43. *The Constitution of Yeast Ribonucleic Acid. Part IX. Alkali-labile Linkages.*

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Evidence has now accumulated that yeast ribonucleic acid does not contain phospho-diester groups involving the hydroxyl at C_5 of the sugar. The suggestion, previously made tentatively, cannot be sustained, namely, that the observed liberation of inorganic phosphate by Russell's viper venom occurred as a result of the united action

of its phosphodiesterase and highly specific 5-nucleotidase on such groups. The explanation of the dephosphorylation is found in the combined results of fission of phospho-monoesters (i) by a very slow enzyme action and (ii) by alkaline hydrolysis. Thus, enzymic experiments have permitted the demonstration of the presence in yeast ribonucleic acid of alkali-labile ester groups, of which the existence has hitherto been inferred by reason of the alkaline fission of the acid to nucleotides. The site of the labile linkages in the polynucleotide is discussed and considered to be at C_3 of the sugar.

During the first, rapid stage of enzymic dephosphorylation, the liberated phosphate originated from the purine nucleotides. Subsequently, both purine and pyrimidine nucleotide phosphates were set free, the latter more rapidly than the former.

RUSSELL'S viper venom, which contained a phosphodiesterase and 5-nucleotidase but no apparent non-specific phosphomonoesterase (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 590, 597), effected partial dephosphorylation of yeast ribonucleic acid (Gulland and Jackson, J., 1938, 1492). The possibility was stated tentatively that the nucleic acid might contain doubly esterified phosphoryl groups linked to hydroxyls at C_3 and C_5 of the sugar radicals, and that these groups were set free as inorganic phosphate by 5-nucleotidase following hydrolysis of the C_3 link by the diesterase. It was, however, pointed out that a decision on this point would have to be deferred until knowledge became available of the action of 5-nucleotidase on nucleoside-5-phosphates other than muscle adenylic and inosinic acids, the only substrates known for this highly specific enzyme (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 597).

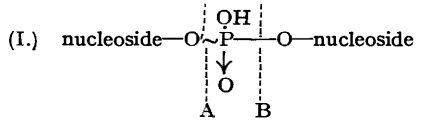
Evidence against the presence of phosphoryl groups at C_5 in yeast ribonucleic acid has accumulated from two directions. First, uridine-5-phosphate and guanosine-5-phosphate, both possible substrates on the preceding line of argument, were prepared by Gulland and Hobday (J., 1940, 746) and are not attacked by 5-nucleotidase. Second, the products of hydrolysis of the nucleic acid by cold 1% sodium hydroxide solution are the 3-phospho-esters guanylic, adenylic, cytidylic and uridylic acids. It is, however, a sound assumption that, if 5-phospho-ester groups were present in the molecule, they would resemble the 3-phospho-esters in surviving this treatment, since adenosine-5-phosphate, for example, is resistant to cold 1% sodium hydroxide solution and is only slightly more susceptible to drastic conditions of alkaline hydrolysis than is adenosine-3-phosphate (Gulland and Jackson, J., 1938, 1492). It thus seems that yeast ribonucleic acid does not contain 5-phospho-ester groups and that the explanation of the dephosphorylation by Russell's viper venom must be sought in other directions.

In the present investigation, made with purified yeast ribonucleic acid from two B.D.H. samples, measurements of inorganic phosphate were made (i) at intervals during the action of the venom on the purified nucleic acid; (ii) at intervals during the action of the venom on the mixed nucleotides resulting from the hydrolysis of the acid by cold 1% sodium hydroxide solution; (iii) after the action of cold 1% sodium hydroxide solution on the products formed when the venom acts on the acid for various periods of time.

The venom liberated about 25% of the total phosphate from the nucleic acid with relative rapidity. The rate then slackened, but dephosphorylation continued steadily for several days and finally appeared to cease when 50—55% of the total phosphate had been mineralised. The slow liberation of phosphate in the second stage proceeded at a rate closely similar to that of the dephosphorylation under comparable conditions of the mixed nucleotides produced by cold alkaline hydrolysis of the same quantity of nucleic acid. It was concluded that in addition to its pronounced 5-nucleotidase and phospho-diesterase activities the venom also exhibited a very weak or slow phosphomonoesterase activity towards the nucleotides, and this was confirmed by experiments with the mixed nucleotides and with guanylic, adenylic, cytidylic, and uridylic acids.

When sodium hydroxide, in amounts sufficient to make a 1% solution, was added to the products of the action of venom on purified nucleic acid, inorganic phosphate was set free in addition to that liberated during the enzyme action. This was not due to the further action of the venom phosphatases, since it occurred in presence of cyanide ions, which were shown to inhibit the action of these enzymes on a variety of substrates, as will be described elsewhere. Since phosphate was not liberated by cold 1% sodium hydroxide solution in control experiments with the nucleic acid or the mixed 3-phospho-nucleosides, it follows that alkali-unstable phospho-esters were formed during the action of the venom on the nucleic acid. These tended to accumulate during the early stage of the venom action and later underwent hydrolysis, either gradually in the alkaline (pH 8·6) medium of the experiments or more rapidly in 1% sodium hydroxide solution. By the end of the period of enzymic hydrolysis, the quantity of alkali-labile esters in the solution had become extremely small.

These facts suggest that, using as a basis of discussion a polymer of the hypothetical tetranucleotide (Fletcher, Gulland, and Jordan, J., 1944, 33), one or both of the diester groups associated with each four atoms of phosphorus is in the form (I), where A represents an alkali-unstable link and B the alkali-stable 3-phospho-ester

(I.) 

link found in the individual nucleotides as obtained by alkaline hydrolysis of the nucleic acid. Enzymic fission could occur entirely at B in one such group or concurrently at A and B in both groups; the resulting ester groups with the A link unattacked would then undergo further hydrolysis by the alkali.

This liberation of inorganic phosphate by alkali after inhibition of the enzymes with cyanide forms the first demonstration of the independent existence of alkali-unstable phosphoryl groups in the fission products

of yeast ribonucleic acid. Their presence in the acid itself has for many years only been inferred from the ease with which alkaline hydrolysis effects fission into the four constituent nucleotides.

The following considerations are relevant in assigning positions to these groups in the samples under investigation. Yeast ribonucleic acid is a polynucleotide considerably larger than a tetranucleotide (Loring, *J. Biol. Chem.*, 1939, **128**, *Sci. Proc.*, **33**, 61; Fischer, Böttger, and Lehmann-Echternacht, *Z. physiol. Chem.*, 1941, **271**, 246; Fletcher, Gulland, Jordan, and Dibben, J., 1944, 30). The hydroxyls at C_3 (presence of phosphoryl group) and at C_5 of the sugar radicals are excluded. The hydroxyls of the purines and pyrimidines are not substituted, since they were titratable (Fletcher, Gulland, and Jordan, J., 1944, **33**). The amino-groups were unsubstituted, since they underwent deamination without diminution of the molecular size (Fletcher, Gulland, Jordan, and Dibben, *loc. cit.*). It thus seems that the hydroxyls at C_2 of the sugars alone remain as possible sites, a view which is not novel (Levene and Tipson, *J. Biol. Chem.*, 1935, **109**, 623; Makino, *Z. physiol. Chem.*, 1935, **236**, 201; Bredereck, " Fortschritte d. Chemie Org. Naturstoffe," 1938, **1**, 152), although previous conjectures were based on the hypothesis that the molecule is a simple tetranucleotide. This view cannot be accepted unreservedly until positive, rather than negative, evidence becomes available, since so far as we are aware the literature does not record for substituents in the hydroxyls of sugars differences in stability so marked in degree as to explain the complete fission of an ester at C_2 whilst a similar linkage at C_3 remains wholly unattacked.

A working hypothesis is as follows: Pentose polynucleotides, which suffer alkaline fission into nucleotides and of which the molecular weight is not diminished by deamination, are built up by a series of phospho-ester linkages at C_2 and C_3 of the sugars. Alkali-labile phospho-amide groups may occur in some samples of nucleic acids, guanine-uridylic acid, for example, being obtainable from some, but not all, samples of yeast ribonucleic acid (Falconer, Gulland, Hobday, and Jackson, J., 1939, 907; Bredereck, Berger, and Richter, *Ber.*, 1941, **74**, 338), but such groups would undergo fission and deamination by nitrous acid (Falconer *et al.*, *loc. cit.*; Gulland, *Chem. and Ind.*, 1940, **59**, 312; Fischer, *Naturwiss.*, 1942, **30**, 377) and diminution of molecular size would then occur. It is possible that phospho-amide linkages may unite polynucleotides of intermediate size to form large molecules of the nucleic acid in its native state; if so, they might undergo, or survive, hydrolysis in the alkaline media at present used to extract the acid from yeast.

The phosphate of yeast nucleic acid or of a mixture of its nucleotides may be estimated differentially as purine nucleotide phosphate and pyrimidine nucleotide phosphate by the method of Jones (*J. Biol. Chem.*, 1916, **24**, *Sci. Proc.* iii). During the action of Russell's viper venom on the acid, samples were analysed by his method for the amounts of purine and pyrimidine nucleotide phosphate remaining in organic combination. During the first stage of the enzyme action (comparatively rapid dephosphorylation) all the pyrimidine nucleotide phosphate originally present remained combined, *i.e.*, the phosphate liberated, about 25% of the total, originated from the purine nucleotides. During the second stage (slow dephosphorylation) both purine and pyrimidine nucleotide phosphate were set free, the latter more rapidly than the former. The phosphate liberated during this stage resulted from the alkaline hydrolysis of the alkali-labile phosphoryl groups and the action of the non-specific phosphomonoesterase on the nucleotides present.

The integration of results of this nature with analogous observations of others (for example, Bolomey and Allen, *J. Biol. Chem.*, 1942, **144**, 113; Bredereck, Berger, and Richter, *Ber.*, 1941, **74**, 338) may in time throw light on the relative orientation of the nucleotides in the polynucleotide.

Experimental.

Two samples of B.D.H. nucleic acid were used; one was obtained from Fletcher, Gulland, and Jordan (*loc. cit.*), the other was purified as described by these authors. Estimations of inorganic phosphate were made colorimetrically by the Bell–Doisy–Briggs method.

Action of Russell's Viper Venom on Purified Yeast Ribonucleic Acid and on the Mixed Nucleotides obtained by Cold Alkaline Hydrolysis.—Substrate A: Purified yeast ribonucleic acid (P = 9·2%) (0·2 g.) was dissolved in a little water with sufficient aqueous sodium hydroxide to yield a solution faintly pink to phenolphthalein, and the solution diluted with water to 20 c.c. Substrate B: The same sample of nucleic acid (0·2 g.) was dissolved as before, 10% sodium hydroxide solution (2 c.c.) added, and the whole made up to 20 c.c. with water. After 24 hours at 20°, glacial acetic acid was added until the solution was faintly pink to phenolphthalein. Enzyme solution: Russell's viper venom (100 mg.) in water (10 c.c.). Mixtures of substrate solutions A and B respectively (2·0 c.c.), Clark and Lubs pH 8·6 borate buffer (10 c.c.) and water (5 c.c.) were warmed to 37°, enzyme solution (2·0 c.c.) added to each, and the volumes adjusted to 20 c.c. A few drops of toluene were added, and the mixtures incubated at 37° and pH 8·6. The phosphorus concentrations were respectively: Expt. A, 0·086 mg. per c.c.; Expt. B, 0·083 mg. per c.c. Samples (1·0 or 2·0 c.c.) were withdrawn during the course of the incubation for the determination of inorganic phosphate.

Table I.

Time, hours.		0.	2.	5.	8.	26.	50.	75.	100.	120.
PO_4 liberated, %.	A	0	6·1	13·2	17·2	27·5	33·6	39·4	46·1	49·8
,, ,,	B	0	0	0	+	6·2	10·5	16·0	20·5	28·0

No free phosphate was liberated from the mixed nucleotides during the first 5 hours and only a trace was detected after 8 hours' incubation, but the quantity was too small to permit accurate determination. This apparently delayed action of the non-specific phospho-monoesterase in the venom is a definite characteristic and has been observed in numerous experiments carried out with various concentrations of enzyme and substrate. Enzymic hydrolysis then proceeds steadily for several days, after which time the activity appears to increase. This increase in activity of the enzyme after prolonged incubation with the substrate is also characteristic and similar curves have been obtained in numerous

experiments. It is hoped to discuss possible explanations elsewhere; toluene was present throughout the experiments and in no case was bacterial contamination observed.

Action of Russell's Viper Venom on the Individual Nucleotides of Yeast Ribonucleic Acid.—Solutions of the sodium salts of each of the four nucleotides were incubated separately with borate buffer (7·5 c.c.) and Russell's viper venom (15 mg.) at 37° and pH 8·6 in the presence of toluene, the volumes being 15 c.c., and phosphorus concentration 0·082 mg. per c.c. in each case. Samples (2·0 c.c.) were withdrawn for the determination of inorganic phosphate. Solutions of the substrates and buffer incubated at 37° and pH 8·6 without enzyme liberated no phosphate during the time of the experiment.

TABLE II.

Phosphate liberated, %.

Time, hours.	Yeast adenylic.	Guanylic.	Cytidylic.	Uridylic.	Time, hours.	Yeast adenylic.	Guanylic.	Cytidylic.	Uridylic.
0	0	0	0	0	93	16·5	28·5	22·5	7·3
40	6·9	15·8	10·6	0	113	19·0	32·7	25·2	10·1
66	11·7	22·6	17·0	3·6	164	25·0	41·0	33·6	15·2

The Action of Cold Aqueous 1% Sodium Hydroxide on the Products of the Action of Russell's Viper Venom on Purified Yeast Ribonucleic Acid.—A mixture (20 c.c.) of the acid (P = 9·2%) (*ca.* 20 mg.), dissolved in water with sufficient aqueous sodium hydroxide to effect solution, Clark and Lubs borate buffer (10 c.c.), Russell's viper venom (20 mg.), and water was incubated in the presence of toluene at 37° and pH 8·6. The phosphorus concentration was 0·0997 mg. per c.c. Samples (1·0 or 2·0 c.c.) were withdrawn for the estimation of inorganic phosphate (Table III, col. A).

As each sample was taken for immediate determination of phosphate, a similar sample was at once diluted with 1 vol. of 4% sodium hydroxide solution and 2 vols. of water, a few drops of 0·5M-cyanide added, and the alkaline solution kept at 20° for 24 hours in a tightly corked tube. After neutralisation of the excess of alkali, the inorganic phosphate was determined by the usual procedure (Table III, col. C). After 22½ hours and 49½ hours samples (1 c.c.) were also taken, and phosphatase action arrested by addition of cyanide; the samples were then incubated at 37° and pH 8·6 for 24 hours, and the phosphate determined (Table III, col. B).

TABLE III.

Phosphate liberated, %.

Time, hours.	A.	B.	C.	Time, hours.	A.	B.	C.
0	0	0	0	49½	34·5	37·8	45·1
4	9	—	12	73½	42·7	—	49·5
8	15	—	19	120	53	—	54
22½	25·5	27·7	32·7				

Ratio of Purine to Pyrimidine Nucleotide Phosphate set free by Russell's Viper Venom acting on Purified Yeast Ribonucleic Acid.—A mixture (25 c.c.; P, 0·100 mg. per c.c.) of the acid (*ca.* 28 mg.), dissolved in a little water and sufficient dilute alkali to effect solution, borate buffer (15 c.c.), venom (20 mg.), and water was incubated with toluene at pH 8·6 and 37°. Samples (1·0 or 2·0 c.c.) of the above mixture were withdrawn at intervals for the determination of inorganic phosphate. Simultaneously two 1 c.c. samples were taken and mixed with an equal volume of 2N-hydrochloric acid; one was heated for 1 hour, the other for 2 hours, at 100°. The inorganic phosphate in each was then determined. All purine nucleotide phosphorus is hydrolysed completely to inorganic phosphate by heating in N-acid for 1 hour at 100°, whereas the pyrimidine nucleotides are but slowly attacked. The amount of pyrimidine nucleotide phosphorus set free per hour under these conditions is a constant (Jones, *loc. cit.*), so that the amount set free during the 1-hour hydrolysis is equal to that set free during the second hour of the 2-hour hydrolysis, *i.e.*, (2 hr. value − 1 hr. value). The difference between this quantity and the 1-hour value is equal to the sum of the purine nucleotide phosphorus in organic combination and the phosphorus present as inorganic phosphate, *i.e.*, [1 hr. value—(2 hr. value—1 hr. value)]. The pyrimidine nucleotide phosphorus remaining in organic combination can therefore be calculated from the expression Total phosphorus—[1 hr. value—(2 hr. value—1 hr. value)].

In this way the amounts of purine and pyrimidine nucleotide phosphate present in organic combination in the enzyme–nucleic acid mixture may be determined throughout the enzyme action. It follows that the phosphate set free by the venom may be differentiated into phosphate of purine and of pyrimidine nucleotide origin.

TABLE IV.

Inorganic phosphate expressed as percentage of total phosphorus present in the mixture.

Time, hours.	0.	8.	22.	50.	76.	118.	140.
I. Phosphate set free by enzyme action	0	16	23	31·1	37·9	49·7	54
II. Total purine phosphate and free phosphate [1 hr. value—(2 hr. value—1 hr. value)]	49·7	—	49·7	55·4	59·3	65·2	68·1
III. Remaining pyrimidine phosphate (total P—II)	50·3	—	50·3	44·6	40·7	34·8	31·9
IV. Pyrimidine phosphate set free by enzyme action (total pyrimidine P—III)	0	0	0	5·7	9·6	15·5	18·4
V. Purine phosphate set free by enzyme action (I—IV)	0	16	23	25·4	28·3	34·2	35·6

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