16. *The Constitution of Yeast Ribonucleic Acid. Part VII. Diffusion Coefficients and Molecular Weights.*

By W. E. Fletcher, J. Masson Gulland, D. O. Jordan, and in (part) H. E. Dibben.

From measurements of diffusion coefficients it is concluded that yeast ribonucleic acids of different origin have molecular weights ranging between those corresponding to 8 and 18 hypothetical tetranucleotides. Deamination of B.D.H. yeast ribonucleic acid under special conditions does not diminish the molecular weight, confirming the conclusion already reached that phospho-amide groups do not play an essential part in linking nucleotides in the acid from that source. Less controlled conditions cause extensive molecular degradation.

THE values of the molecular weight of yeast ribonucleic acid previously recorded show considerable disagreement. Myrbäck and Jorpes (*Z. physiol. Chem.*, 1935, **237**, 159), using the method of free diffusion described by Ohölm (*Z. physikal. Chem.*, 1904, **50**, 309), determined the molecular weight of a sample of yeast ribonucleic acid prepared by Merck, and obtained values between 1300 and 2150 in the presence of 5% sodium chloride. Loring (*J. Biol. Chem.*, 1939, **128**, Sci. Proc., **33**, 61), using the method of Northrop and Anson (*J. Gen. Physiol.*, 1928—29, **12**, 543), obtained values for the diffusion coefficient of 0·13—0·11 cm.² per day, giving values for the molecular weight of 17,000—28,000. Fischer, Böttger, and Lehmann-Echternacht (*Z. physiol. Chem.*, 1941, **271**, 246), using Jander and Spandau's (*Z. physikal. Chem., A*, 1939, **185**, 325; 1940, **187**, 13) modification of Brintzinger's dialysis method (see *ibid.*, p. 317), obtained a value of 10,300 for the molecular weight of Boehringer yeast ribonucleic acid. Bredereck and Hoepfner (*Ber.*, 1942, **75**, 1086), using the same method, give the value of 1177 for the product isolated after the mild alkaline hydrolysis of yeast ribonucleic acid. This material they consider to be the fundamental tetranucleotide unit.

In Table I are given the diffusion coefficients (D) at 25° for various samples of yeast ribonucleic acid in N-sodium sulphate, calculated by using Mehl and Schmidt's equation (*Univ. Cal. Pub. Physiol.*, 1937, **8**, 165). The constancy of the values with decreasing concentration of the nucleic acid indicates the absence of abnormal diffusion processes.

TABLE I.

Sample of yeast nucleic acid.	Cell.	Nucleic acid concn., %.	Buffer.	Time, hrs.	D, cm.²/day.
B.D.H. Y21	1	0·66	Borate pH 8·0	1	0·116
	1	0·97	,,	1	0·114
	1	1·20	,,	1	0·116
	4	0·97	,,	5	0·115
	4	0·97	,,	24	0·116
	4	1·90	,,	43·5	0·117
B.D.H. Y8	4	1·53	Acetate pH 5·8	17	0·105
	4	1·53	,,	23	0·106
	4	1·53	,,	26	0·105
	1a	1·04	(None) pH 7·0	4·5	0·099
	1a	0·62	,,	3	0·102
Boehringer BY1	1	1·40	Borate pH 8·0 *	1	0·139
	1	0·63	,,	1	0·124
	1	0·11	,,	2	0·138
Eastman Y9	1a	0·71	(None) pH 6·9	16	0·105
	1a	0·71	,,	5	0·102
	1a	0·71	,,	17	0·100
Merck Y10	4	0·76	(None) pH 7·0	22	0·104
	4	0·76	,,	23	0·107
	4	0·76	,,	29·5	0·115
Product of mild alkaline hydrolysis	1	0·80	Borate pH 8·0	1	0·162
Deaminated B.D.H. DY33	1	0·80	,,	1	0·114
DY5	4	1·33	Acetate pH 5·8	22	0·105
	4	1·33	,,	23	0·096
	4	1·33	,,	47·5	0·102
Boiled sample of DY5	4	0·44	(None) pH 7·0	19	0·227
	4	0·44	,,	22·5	0·242
	4	0·44	,,	22·5	0·256

* N-Sodium chloride in place of N-sodium sulphate.

By application of the Stokes–Einstein equation to the values of the diffusion coefficient, a value of the molecular radius may be obtained on the assumption that the diffusing ion is spherical—an assumption which is justifiable provided that the asymmetry of the molecule be not too great. The molecular weight may then be directly obtained from this value of the molecular radius and the specific weight of the diffusing particle. In Table II the approximate values of the molecular weights determined from the mean diffusion coefficients are given. The value of the specific weight of yeast ribonucleic acid and of deaminated yeast ribonucleic acid was taken as 1·70 g. per c.c.

TABLE II.

Sample of yeast ribonucleic acid.	D, cm.²/day.	M.	Sample of yeast ribonucleic acid.	D, cm.²/day.	M.
B.D.H. Y21	0·116 [5]	15,830	Product of mild alkaline hydrolysis [1]	0·162 [5]	5,810
Y8	0·102 [6]	22,550	Deaminated B.D.H. DY33 [2] ...	0·114 [5]	16,690
Boehringer BY1	0·134 [5]	10,280	DY5 [3]	0·102 [6]	22,550
Eastman Y9	0·102 [5]	23,250	Boiled sample of DY5 [4]	0·242 [7]	1,830
Merck Y10	0·108 [5]	19,630			

[1] Prepared according to the method of Bredereck and Hoepfner (*loc. cit.*). [2] Prepared from B.D.H. Y21.
[3] Prepared from B.D.H. Y8. [4] Contained a trace of inorganic phosphorus. [5] Borate buffer at pH 8·0.
[6] Acetate buffer at pH 5·8. [7] Unbuffered N-Na_2SO_4 solution at pH 7·0.

It is to be concluded from the data in Table II that the preparations of yeast ribonucleic acid investigated are polynucleotides, built up of 8—18 tetranucleotide units, although it must be emphasised that there is as

yet little evidence for the existence of the tetranucleotide as a chemical entity. The isolation of a product having a molecular weight of 5810 in place of the product of molecular weight 1177 obtained by Bredereck and Hoepfner (*loc. cit.*), after the mild alkaline hydrolysis of Boehringer yeast ribonucleic acid, suggests that the nature of the hydrolysis product is very dependent on the conditions of hydrolysis, and possibly also on the molecular size of the starting material.

The diffusion coefficients and molecular weights of deaminated B.D.H. yeast ribonucleic acid are of the same order (Table II) as those of the original acid. This may be interpreted as indicating that both these substances are similarly constituted; and that a P–N linkage involving a primary amino-group is not an essential structure in the polynucleotide, thus confirming the conclusions of Falconer, Gulland, Hobday, and Jackson (J., 1939, 907).

Experimental.

Determination of the Diffusion Coefficient.—The diffusion coefficient of molecules possessing high molecular weight may be determined by the porous disc method of Northrop and Anson (*loc. cit.*), who in applying Fick's law ($dQ/dt = Ddc/dx$, where dQ is the amount of substance diffusing across an area of 1 cm.^{2} in time dt under a concentration gradient dc/dx) assume that $dc/dx = (c' - c'')/h$, where c' and c'' are the concentrations on either side of the porous disc in the diffusion cell, and h is the effective thickness of the porous disc. This assumption introduces the restriction that the variation of c' and c'' during the experiment must be small; Northrop and Anson suggest that the maximum permissible variation in c' is 3%. McBain and Liu (*J. Amer. Chem. Soc.*, 1931, **53**, 59), using the same experimental method, assume that the variation of the concentration within the cell over a considerable time period is negligible. The technique of the method has been improved by Mehl and Schmidt (*loc. cit.*), who, by changing the outer solution of the diffusion cell at increasingly frequent intervals during a preliminary period when the diffusion gradient is being established, commence the measured diffusion period with the concentration of the solution at the lower surface of the porous disc very nearly zero. To determine the diffusion coefficient, Mehl and Schmidt use the following equation, which is derived from Fick's law by integration over the time interval t and is therefore valid for any value of t irrespective of the magnitude of the change in concentration:

$$D'kt = [V''V'/(V'' + V')]\{\ln c_0'' - \ln [c_0'' - (1 + V'/V'')c']\}$$

where D' is the integral diffusion coefficient representing a mean value over a range of concentration, k the cell constant, and t the time interval; V'' is the volume and c_0'' the original concentration of the upper solution, V' is the volume of the lower solution and c' the concentration after diffusion over the time period t.

During the diffusion of ions of high molecular weight, an electrical potential may be established by the rapid diffusion of the more mobile ions of opposite charge. It is therefore necessary (see, *e.g.*, Hartley and Robinson, *Proc. Roy. Soc., A*, 1931, **134**, 20; Valko, *Trans. Faraday Soc.*, 1935, **31**, 230), in order to obtain a value for the diffusion coefficient which may be used in the Stokes–Einstein equation for the evaluation of the particle radius, to carry out the determination of the diffusion coefficient in the presence of a considerable excess of electrolyte.

Apparatus.—The diffusion cells used were similar to those described by Northrop and Anson (*loc. cit.*). The porous diaphragms were sintered glass discs, porosity number 4, and had the following dimensions: cell 1 and 1a, 50 mm. in diam., 0·5 mm. thick; cell 4, 30 mm. in diam., 2 mm. thick. In order to prevent evaporation of the lower solution, a rubber ring was fitted between the cell and the outer vessel as described by McBain and Liu (*loc. cit.*). To reduce vibration to a minimum, the cells were mounted on a slate bench built into a main structural wall. The air temperature was maintained at 25° ± 0·2°.

The cell constant was determined by using 2N-sodium chloride as the diffusing solution. The diffusion constant of sodium chloride at this concentration has been given by Anson and Northrop (*J. Gen. Physiol.*, 1937, **20**, 575) as 1·26 cm.^{2}/day. This solution has the advantage that the diffusion coefficient remains constant when the concentration is decreased. The method of manipulation was carried out as described by Mehl and Schmidt (*loc. cit.*). The outer solution was analysed for chloride by titration with 0·1N-silver nitrate. The values for the cell constants were: cell 1, 30·0; cell 1a, 30·5; cell 4, 2·62.

The diffusion of yeast ribonucleic acid was carried out in Clark and Lubs' borate buffer, pH 8, and in acetate buffer, pH 5·8, containing added electrolyte; and also in unbuffered N-sodium sulphate solution. The nucleic acid concentration in the outer solution was determined by estimating the phosphorus content by Briggs' modification (*J. Biol. Chem.*, 1922, **53**, 13) of Bell and Doisy's colorimetric method (*ibid.*, 1930, **44**, 55). The nucleic acid before dissolution was dried in a vacuum over phosphoric oxide at 100° to constant weight, and analysed by the same method and by the strychnine phosphomolybdate method [Embden, *Z. physiol. Chem.*, 1920, **113**, 138, modified by Still (unpublished)] which gave identical results.

The determination of the specific weight of the samples of nucleic acid was carried out by two methods. (1) The specific weight of the solid nucleic acid was determined by the usual method using a specific gravity bottle (10 c.c.) and pure, dry benzene as the inert liquid. (2) The specific volume of the nucleic acid ion was determined pycnometrically and calculated from the formula $v_2 = [n_0 - (n_1 - n_2)]/d_0n_2$, where v_2 is the specific volume of the nucleic acid ion, n_0 and n_1 are the weights of solvent and solution respectively contained in the pycnometer, n_2 is the weight of solute present in n_1 g. of solution, and d_0 is the density of the solvent. The specific weight is the reciprocal of the specific volume. The values obtained by the two methods were in agreement, although variations from 1·65 to 1·80 g. per c.c. were obtained. These variations may be attributed to the hygroscopic nature of nucleic acid which introduces an inaccuracy during weighing.

The measurement of the viscosity of the nucleic acid solutions, required in the Stokes–Einstein equation, was determined at 25° by means of an Ostwald viscometer.

Purification of Yeast Ribonucleic Acid.—The samples of this acid were supplied by B.D.H., Boehringer, Merck, and Eastman. The commercial samples were purified by a method similar to that described by Makino (*Z. physiol. Chem.*, 1935, **236**, 206). Yeast ribonucleic acid (60 g.) was suspended in cold water (1650 c.c.) and dissolved by the addition of 2N-ammonium hydroxide (80 c.c.) with mechanical stirring. This solution had pH *ca.* 5·8. After being filtered, it was poured with vigorous stirring into alcohol (3460 c.c.) containing concentrated hydrochloric acid (20 c.c.). The final pH of this solution was 2·2. The white gelatinous precipitate of nucleic acid was collected, stirred with 66% alcohol (500 c.c.), and again collected. The nucleic acid was resuspended in cold water (1000 c.c.) and dissolved by the slow addition of 2N-ammonium hydroxide (65 c.c.). The resulting solution was filtered and poured slowly into alcohol (2130 c.c.) containing concentrated hydrochloric acid (17 c.c.); the final solution had pH 2·0. The precipitated nucleic acid was collected, repeatedly washed by being vigorously dispersed in 66% alcohol until free from chloride ions, and finally collected and washed successively with 80% alcohol, 100% alcohol, and ether, and dried in a vacuum over phosphoric oxide and potassium hydroxide. The yield was 40 g. (Found, in samples dried at 110° in a vacuum over phosphoric oxide: B.D.H., Y1: N, 16·2; P, 8·8. Y2: N, 16·0; P, 8·6. Y21: N, 16·0; P, 8·8. Y8: N, 16·3; P, 8·9. Boehringer, BY1: N, 15·8;

P, 9·1. Merck, Y10: N, 16·1; P, 9·3. Eastman, Y9; N, 15·7; P, 8·9. Calc., for a tetranucleotide containing 1 mol. of the nucleotides, adenylic, guanylic, uridylic, and cytidylic acids, $C_{38}H_{47}O_{28}N_{15}P_4$: N, 16·3; P, 9·5%).

A specimen of nucleic acid having a lower molecular weight was prepared by the mild alkaline hydrolysis of purified Boehringer yeast ribonucleic acid, as described by Bredereck and Hoepfner (*loc. cit.*) (Found, in sample dried at 110° in a vacuum: N, 15·6; P, 8·8%).

Deamination of Yeast Ribonucleic Acid (with H. E. DIBBEN).—A method of deaminating yeast ribonucleic acid has been described by Bredereck, Köthnig, and Lehmann (*Ber.*, 1938, **71**, 2613) in which the acid, in neutral solution, was treated with sodium nitrite and acetic acid at room temperature, and the deaminated nucleic acid subsequently precipitated as the lead salt. This was suspended in water and decomposed by hydrogen sulphide; these authors do not record the temperature at which this process was carried out, but we have found that the lead sulphide is coagulated satisfactorily only when the treatment is in hot water; cold decomposition of the salt proved quite unsuccessful. The filtrate from lead sulphide was then concentrated to small volume under reduced pressure, and the deaminated nucleic acid precipitated by the addition of alcohol, collected, and dried.

The electrometric titration (Part VIII; following paper) of samples of deaminated acid, prepared as described by Bredereck, Köthnig, and Lehmann and including heat decomposition of the lead salt, suggested that the number of acidic groups of the product was dependent on the length of time for which the solution was heated during the precipitation of the lead sulphide.

We therefore consider the method of Bredereck *et al.* to be unsatisfactory, owing to the difficulty of removing the last traces of sodium acetate and the probability of decomposition occurring during the treatment with hydrogen sulphide. An attempt was therefore made to bring about deamination by using amyl nitrite in acetic acid solution at room temperature and at 40° for various lengths of time, followed by alcohol precipitation. Determination of the amino-nitrogen by the van Slyke method, however, showed that the maximum deamination obtained was only 10% of the theoretical.

The following method was finally adopted for the deamination of yeast ribonucleic acid; precipitation as the lead salt can be avoided if potassium salts are used, because potassium acetate is sufficiently soluble in alcohol to escape precipitation along with the deaminated acid. Purified acid (10 g.) was suspended in water (20 c.c.) and dissolved by adding slowly 0·5N-potassium hydroxide (44 c.c.); the pH of the solution remained below 6·0 throughout this operation. The solution was transferred to a narrow-necked flask, and potassium nitrite (20 g.) dissolved in water (10 c.c.) added, together with a few drops of capryl alcohol to prevent frothing. The flask was closed by a Bunsen valve, and glacial acetic acid added slowly over a period of 2 hours. The mixture stood at room temperature for 48 hours; nitrous acid was then still present. The solution was then poured into alcohol (150 c.c.) containing concentrated hydrochloric acid (6 c.c.). The precipitated deaminated ribonucleic acid was collected, washed with 66% alcohol, suspended by stirring in cold water (30 c.c.), and dissolved by slowly adding 2N-ammonium hydroxide (12 c.c.); the solution had a pH value of 5·0. The deaminated acid was then finally precipitated by pouring this solution into alcohol (78 c.c.) containing concentrated hydrochloric acid (4 c.c.); the final pH of this solution was 1·6. The precipitate was collected, washed with 75% alcohol until free from chloride, nitrite, and nitrate ions, then with 80% alcohol, 100% alcohol, and ether, and dried in a vacuum over phosphoric oxide and potassium hydroxide. The yield was 4 g. (Found, in samples dried at 110° over phosphoric oxide in a vacuum; DY33: N, 13·3; P, 8·9. DY 5: N, 13·1; P, 8·9. Calc. for deaminated tetranucleotide containing 1 mol. of each of the nucleotides inosinic and xanthylic acids and 2 mols. of the nucleotide uridylic acid, $C_{38}H_{44}O_{31}N_{12}P_4$: N, 12·9; P, 9·5%).

Deaminated yeast ribonucleic acid undergoes hydrolysis when an aqueous solution of the acid is heated; it has been shown by electrometric titration (see Part VIII) that this hydrolysis is complete after heating at 100° for 10 minutes. The isolation of the hydrolysis product was attempted by concentrating the solution under reduced pressure and precipitating it by addition of alcohol containing hydrochloric acid. The product was contaminated by free purines; for this reason the determination of the diffusion coefficient of the hydrolysis product was carried out on the solution obtained after hydrolysis, which contained only a trace of free purine.

The microanalyses were carried out by Mr. J. E. Still. It is a pleasure to record the help given by Imperial Chemical Industries Ltd. in the form of grants for chemicals and apparatus.

UNIVERSITY COLLEGE, NOTTINGHAM. [*Received, November 19th*, 1943.]