**, 344). The analytical data and properties of these preparations will be recorded in a subsequent communication, and the present account refers only to the results obtained with the commercial samples of thymus sodium deoxyribonucleate. The use of the commercial (partly degraded) nucleic acid in these experiments appeared justified, since one of the objects of the present work was the production of polynucleotides of small molecular weight for structural and other investigations.

Since the commercial thymus sodium deoxyribonucleate contained free inorganic phosphate ions and traces of magnesium ions, it (7 g.) was purified by dissolution in the minimum amount of water followed by dialysis for 6 hours against water (4 l.) containing 5N-ammonia solution (2 c.c.). The sodium deoxyribonucleate was recovered by pouring the filtered solution into ethanol (2 l.) containing 0·85% (w/v) sodium chloride solution (50 c.c.). The precipitate was collected by filtration and dried at room temperature. The resulting white powder (4·5 g.) was free from inorganic phosphate and magnesium ions (Found : N : P, 1·6; Purine-N : Pyrimidine-N, 1·57).

A preliminary determination of the activity of the enzyme [1·0 c.c. of a 0·5% (w/v) solution in water containing magnesium sulphate (0·05%)] against 1·0% (w/v) thymus sodium deoxyribonucleate (2·0 c.c.) at pH 7·0 (0·2M-veronal buffer, 1·0 c.c.) showed that the preparation, under these conditions, completely degraded this nucleic acid to acid-soluble material [as shown by the addition of 5N-hydrochloric acid (0·01 c.c.)] within 12 hours at 37° without the liberation of inorganic phosphate.

(a) *Activation by magnesium ions.* Magnesium sulphate solutions (1·0 c.c.) of increasing concentrations were added to a series of tubes each containing a solution of purified sodium deoxyribonucleate (1·0%, w/v; 2·0 c.c.), 0·2M-veronal buffer (pH 7·0; 1·0 c.c.), distilled water (0·5 c.c.), and 1·0% (w/v) deoxyribonuclease in water (0·5 c.c.). The tubes were incubated at 37° for 18 hours, and then an aliquot (4·0 c.c.) of each solution was transferred to a tared centrifuge tube. The acid-insoluble material remaining in each tube was precipitated by the addition, from a pipette, of 5N-hydrochloric acid (2 drops). The precipitates were collected by centrifuging, dehydrated with ethanol, and washed by centrifuging them first with ethanol and then with ether. After being dried *in vacuo* the tubes were re-weighed, and the influence of the magnesium ion on the activity of the enzyme, as measured by the change in weight of the acid-insoluble material, was determined :

Log of molar concn. of Mg^{++} added	−1	−2	−3	−4	−5	−6
% Nucleic acid recovered	3·6	6·6	68	86	92	94

[McCarty (*loc. cit.*) found 0·003M-Mg^{++} to be the optimal concentration for the activation of systems containing varying amounts of enzyme but a fixed amount of nucleic acid.] Control experiments showed that this method of determining enzyme activity was reproducible and that its accuracy was within 5%.

(b) *Optimum* pH *for the activity of deoxyribonuclease.* A series of tubes containing purified sodium deoxyribonucleate (1%; 2 c.c.), 0·1M-magnesium sulphate (1·0 c.c.), distilled water (0·5 c.c.), and deoxyribonuclease solution (0·1%; 0·5 c.c.) were adjusted to the pH values shown below with 0·2M-phosphate

buffers (1·0 c.c.). The tubes were incubated at 37° for 12 hours and the extent of the enzymic hydrolysis was then determined in aliquots of each solution by acid precipitation as described above. (Average values of several determinations are quoted in this and subsequent experiments.)

pH	3·8	4·5	5·9	6·1	7·2	7·9	9·4
Nucleic acid degraded (%)	44	64	78	79	95	94	70

Determination of the Minimum Enzyme Concentration Required for Degradation of the Nucleic Acid Sample.—Solutions of varying enzyme concentrations (0·5 c.c.) were added to a series of tubes containing thymus sodium deoxyribonucleate solution (1%; 2·0 c.c.), 0·1M-veronal buffer (pH 7·0; 1·0 c.c.), 0·1M-magnesium sulphate (1·0 c.c.), and distilled water (0·5 c.c.). After 12 hours at 37°, the acid-precipitable material was determined in the usual way. Results were:

Log of (final) enzyme concn.	−1	−2	−3	−4	−5	−6	−7
Nucleic acid recovered (%)	—	5	5	8	48	82	90

Inactivation of Deoxyribonuclease by Heat.—Solutions of deoxyribonuclease (1%), buffered at pH 7·0, were maintained for 1 hour at the temperatures shown below. After they had cooled to room temperature their activities against thymus sodium deoxyribonucleate at pH 7·0 were determined in the usual way:

Temp. to which enzyme had been heated	50°	60°	70°	80°	90°	100°
Nucleic acid recovered (%)	—	—	5	15	88	92

Determination of the Acid Groups liberated by the Action of Deoxyribonuclease on Thymus Deoxyribonucleic Acid.—An aqueous solution of sodium deoxyribonucleate (96 c.c., containing 0·592 mg. of organic P per c.c.), together with 0·1M-magnesium sulphate (19 c.c.), was adjusted to pH 7·5 (glass electrode) with 0·01N-sodium hydroxide. The solution was diluted (final volume, 120 c.c.) and divided into two equal portions (I & II). To (I) deoxyribonuclease solution [15 c.c.; 0·002% (w/v)] was added, and to (II) the same enzyme solution (15 c.c.) previously inactivated by heating it for 1 hour at 100° was added. Each solution was diluted to 100 c.c. and incubated at 37° for 18 hours (time for completion). Aliquots (10 c.c.) of each solution were then titrated with standard alkali (phenolphthalein). The difference between the mean titres of (I) and (II) (1·268 c.c. of 0·0091N-NaOH) was a measure of the acid groups liberated by the action of deoxyribonuclease, since control experiments in which aliquots (15 c.c.) of the above enzyme solution, adjusted to pH 7·5 and diluted to 100 c.c., were titrated initially and again after 18 hours at 37° showed that no self-hydrolysis of the enzyme, with the liberation of titratable acid groups, occurred under these conditions. The results show that 0·503 acid equivalent per 4 atoms of phosphorus are liberated by the enzymic action.

Variation of Deoxyribonuclease Activity with Time.—A series of tubes, each containing 1% sodium deoxyribonucleate (2·0 c.c.), 0·1M-veronal buffer (pH 8·0; 1·0 c.c.), 0·1M-magnesium sulphate (1·0 c.c.), and 0·001% (w/v) deoxyribonuclease solution (1·0 c.c.), was incubated at 37°. At suitable time intervals, duplicate tubes were withdrawn and the acid-precipitable material was determined in the usual manner. The results are shown in Fig. 1. If the concentration of the enzyme solution was increased to 0·1%, the substrate concentration remaining unchanged, equilibrium was reached in 1·5 hours.

Viscosity determinations were carried out initially at 36° on 2 c.c. of solution in the viscometers described by Koch, Orthmann, and Degenfelder (*J. Amer. Leather Chem. Soc.*, 1939, **34**, 489). The substrate solution used contained 1% sodium deoxyribonucleate (2·0 c.c.), 0·2M-veronal buffer (pH 6·9; 1·0 c.c.), 0·1M-magnesium sulphate (1·0 c.c.), and distilled water (0·5 c.c.). The solution was brought to 36° and a solution of deoxyribonuclease in water (0·002%, 0·5 c.c.) at 36° was added. After thorough mixing, an aliquot (2 c.c.) was transferred to the viscometer. Later experiments were carried out in Ostwald viscometers on 5 c.c. of solution. The buffered substrate solution (4·5 c.c.) described above was introduced into the viscometer supported in a thermostat at 36°, and the temperature allowed to equilibrate. The deoxyribonuclease solution (0·5 c.c. of a 0·002% solution), preheated to 36°, was then added and measurements were commenced immediately after thorough mixing. Results are shown in Fig. 2. After a slight fall in viscosity owing to dilution, no further change occurred in the viscosity of a solution containing 1% sodium deoxyribonucleate (2·0 c.c.) and water (0·5 c.c.), buffered to pH 6·92 with 0·2M-veronal buffer (1·0 c.c.), followed the addition of 0·1M-magnesium sulphate solution (1·0 c.c.), but a rapid decrease in the relative viscosity of the system followed the addition of 0·002% deoxyribonuclease solution (0·5 c.c.) which was free from metallic ions. If the above experiment was carried out with the water (0·5 c.c.) replaced by 0·1N-sodium arsenate (0·5 c.c.) there was no decrease in viscosity. Results are shown in Fig. 3.

Large-scale Hydrolysis of Deoxyribonucleic Acid with Deoxyribonuclease, and Fractionation of the Resulting Degradation Products.—A solution of commercial thymus sodium deoxyribonucleate (B.D.H. Ltd.; ash, 14%; moisture, 26·5%) (100 g.) in water (2 l.) and 0·2M-magnesium sulphate (125 c.c.) was buffered with 0·2M-veronal buffer (pH 7·0; 625 c.c.) and incubated for 30 hours at 37° with deoxyribonuclease (0·4 g.). The solution was maintained at neutral pH by the addition of 0·1N-sodium hydroxide at suitable intervals. During the hydrolysis a sticky solid separated which adhered to the walls of the containing vessel and was isolated by decantation of the clear supernatant solution. After being dried with ethanol and ether, this residual solid (10 g.) gave the usual protein reactions, was soluble in dilute acids, but was sparingly soluble in dilute sodium hydroxide solution. It gave a weak Molisch reaction and appeared to consist mainly of a basic protein derived from the commercial sample of deoxyribonucleic acid. It was therefore discarded.

The supernatant solution containing the degradation products of the deoxyribonucleic acid was concentrated by pervaporation at room temperature to 750 c.c. During this procedure, veronal crystal-

lised both in the solution and on the outer surface of the cellophane sac; the polynucleotides, however, remained in solution and did not diffuse through the membrane during the evaporation. The concentrated solution was adjusted to pH 2·9 with 5N-hydrochloric acid and clarified by filtration. The filtrate was made slightly alkaline (pH 8) with N-sodium hydroxide, and the precipitate of magnesium hydroxide collected at the centrifuge. This precipitate contained some organic (polynucleotide) material which was recovered by solution in 0·25N-hydrochloric acid and re-precipitated with MacFadyen's reagent (uranyl acetate in trichloroacetic acid) (*J. Biol. Chem.*, 1934, **107**, 299). The precipitate was dissolved in 0·1M-sodium carbonate (2 c.c.) and diluted with water (10 c.c.), and ethanol (36 c.c.) was added. The solid which separated was dehydrated with ethanol and after being dried with ethanol and ether was obtained as a white powder (0·67 g.).

A preliminary fractionation of the above solution (pH 8), containing the main bulk of the mixed polynucleotides, was attempted by dialysis against distilled water (3 l.) for 3 days at 0°. The solution remaining inside the cellophane sac was then added to ethanol (2 vols.), and the resulting precipitate (6·6 g.; fraction R) dried with ethanol and ether. The dialysate (3 l.) was concentrated under diminished pressure at 40° to 750 c.c. and then precipitated with uranyl acetate in trichloroacetic acid (MacFadyen, *loc. cit.*). The precipitate was collected at the centrifuge and redissolved in 0·1M-sodium carbonate (200 c.c.), and the solution added to absolute ethanol (5 vols.). The mixture of polynucleotides precipitated was dehydrated with ethanol and ether, to yield a pale yellow powder (38 g.; fraction S). From evidence obtained later it was apparent that dialysis under the conditions above effected little if any separation of the polynucleotides and merely distributed them into the two fractions S and R of essentially similar composition.

Partial Fractionation of Fraction S.—(a) *Dialysis of mixed polynucleotides* (S). A solution of S (5 g.) in distilled water (200 c.c.) was dialysed at 1° against distilled water (3 l.) changed at intervals. Each dialysate was evaporated under diminished pressure at 40° to 50 c.c. and then added to ethanol (250 c.c.). The resulting precipitate was collected at the centrifuge after 12 hours, washed with ethanol, and dried with ethanol and ether. Dialysis was continued until the final dialysate after concentration afforded no precipitate when added to ethanol. In this way, seven fractions, including the non-dialysable fraction S_7, were collected. S_1 and S_2 were both obtained after 3 days. S_1 was an inorganic fraction

Time of dialysis (days)	3	6	9	13	17	—
Fraction	S_1 & S_2	S_3	S_4	S_5	S_6	S_7 (residue)
Weight (g.)	0·052 & 1·92	0·49	0·23	0·13	0·02	0·35

which crystallised from the concentrated dialysate. S_3 and S_4 were readily soluble in water, dilute acids, and sodium hydroxide. S_5 was soluble in water and acids. Dilute solutions were completely precipitated by molybdate in acid solution. S_7 was a non-dialysable residue, soluble in water; aqueous solutions, in contrast to fractions S_2—S_6, frothed readily on shaking, and gave a slight precipitate with acetic acid; ammonium molybdate in acid solution gave a copious precipitate.

Some further fractionation of S_2 (0·82 g.) was obtained by adjusting its solution in water (20 c.c.) to pH 4 with 5N-acetic acid and adding 10% ammonium molybdate (6 c.c.). The precipitate formed was removed (centrifuge) and the supernatant liquor treated with 10N-acetic acid (3 c.c.). A further precipitate separated. The precipitates (fraction M; 0·47 g.) were combined, washed with N-acetic acid containing 1% ammonium molybdate, and dried with ethanol and ether. The addition of both ammonium molybdate and acetic acid to the supernatant solution failed to produce further precipitation, but the addition of 0·25% uranyl acetate in 2·5% trichloroacetic acid (10 c.c.) precipitated further polynucleotide material (0·46 g., after drying with ethanol and ether) since the precipitate gave a positive test for deoxyribose with the Dische diphenylamine reagent.

(b) *Controlled precipitation with ethanol.* The direct addition of increasing amounts of ethanol to a solution of S (10 g.) in water (100 c.c.) initially gave precipitates which contained little polynucleotide material and were mainly inorganic in nature. After complete removal of the inorganic fractions by the total addition of 225 c.c. of ethanol, no further precipitation occurred until the ethanol concentration reached 80%; fraction W_7 (0·35 g.) was then obtained. The volume of the solution at this stage was such that further fractionation could not be conveniently achieved. Accordingly a solution of S (20 g.) in water (200 c.c.) was added to ethanol (400 c.c.), and the resulting precipitate removed by filtration. The filtrate was concentrated at 40° (bath-temp.) to 70 c.c. and an equal volume of ethanol added. The precipitate (O_1; 0·35 g.) which slowly separated was centrifuged off, washed with ethanol, and dried with ethanol and ether. The precipitate (O_2; 1·6 g.) which separated on addition of ethanol (70 c.c.) to the supernatant liquid was collected after 18 hours and dried as above. Repetition of this procedure yielded fractions O_3 (0·1 g.) and O_4 (0·75 g.). Fraction O_5 (0·05 g.) was obtained by the addition of excess of ethanol (6 vols.) to the supernatant liquid remaining after the isolation of O_4.

Diffusion Measurements on the Degradation Products.—The experiments were carried out in the apparatus described by Gage (*Trans. Faraday Soc.*, 1948, **44**, 253) with filter paper (Whatman No. 54) as the diffusion membrane. A solution (0·5%, w/v) of the given fraction (8·0 c.c.) was introduced into the cell, and diffusion allowed to proceed for 17 hours at 36° against distilled water (1·25 l.). The initial solution used and the solution remaining in the cell were then analysed for organic phosphorus by Allen's method (*Biochem. J.*, 1940, **34**, 858), and the percentage diffusion determined. (A control experiment was made using adenylic acid.)

Fraction	Control (adenylic acid)	S_2	S_3	S_4	S_{7B}	R	O_2	O_3	O_4
Mean diffusion (%)	93·4	43·0	20·4	*ca.* 5·0	64	79·1	86·6	86·9	58·6

Action of Prostate Phosphatase on the Fractionated Degradation Products.—The acid phosphatase was isolated from surgically enucleated human prostate glands by essentially the method of Kutscher and Wolbergs (*Z. physiol. Chem.*, 1935, **236**, 237). The minced glands were suspended in water (4 vols.) and allowed to autolyse under toluene for 4 days at 37°. The suspension was then centrifuged and the acid phosphatase removed from the clear supernatant liquid by adsorption on Cγ alumina [≡20 mg. of $Al(OH)_3$]. The adsorbent was collected (centrifuge) after 10 minutes and washed with water, and the enzyme eluted with 0·5M-citrate buffer (pH 5·6; 2 × 5 c.c.). The combined eluates were saturated with ammonium sulphate, and the resulting precipitate was collected (centrifuge) after 8 hours and dissolved in water (10 c.c.). The solution was dialysed for 70 hours against running tap-water and then dried in the frozen state. A series of tubes, each containing a solution (2%, w/v; 2·0 ml.) of the polynucleotide fraction, 0·2M-acetate buffer (pH 5·0; 1·0 c.c.), and a 0·02% (w/v) solution of prostate phosphatase (1·0 c.c.), was incubated at 37°. At suitable intervals, the contents of one tube were precipitated with MacFadyen's reagent (*J. Biol. Chem.*, 1934, **107**, 299) (4·0 c.c.), and the inorganic phosphate was determined colorimetrically in an aliquot (4·0 c.c.) of the filtrate by Allen's method (*loc. cit.*). The results obtained with the polynucleotide fragments and two reference compounds (adenylic acid and adenosine–cytosine dinucleotide) are shown in Fig. 6.

Further Action of Deoxyribonuclease on S_7.—A solution of S_7 (0·22 g.) in distilled water (20 c.c.) was added to a mixture of 0·1M-magnesium sulphate (5·0 c.c.), 0·2M-veronal buffer (pH 7·6; 10 c.c.), and a 0·002% (w/v) solution of deoxyribonuclease (10 c.c.). After 24 hours at 37°, an aliquot of the digest (40 c.c.) was dialysed at 2° against frequent changes of distilled water until a sample of the dialysate left no residue on evaporation (72 hours). The combined dialysates were concentrated at 40° (bath-temp.) to 30 c.c. and absolute ethanol (4 vols.) was added. The precipitate (S_{7A}) which separated was collected in a tared centrifuge tube, washed with ethanol, and dried with ethanol and ether, to give a white powder (0·065 g.) which contained magnesium sulphate (0·021 g.). The solution remaining in the dialysis sac was added to ethanol (4 vols.), and the precipitate (S_{7B}; 0·095 g.) isolated as above. Thus losses incurred during the experiment being neglected, *ca.* 27·5% of S_7 was rendered dialysable by the further action of deoxyribonuclease. S_{7B} was completely resistant.

Identification of Mononucleotides in Fraction S_{7A}.—The experimental procedure finally adopted was as follows. Fraction S_{7A} (10 mg.) was dissolved in 0·1M-acetate buffer (pH 5·0; 3·0 c.c.), and 1·0 c.c. of this solution diluted with distilled water so that the concentration of S_{7A} was 500 μg./c.c. The remaining 2·0 c.c. of the solution were incubated at 37° for 5 hours with a solution of prostate phosphatase (0·02%, w/v; 0·5 c.c.) and then similarly diluted. Some of each solution (0·1 c.c. ≡50 μg. of S_{7A}) was deposited in lanes (2-cm. wide) on a strip (12 × 40 cm.) of Whatman No. 1 filter paper according to the method described by Hotchkiss (*J. Biol. Chem.*, 1948, **175**, 315). The chromatogram was run with *n*-butanol saturated with water, as solvent, in an atmosphere of butanol, water, and ammonia (cf. Hotchkiss, *loc. cit.*). After 18 hours at 20° the position of the solvent front was marked, and the paper strip inverted and dried initially at room temperature and then in an oven at 110° for 20 minutes. The position of the nucleosides on the chromatogram could not be developed by any of the methods used to demonstrate the free purines and pyrimidines (Vischer and Chargaff, *J. Biol. Chem.*, 1948, **176**, 705, 715). Accordingly the individual lanes were cut into sections which were then cut into small pieces and separately extracted with water (4·0 c.c.). After the paper pulp had been centrifuged off, the clear supernatant solutions were examined in the Beckman photoelectric quartz spectrophotometer. No material which absorbed ultra-violet light was demonstrated in the eluates from the control runs of S_{7A} alone. In the case of S_{7A} treated with prostate phosphatase, the presence in the eluates of two absorbing substances with maximum absorption at 266—267 and 258 mμ. was demonstrated at R_F values of 0·42 and 0·67. In order to characterise further the nucleoside fractions separated above, the eluates from three chromatograms which exhibited the same absorption maxima were combined. The resulting solutions (absorption max., 266—267 and 258 mμ. respectively) were evaporated to small volume (2—3 c.c.) and hydrolysed with concentrated sulphuric acid (0·4 c.c.) in sealed tubes at 175° for 2·5 hours. According to the method of Woodhouse (*Biochem. J.*, 1949, **44**, 185), the acid solutions were then brought to pH 9 by the addition of hot saturated barium hydroxide solution and filtered from the precipitated barium sulphate. After evaporation the solutions (5—10 c.c.) were acidified to pH 6 with 5N-nitric acid, and 5% silver nitrate (1 c.c.) was added to each. As in each case, no precipitate was formed (absence of purines), the solutions were made alkaline to pH 9 with barium hydroxide solution, and the resulting precipitates collected (centrifuge) after 18 hours. These were suspended separately in N-hydrochloric acid (3 c.c.), and the suspensions heated at 80° for 30 minutes. After centrifugation, the clear supernatant solutions were neutralised with sodium carbonate and tested for the presence of thymine by Hunter's method (*Biochem. J.*, 1936, **30**, 745) as adapted to micro-quantities by Woodhouse (*loc. cit.*). A positive reaction was given by the solution of the base separated from the nucleoside fraction with absorption maximum at 258-mμ.

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