Nucleotides. Part XXXI. The Stepwise Degradation of Polyribonucleotides: Model Experiments.*

By D. M. Brown, M. Fried, and Sir Alexander R. Todd.

[Reprint Order No. 6111.]

The fundamental requirements for a stepwise degradation of polyribonucleotides are delineated and a method is evolved, in model experiments, depending on the easy base-catalysed elimination reaction undergone by esters of β-hydroxy-carbonyl compounds. Oxidation of adenosine-5′ phosphate and adenosine-5′ benzyl phosphate by periodate to the corresponding dialdehydes, followed by very mild treatment with alkali, causes rapid elimination of inorganic phosphate and monobenzyl phosphate respectively. Under the conditions of the reaction no appreciable hydrolysis of the internucleotide linkages in yeast ribonucleic acid occurs.

Recent work in these and other laboratories has led to general structures for the ribonucleic acids. They have been shown to consist of chains of nucleoside residues linked at the 3′- and 5′-positions by phosphodiester groups as in (X) (see, *inter alia* Brown and Todd, *J.*, 1952, 52; 1953, 2040; Brown, Heppel, and Hilmoe, *J.*, 1954, 40; Todd, *Proc. Nat. Acad. Sci. U.S.A.*, 1954, **40**, 748). Two problems are still, however, outstanding. The first relates to the question of chain branching (see, *e.g.*, Brown and Todd, *locc. cit.*), and the second to the sequence of the individual nucleotide residues in ribonucleic acids. A successful solution to the latter problem could only be achieved if the homogeneity of a given ribonucleic acid were proved. If, as seems certain, the ribonucleic acids, like the deoxyribonucleic acids, are mixtures of different molecular species efforts to determine end-groups or nucleotide sequence would be of little value, except possibly as a criterion of homogeneity, during fractionation. Nevertheless a method of stepwise degradation would be of considerable value quite apart from its application to intact ribonucleic acids, since only by its use could the structures of the larger oligonucleotides (see, *e.g.*, Volkin and Cohn, *J. Biol. Chem.*, 1953, **205**, 767) be determined. A method has now been evolved and has been briefly reported (Brown, Fried, and Todd, *Chem. and Ind.*, 1953, 352) which, it was pointed out, could in theory also give information regarding the first problem, namely, the nature and extent of any chain-branching present in a given polynucleotide.

The fundamental problem in the determination of residue sequence in a polynucleotide is the discovery of a method of differentiating the terminal phosphodiester linkage from all other similar linkages in the molecule, so as to allow the removal of the terminal residue, while leaving the rest of the polynucleotide intact and ready for a repetition of the same process. It is clear from present knowledge of the chemistry of the ribonucleic acids, or more generally of alkyl esters of ribonucleotides (Brown and Todd, *locc. cit.*), that the great lability towards alkaline or acid reagents of a phosphodiester system carrying a vicinal hydroxyl function, severely limits the choice of methods. Indeed, it appears, at present, that little, if any, differentiation between the stabilities towards hydrolysis of the various phosphodiester linkages comprising the polynucleotide chain can be made. In any event graded hydrolysis could not be expected to lead solely to removal of terminal residues.

On these grounds, we were led to consider whether an elimination, rather than a hydrolytic process could be used, and how the necessary activating function could be introduced. The ease with which esters of β-hydroxy-carbonyl derivatives undergo base-catalysed elimination reactions is well known. Thus Linstead, Owen, and Webb (*J.*, 1953, 1211) have considered some earlier examples of the reaction and have themselves studied quantitatively the alkali-catalysed elimination of toluene-*p*-sulphonyloxy-, acetoxy-, benzoyloxy-, and, in particular, diphenylphosphonyloxy- and phosphonyloxy-anions from a series of the corresponding β-acyloxy-butyric and -β-phenylpropionic esters (*e.g.*, I → II). Nearer to our theme is the conversion of glyceraldehyde-3 phosphate (III) into lactic acid in very

* Part XXX, *J.*, 1955, 808.

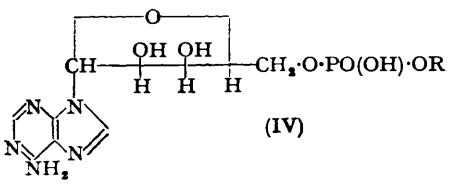
mild alkali and into pyruvaldehyde in presence of acid (Meyerhof and Lohmann, *Biochem. Z.*, 1934, **271**, 89; Baer and Fischer, *J. Biol. Chem.*, 1943, **150**, 223).

$$\underset{\text{(I)}}{>C(OR)\cdot CHR'\cdot CO\cdot R''} + OH^- \longrightarrow \underset{\text{(II)}}{>C{=}CR'\cdot CO\cdot R''} + RO^- + H_2O$$

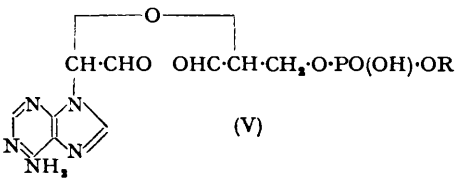
$$OHC\cdot CH(OH)\cdot CH_2\cdot O\cdot PO_3H_2 \xrightarrow[\text{(III)}]{H^+ \text{ or } OH^-} H_3PO_4 + OHC\cdot C(OH){=}CH_2 \xrightarrow{OH^-} HO_2C\cdot CH(OH)\cdot CH_3$$

Zittle (*J. Franklin Inst.*, 1946, **242**, 221) observed that no acid groups are liberated from yeast ribonucleic acid in alkali below pH 10·6. Above that, hydrolysis of the internucleotidic linkages begins. In confirmation, we found that when a solution of yeast ribonucleic acid was brought carefully to pH 10·5, no drop in pH occurred over a considerable period at room temperature, indicating absence of hydrolytic degradation of the polynucleotide. In subsequent experiments on the elimination reaction pH values in the range 10—10·5 were adhered to, as satisfying the main criterion expressed above.

As model substances in the nucleotide series, adenosine-5′ phosphate (IV; R = H) and adenosine-5′ benzyl phosphate (IV; R = CH_2Ph) were chosen, since oxidation to the corresponding dialdehydes (V; R = H and CH_2Ph) can be carried out quantitatively at room temperature in the pH range 5—6. The phosphoryl group in the latter substances is in the β-position with respect to an aldehyde function so that it was expected, by analogy with the examples cited above, that treatment with base would split off phosphate and monobenzyl phosphate respectively. This was found to be the case, when the reaction was studied by paper chromatography, by phosphate estimation, and by titration of liberated acid groups as a function of time.



(IV)



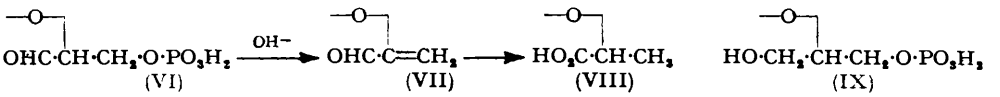
(V)

The dialdehydes were not isolated because of their instability, and so the solutions were studied directly. On paper chromatograms they had a marked tendency to trail. When brought to pH 10·5 with glycine buffer or by careful addition of dilute ammonia solution a rapid reaction appeared to ensue since chromatograms showed that the spot absorbing in the ultraviolet (due to the adenine residue) no longer coincided with that containing phosphorus. In several solvent systems the phosphorus-containing substance from the aldehyde (V; R = H) was identified with inorganic phosphate, and that from the ester (V; R = CH_2Ph) with monobenzyl phosphate. The major ultraviolet-absorbing spot in each case corresponded in R_F value with adenine, and not with the periodate oxidation product from adenosine, which might have been expected had the phosphate residue been removed by hydrolysis rather than elimination. Further work is in progress to elucidate the mechanism of the formation of adenine. In most experiments assessed by chromatography liberation of phosphate or benzyl phosphate appeared complete within a few hours, although, in some, traces of ultraviolet-absorbing material still containing phosphorus remained. Adenosine-5′ phosphate and adenosine-5′ benzyl phosphate were, as expected completely stable at pH 10·5.

In order to obtain a clearer idea of the rate and extent of reaction, the dialdehyde (V; R = H) was brought to pH 10·5 (glycine buffer) and the rate of liberation of inorganic phosphate followed. Reaction appeared to be essentially complete in three hours after 85% of the bound phosphate had been liberated. In another experiment reaction proceeded to the extent of 90%. With the dialdehyde (V; R = CH_2Ph), benzyl phosphate could not be estimated directly. Catalytic debenzylation failed and recourse was had to the specific prostate phosphomonoesterase to hydrolyse the ester to inorganic phosphate which was then estimated. By this means, it was found that at least 71% of benzyl phosphate had been liberated. This figure should be considered as a lower limit in view of the chromatographic

evidence and the possibility of enzyme inhibition. Leaving out any of the above steps, *viz.*, periodate oxidation, treatment at pH 10·5, or enzyme hydrolysis, led to the liberation of negligible amounts of phosphate.

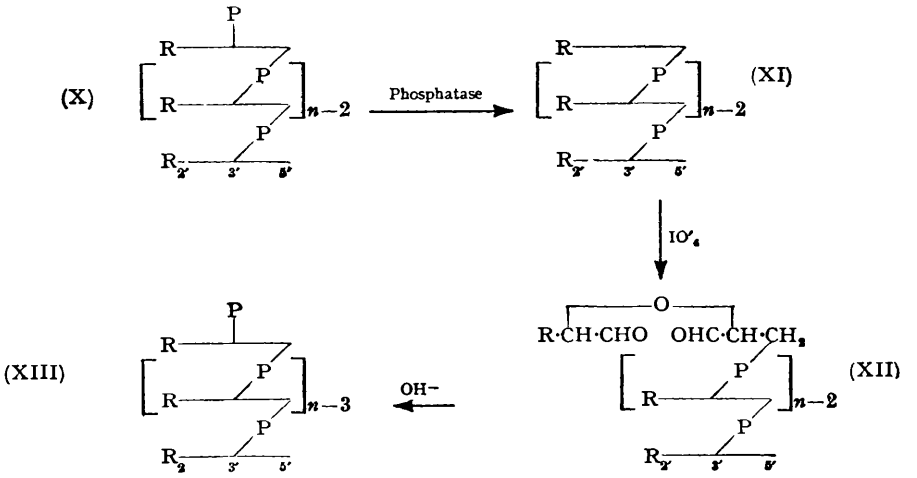
In a further attempt to assess the extent of reaction, solutions of the dialdehydes (V; R = H and CH_2Ph) were brought to pH 10·5 and held at that pH by addition, at intervals, of dilute sodium hydroxide solution. It was observed that over a period of ten hours, 2·04 and 1·9 mols. respectively of alkali were consumed. The reason for the excess of approximately one mol. over that required for the elimination of a phosphate or benzyl phosphate anion is not yet clear. Possible explanations would involve the formation of adenine (pK_a 9·8; Taylor, *J.*, 1948, 765) after the elimination reaction, for which there is already evidence, or an oxidoreduction in the eliminated residue (VI → VIII), or an internal Cannizzarro reaction (cf. Fry, Wilson, and Hudson, *J. Amer. Chem. Soc.*, 1942, **64**, 872). The last reaction would also explain why elimination was incomplete : for if a small amount



(VI) (VII) (VIII) (IX)

of material having the structure shown in the partial formula (IX) were formed by a Cannizzaro reaction, the activation due to the carbonyl function would be lost and elimination would not then proceed. A more detailed study of this aspect of the reaction is being carried out, but it is sufficient at present that the elimination occurs readily at room temperature at a pH of 10·5 to the extent of 85—90%.

The bearing of these model experiments on sequence determination in polyribonucleotides was discussed briefly in a preliminary note (Brown, Fried, and Todd, *loc. cit.*). A polyribonucleotide in which the nucleoside residues are linked at the 3'- and 5'-positions through phosphodiester groups must have either a terminal α-glycol system or a phosphate residue at the 3'-position. If a polynucleotide (X) of the latter type containing *n* residues is considered, removal of the terminal phosphate by phosphomonoesterase treatment will



(In these representations, R represents the heterocyclic nuclei, and the horizontal lines the carbohydrate residues.)

yield (XI) in which only the terminal residue carries an α-glycol system. Oxidation by periodate will then lead to the dialdehyde (XII). That the above reactions can be carried out on polynucleotides has already been shown (Schmidt *et al.*, *J. Biol. Chem.*, 1951, **192**, 715). The dialdehyde (XII) is strictly analogous to the periodate oxidation product (V) from adenosine-5' benzyl phosphate, the benzyl groups being equivalent to the remainder of the polynucleotide chain. Since we have shown that (V; R = CH_2Ph) readily eliminates

benzyl phosphate, treatment of the analogue (XII) at pH 10·5 should split off the terminal residue, leaving the polynucleotide (XIII) having $(n - 1)$ residues, but otherwise unaltered. The whole process can then be repeated, thus effecting a stepwise degradation. This process is feasible since the degradation has been successfully carried out on several dinucleotides ($n = 2$; Whitfeld and Markham, *Nature*, **1953**, **171**, 1151) and trinucleotides ($n = 3$; Whitfeld, *Biochem. J.*, 1954, **58**, 390).

The above method could, in theory, afford evidence as to the presence or absence of chain branching in a ribonucleic acid. The type of branching postulated earlier (Brown and Todd, *J.*, 1952, 52), and for which further evidence has been given (Volkin and Cohn, *J. Biol. Chem.*, **1953**, **203**, 319), involved the attachment of side-chains at $C_{(2')}$ of nucleoside residues in the main chain. The presence of such a branch would be revealed if, during a stepwise degradation, phosphomonoesterase removed the terminal phosphate residue giving a molecule which was not oxidisable by periodate, since the side chain at $C_{(2')}$ would preclude the formation of a terminal α-glycol system.

Experimental

Paper-chromatography Studies.—For the paper-chromatographic study of the elimination reaction, solutions were prepared so that a final concentration of *ca.* 10 mg./c.c. was obtained.

Adenosine-5′ phosphate (34·7 mg.) was oxidised with sodium metaperiodate (21·6 mg., 1·1 mol.) in water (1 c.c.) for 24 hr. This solution (0·1 c.c.) was brought to pH 8, 0·25M-glycine buffer (0·2 c.c. of pH 10·5; Internat. Crit. Tables, Vol. I, p. 83) added, and the solution kept at room temperature overnight.

The same procedure was applied to adenosine-5′ benzyl phosphate (Baddiley and Todd, *J.*, 1947, 648).

The solutions were studied on ascending chromatograms (Whatman No. 1 paper), together with standard substances, and some representative results are collected in Table 1. The R_P values of phosphorus-containing (P) and ultraviolet-absorbing (U.V.) spots are recorded. Phosphates were detected by the spray reagent of Hanes and Isherwood (*Nature*, 1949, **164**, 1107). Solvent systems used were: A, *n*-butanol–acetic acid–water (5 : 1 : 4); B, propan-2-ol–water–trichloroacetic acid–ammonia (Abel, *Bull. Soc. chim. France*, 1953, 1089); C, propan-2-ol–water–ammonia (*d* 0·88) (70 : 20 : 10).

Table 1. *R_P values in solvent systems.*

	A		B		C	
Substance	P	U.V.	P	U.V.	P	U.V.
Adenosine-5′ phosphate	0·02	0·02	0·03	0·03	0·06	0·06
Inorg. phosphate	0·072 *	—	0·53 *	—	0·03 *	—
Adenine	—	0·36	—	0·49	—	—
Dialdehyde (V; R = H)	0·13	0·13	0·46	0·46	0·03	0·03
Ditto, treated at pH 10·5	0·07 *	0·37	0·53 *	0·49	0·03 *	—
Adenosine-5′ benzyl phosphate ...	0·42	0·42	0·58	0·58	0·57	0·57
Benzyl dihydrogen phosphate	0·55	—	0·86	—	0·27	—
Adenine	—	0·54	—	0·62	—	0·54
Dialdehyde (V; R = CH_2Ph)	0·60	0·60	0·75	0·75	0·37	0·37
Ditto, treated at pH 10·5	0·55	0·48—0·65	0·86	0·62	0·27	0·54, 0·63

A, B, and C. The dialdehydes gave elongated spots; R_P values refer to the mid-point of the spot. The spots marked with an asterisk developed a yellow colour immediately on spraying, giving added confirmation of the presence of inorganic phosphate.

The R_P values recorded in Table 1 are those of the major spots. In some experiments faint, diffuse streaks were apparent, containing both phosphorus and ultraviolet-absorbing material, suggestive of incomplete reaction. A material with the same R_P value as adenine was the main ultraviolet-absorbing product of the reaction but other products also appeared to be present. Oxidation of adenosine with periodate and treatment at pH 10·5 gave solutions which streaked on chromatograms and evidently contained several substances, but the main spot again corresponded to adenine.

In similar experiments in which very dilute ammonia solution was used to bring the solutions of oxidised adenosine-5′ phosphate to pH *ca.* 11, paper chromatography showed that the reaction was almost complete within 1—10 min.

Eliminations.—Examination by phosphate analysis. (*a*) Adenosine-5′ phosphate. Adenosine-5′ phosphate (anhydrous; 68·1 mg.) was dissolved in 0·025M-sodium metaperiodate

(12·5 c.c.) and set aside for 20 hr. Aliquot parts (2 c.c.) were withdrawn and the excess of periodate was back-titrated with arsenite (cf. Lythgoe and Todd, *J.*, 1944, 592). The uptake of periodate (1·01, 0·97 mol.) showed complete oxidation. To the remaining solution were added 20 c.c. of 0·25M-glycine buffer (pH 10·3), the solution was diluted to 50 c.c., and aliquot parts (2 c.c.) were removed at intervals for estimation of inorganic phosphate and total phosphorus (Allen, *Biochem. J.*, 1946, **34**, 858).

The release of inorganic phosphate as a percentage of total phosphate was: 53 (5 min.); 64 (1 hr.); 85 (3 hr.); 86 (19 hr). In another experiment the results were: 53 (6 hr.); 70 (24 hr.); 82 (48 hr.); 89 (72 hr.). The rate of liberation of phosphate was unaltered when ethylene glycol was added before the buffer, to reduce excess of periodate.

When a solution of periodate-oxidised adenosine-5′ phosphate prepared as above was brought to pH 4·7 by addition of 0·2M-acetate buffer, the amount of phosphate liberated was negligible (only 5·3% in 21 hr.).

In control experiments, the addition of iodate, periodate, or glycine buffer did not affect phosphate analyses.

(*b*) Adenosine-5′ benzyl phosphate. The ester (14·7 mg.) and sodium metaperiodate (14·8 mg., 2·05 mol.) were suspended in water (4 c.c.), and the solution was shaken for 20 hr. to effect oxidation. 0·25M-Glycine buffer (5 c.c.) was added and the solution made up to 10 c.c. Aliquot parts (1 c.c.) were taken at intervals for determination of inorganic phosphate (none found) and total phosphate, and for treatment with prostate phosphatase followed by phosphate determination. The last process was carried out by adding to each aliquot part the enzyme solution [1 c.c.; containing 5 mg. lyophylised prostate extract/10 c.c. (Loring *et al.*, *J. Biol. Chem.*, 1952, **196**, 821)] and 0·2M-acetate buffer (2 c.c.; pH 4·45) (final pH 5·0). The solution was set aside overnight before analysis for inorganic phosphate. Release of phosphate as a percentage of total phosphate was: 20 (5 min.); 37 (1 hr.); 46 (3 hr.); 56 (7 hr.); 60 (23 hr.). In another experiment the results were: 25 (5 min.); 71 (24 hr.).

The enzyme solution (4 c.c.) completely hydrolysed *cyclo*hexylammonium benzyl phosphate (5·0 mg.) in less than 6 hr.

Titration studies. (*a*) Adenosine-5′ phosphate (11·9 mg.) and sodium metaperiodate (11·6 mg., 1·6 mol.) were dissolved in water (5 c.c.) and shaken at room temperature for 21 hr. The solution was brought to pH 10·5 with 0·0108N-sodium hydroxide (Cambridge pH Meter) and then at suitable intervals the amount of sodium hydroxide necessary to maintain that pH was determined. Experiments were carried out with the solution under carbon dioxide-free nitrogen. The titration figures are given in Table 2 together with the calculated values (mol.) for liberation of titratable acidity.

(*b*) Similarly adenosine-5′ benzyl phosphate (9·5 mg.) was oxidised with sodium metaperiodate (10·8 mg., 2·3 mol.) and the experiment carried through as above. The results are given in Table 2.

TABLE 2

Oxidised adenosine-5′ phosphate (0·0343 milliequiv.)				*Oxidised adenosine-5′ benzyl phosphate* (0·0217 milliequiv.)			
Time (hr.)	NaOH (c.c.)	NaOH (milliequiv.)	Acid liberated (mols.)	Time (hr.)	NaOH (c.c.)	NaOH (milliequiv.)	Acid liberated (mols.)
0·25	0·22	0·0024	0·07	0·25	0·39	0·0042	0·19
0·50	0·50	0·0054	0·16	0·50	0·70	0·0076	0·35
0·75	0·75	0·0081	0·24	0·75	0·89	0·0096	0·44
1·0	0·95	0·0103	0·30	1·0	1·04	0·0112	0·52
2·5	1·87	0·0202	0·59	2·0	1·55	0·0167	0·77
4·0	2·92	0·0315	0·92	3·0	2·15	0·0232	1·07
6·0	4·35	0·0469	1·37	5·0	2·95	0·0318	1·47
10·5	6·50	0·0702	2·04	10·0	3·76	0·0406	1·87

In the blank runs on the unoxidised nucleotides, no increase in titratable acidity was found.

(*c*) When a sample of yeast nucleic acid, prepared by dialysis of a commercial product followed by freeze-drying, was dissolved in water and the solution brought to pH 10·5, no further addition of sodium hydroxide was necessary to maintain this pH for the duration of the experiment (3 hours).

One of us (M. F.) thanks the Jane Coffin Childs Fund for Medical Research for a postdoctoral fellowship.

UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE. [*Received, February 9th*, 1955.]