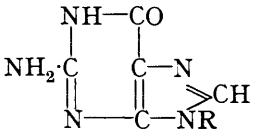
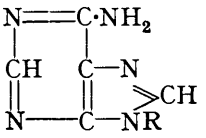
## 284. *The Constitution of Yeast Nucleic Acid.*

By J. Masson Gulland and Elisabeth M. Jackson.

A study of the dephosphorylating action of specific phosphatases on yeast nucleic acid shows that all four phosphoryl groups are doubly linked, thus confirming the conclusion of Takahashi. The degree of dephosphorylation by various mixtures of phosphomonoesterases with phosphodiesterases is 75%, suggesting that one phosphoryl group may be constituted differently from the others. The joint action of phosphodiesterase and 5-nucleotidase liberates 35% of the total phosphorus as phosphate, suggesting that 2 or more phosphoryl groups may be attached at $C_5$ of the ribose radicals in view of the high specificity of 5-nucleotidase for adenosine(inosine)-5-phosphate.

It is generally accepted that the four nucleotides, guanylic (I), adenylic (II), cytidylic (III), and uridylic (IV) acids, into which yeast nucleic acid is quantitatively decomposed by alkaline fission, are united by the elimination of the elements of water, and that some, or all, of the phosphoric acid groups are involved in these unions. Nevertheless, the exact manner in which these nucleotides are mutually combined is still unsettled, and the work now described was instituted to throw light on this question.


(I.)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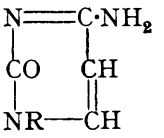
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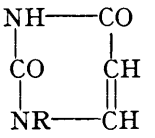
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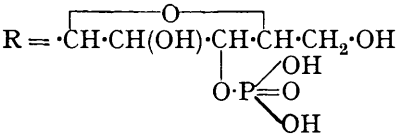
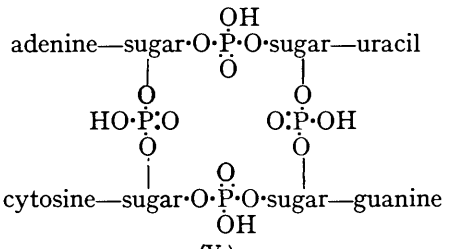


Takahasi (*J. Biochem. Japan*, 1932, **16**, 463) attacked the problem by a study of the actions of the following specific enzymes on yeast nucleic acid: phosphomonoesterases of liver and intestine (Kurata, *ibid.*, 1931, **14**, 25; Takahashi, *ibid.*, 1932, **16**, 447), which liberate phosphoric acid from phosphomonoesters; diphenylpyrophosphatase, or pyrophosphatase of kidney in presence of a diesterpyrophosphatase activator (Kurata, *loc. cit.*; Takahashi, *loc. cit.*), which converts pyrophosphoric esters into two molecules of phosphomonoester but liberates no phosphoric acid; phosphodiesterase of the venom of the Habu, *Trimeresurus flavoviridis* (Uzawa, *ibid.*, 1932, **15**, 19), which transforms phosphodiesters into one molecule each of phosphomonoester and alcohol (phenol) but liberates no phosphoric acid. The first two enzymes had been freed from each other and from diesterase by adsorptive methods, whilst the last occurred in the snake venom uncontaminated by the others.

Takahashi observed that phosphoric acid was not set free from yeast nucleic acid by phosphomonoesterase or by pyrophosphatase, acting either alone or together, in presence of the diester-pyrophosphatase activator, but that it was liberated to the extent of 100% by the joint action of phosphomonoesterase and phosphodiesterase. He therefore concluded that various previously proposed formulæ were unsatisfactory, notably those of Feulgen (*Z. physiol. Chem.*, 1918, **101**, 288) and of Levene and Simms (*J. Biol. Chem.*, 1926, **70**, 327), since by reason of their phosphomonoester or pyrophosphoric ester groups they would have yielded phosphoric acid under enzymic conditions in which experiment showed that none was liberated. Further, because a mixture of monoesterase and diesterase set free all the phosphoric acid in the inorganic state, Takahasi suggested that yeast nucleic acid contains neither phosphomonoester nor pyrophosphoric ester groups and that each phosphoryl group is present as a diester of two nucleosides (dephosphorylated nucleotides). He proposed the structure (V) for yeast nucleic acid, in which the relative positions of the nucleosides were arbitrarily chosen.

Makino (*Z. physiol. Chem.*, 1935, **236**, 201) supported this view by finding only four acidic groups in the yeast nucleic acid molecule when titrated to phenolphthalein, at the $p_H$ of which both primary and secondary dissociating groups of phosphoric acid are neutralised. Baker, Gulland, and Prideaux (unpublished observation) confirmed this conclusion by estimating only four primary dissociations in electrometric titrations of yeast nucleic acid. Klein and Rossi (*ibid.*, 1935, **231**, 104), on the other hand, decided that the cyclic structure of Takahashi had no experimental foundation. They were unable to separate the phosphomonoesterase and phosphodiesterase of kidney, liver, and intestine by the methods used by Takahashi, and they found that a specimen of Habu venom contained both mono- and di-esterases, not diesterase only as stated by Uzawa and by Takahashi.

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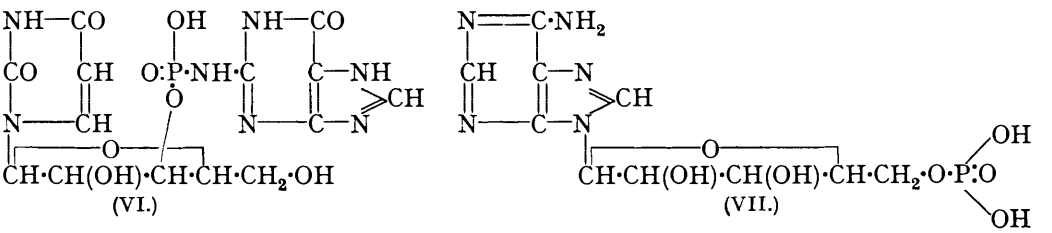


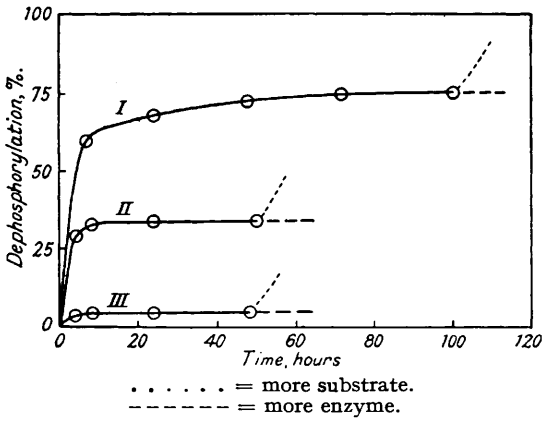

(VI.) (VII.)

These conflicting results called for further investigation, and the continued study of the problem of the constitution of yeast nucleic acid by enzymic methods seemed to be desirable, because at the time this work was begun, owing to the sensitivity of the molecule to chemical reagents, the attack by the more usual procedures during many years had failed to yield any derivatives composed of two nucleotides, or parts thereof, from which deductions might be drawn as to the mode of union. Since then, however, Bredereck and Richter (*Ber.*, 1936, **69**, 1129) have reported the isolation of guanine uridylic acid (VI) from the products of aqueous hydrolysis.

The enzyme preparations used in the present investigation have been described by Gulland and Jackson (*Biochem. J.*, 1938, **32**, 590, 597). Phosphomonoesterase was obtained from sheep and guinea-pig bones and was freed from the accompanying phosphodiesterase by charcoal adsorption; it is non-specific in its hydrolysis of phosphomonoesters, and liberates all the phosphoric acid from the nucleotides (I—IV) and (VII) as inorganic phosphate. Phosphodiesterase could not be recovered from the adsorption just referred to, but it was found to be present in the venom of each of twelve different snakes of the *viperidæ*, *crotalidæ*, and *colubridæ*; six contained both non-specific phosphomonoesterase and phosphodiesterase, whilst six contained phosphodiesterase but no non-specific monoesterase. Phosphodiesterase did not attack the nucleotides (I—IV). Four venoms from the second group not only exhibited diesterase activity but also contained the highly specific phosphomonoesterase 5-nucleotidase, an enzyme which rapidly dephosphorylates adenosine-5-phosphate (VII) and inosine-5-phosphate, but has no action on 15 other phosphomonoesters, including the nucleotides (I—IV) prepared from yeast nucleic acid. The venoms of Russell's viper, *Vipera russellii*, and the water moccasin, *Agkistrodon piscivorus*, which fall in this group, have been used in the investigation. One venom, that of the copperhead, *Agkistrodon mokasin*, contained diesterase, but not non-specific monoesterase. It is presumed not to have contained 5-nucleotidase owing to its different enzymic

behaviour when compared with the venoms of Russell's viper and the water moccasin, but this point has not been tested specifically because of the small amount of venom available and the early stage in the investigation when it was used. Two venoms, those of *Naja naja* and Wagler's pit viper, *Trimeresurus wagleri*, were included in the investigation because they contained both non-specific phosphomonoesterase and phosphodiesterase.

The procedure adopted throughout was to estimate at suitable intervals the inorganic phosphate liberated in a mixture of yeast nucleic acid and enzyme buffered at an appropriate $p_H$ and maintained at a definite temperature, and so to calculate the percentages of total phosphorus liberated as inorganic phosphate. It will be seen later that the conclusions drawn from these experiments rest on the observations that cessation of dephosphorylation occurred at various percentage levels which are less than 100%, and it therefore became imperative to demonstrate that such percentages represent true end-points and are not merely the results of unfavourable experimental conditions. This need has been met in every direction. First, in a large number of representative cases the addition of further quantities of enzyme after dephosphorylation had ceased resulted in no further liberation of phosphate, whereas the addition of more yeast nucleic acid was immediately followed by a rise in the amount of inorganic phosphate, thus proving that dephosphorylation had really ceased and that the enzymes were still active and could still dephosphorylate the substrate, provided this contained labile groups (see figure). Secondly, cessation of dephosphorylation was not due to inhibition of the enzyme by the buffer substance, since the results were not affected by changing from borate to glycine or veronal buffers. Thirdly, changes in temperature between 37° and room temperature did not alter the percentage values. Finally, similar results were obtained by using samples of yeast nucleic acid supplied by British Drug Houses, Merck, and Boehringer.




I. *Mixed purified bone phosphomonoesterase and Russell's viper venom.*
II. *Russell's viper venom alone.*
III. *Purified bone phosphomonoesterase alone.*

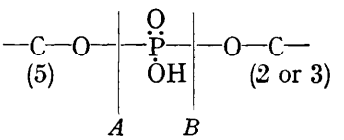
Purified bone phosphomonoesterase (Gulland and Jackson, *loc. cit.*) had little or no action on yeast nucleic acid, never liberating more, and usually much less, than 7% of the total phosphorus. Similarly, phosphodiesterase (copperhead venom) failed to set free inorganic phosphate. Dephosphorylation was readily effected, however, by the following mixtures of monoesterase and diesterase: crude bone phosphatase (Martland and Robison, *Biochem. J.*, 1929, **23**, 237); bone monoesterase and copperhead venom diesterase; the venoms of *Naja naja* or Wagler's pit viper, acting independently; bone monoesterase and the diesterase and 5-nucleotidase of Russell's viper or the water moccasin venoms; kidney phosphatase (Albers and Albers, *Z. physiol. Chem.*, 1935, **232**, 165, 171) and Russell's viper venom. On the basis of these results we concur with Takahashi that yeast nucleic acid contains no phosphomonoester group, and that each phosphorus atom is present as a disubstituted phosphoryl group. Takahashi's further conclusion that each phosphoryl group is a diester may be unjustified in view of the more recent report of the isolation of guanine uridylic acid (VI) by Bredereck and Richter.

The degree of hydrolysis of purified yeast nucleic acid accomplished by these mixtures of enzymes in our experiments averaged 75%, although from less pure specimens of the acid slightly higher values were occasionally recorded. Thus, three only of the four phosphorus atoms were liberated as phosphate. Stringent tests were applied, as already described, to show that this incomplete dephosphorylation was a reliable experimental fact and not merely the result of unfavourable enzymic conditions, and the final proof of its accuracy was obtained in the observation that the mixture of the four nucleotides (I—IV),

prepared by the cold alkaline hydrolysis of yeast nucleic acid, was completely (100%) dephosphorylated by bone phosphomonoesterase in circumstances which resembled as closely as possible the hydrolyses by mixed monoesterases and diesterases. This observation also disposed of the possibility that the concentration of inorganic phosphate formed in these hydrolyses was sufficiently great to interfere with the dephosphorylating action of the monoesterase; phosphate ions are well known to inhibit phosphatase activity.

These results differ from those of Takahashi, who records 100% dephosphorylation by mixed monoesterase and diesterase. Since the sources of the enzymes were different in the two cases, the probable explanation is that our monoesterase preparations or snake venoms did not contain an enzyme which was present in Takahashi's and which has a specific hydrolysing action on one of the doubly-linked phosphoryl groups. The inference is that one of the phosphoryl groups is disposed differently from the other three, or alternatively that all four are similarly constituted and that the specificity of our enzyme preparations did not permit fission of one of them. A knowledge of this inherent difference between the enzymes in Takahashi's preparations and ours should throw light on the nature of this phosphoryl group.

The behaviour of mixtures of phosphodiesterase and 5-nucleotidase (*e.g.*, the venoms of Russell's viper or the water moccasin) towards yeast nucleic acid was unexpected. Either of these venoms liberated about 35% of the total phosphorus as inorganic phosphate (see figure). This suggests that at least two, or more, phosphoryl groups may be attached at $C_5$ of the ribose radicals, in view of the high specificity of 5-nucleotidase for adenosine-(inosine)-5-phosphate (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 597). The degree of hydrolysis, intermediate between 25% and 50%, could result from the competitive diesterase action at *A* and *B* (VIII) on two or more phosphoryl groups; hydrolysis at *A* would not be followed by liberation of phosphate from the resulting phosphoryl group, which would be linked at $C_2$ or $C_3$ of the pentose, whereas hydrolysis at *B* would permit dephosphorylation by 5-nucleotidase.




VIII.)

The incorporation of these suggestions in possible formulæ for yeast nucleic acid is not yet practicable, chiefly owing to lack of knowledge of the behaviour of 5-nucleotidase towards the 5-phosphoric esters of guanosine, cytidine, and uridine (analogous with VII). It is hoped, however, that this knowledge will soon be available from current experiments.

Some implications of the results of this investigation are discussed in the lecture on p. 1722.

## Experimental.

*Purification of Yeast Nucleic Acid.*—The phosphorus content of some commercial samples is low, and purification was effected when needed by the gradual addition of ammonia to the acid (20 g.) suspended in ice water, excess of ammonia being avoided, and the precipitation of the acid from the resulting neutral, aqueous solution (550 c.c.) of its ammonium salt by means of industrial methylated spirit (1·1 l.) containing 3·6% of hydrogen chloride in the form of concentrated hydrochloric acid (compare Makino, *Z. physiol. Chem.*, 1935, **236**, 201). This process was repeated until the correct analytical values were attained (Found, in samples from different sources: Boehringer, P, 9·5; purified Merck, P, 9·3; purified B.D.H., C, 34·9; H, 4·1; N, 16·1; P, 9·2. Calc. for $C_{38}H_{47}O_{28}N_{15}P_4$: C, 35·4; H, 3·7; N, 16·3; P, 9·5%).

*Estimations of Inorganic Phosphate.*—This was determined colorimetrically by the Bell–Doisy–Briggs method. Samples taken from experimental solutions were mixed with the sulphuric acid and molybdate solutions in order to precipitate protein and unchanged nucleic acid. The precipitates were centrifuged, washed once with water by centrifuging, and the clear supernatant liquid and washings were mixed with the sodium sulphite and the quinol solution and adjusted to a standard volume.

In order to test the accuracy of this procedure, correct estimations were made of inorganic

phosphate added to solutions of protein and of intact and partly digested yeast nucleic acid. Further, in many cases the results obtained by the standard method just described were found to be identical with values estimated in duplicate samples by the method of Levene and Dillon (*J. Biol. Chem.*, 1930, **88**, 753), which involves a preliminary precipitation of inorganic phosphate as magnesium ammonium phosphate, and hence its removal from the organic components of the mixture, before the colorimetric determination, which was then carried out by the Bell–Doisy–Briggs method.

On many occasions during the investigation it was necessary to add either fresh enzyme or more substrate to an experimental solution, and then to continue the estimations of inorganic phosphate. Whenever this was done, the necessary corrections for dilution and added phosphate (if any) were made.

All experiments were accompanied by the necessary controls for $p_H$-stability of substrates and non-production of inorganic phosphate by enzymes incubated without substrate.

*Dephosphorylation of Mononucleotides by Bone Phosphomonoesterase.*—The nucleotide (10·0 mg.), dried at 110° in a vacuum and just dissolved in a little dilute sodium carbonate, a solution (10 c.c.) of the enzyme from sheep bone, purified according to Gulland and Jackson (*Biochem. J.*, 1928, **32**, 590), Clark and Lubs's borate buffer at $p_H$ 8·6 (10 c.c.), and M/10-magnesium sulphate solution (1 c.c.) were mixed, diluted with water to 50 c.c. (toluene), incubated at 37° and $p_H$ 8·6, and samples (5 c.c.) were withdrawn at appropriate intervals.

| | Percentage dephosphorylation. | | | |
|---|---|---|---|---|
| Time, hrs. | Guanylic acid, P = 8·3%. | Adenosine-3-phosphate, P = 8·6%. | Adenosine-5-phosphate, P = 8·9%. | Cytidylic acid, P = 9·0%. |
| 3 | 30 | 26 | 25 | 34 |
| 5 | 41 | 35 | 28 | — |
| 23 | 88 | 85 | 88 | 98 |
| 47 | 95 | 100 | 98 | 98 |

Similar results were obtained in hydrolyses by phosphomonoesterase of the mixed nucleotides (I—IV) prepared by the action of 1% sodium hydroxide on yeast nucleic acid (see below).

*Fission of Yeast Nucleic Acid by the Successive Action of Alkali and Phosphomonoesterase.*—A solution of yeast nucleic acid (20·0 mg.) in 1% sodium hydroxide (20 c.c.) was left overnight at room temperature and neutralised carefully with dilute sulphuric acid. Borate buffer at $p_H$ 8·6 (10 c.c.), M/10-magnesium sulphate (1 c.c.), and bone phosphomonoesterase solution (10 c.c.) were added, the mixture was diluted to 50 c.c. with water (toluene), incubated at 37° and $p_H$ 8·6, and samples (4 c.c.) were taken.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (hrs.) ..... | 19 | 26 | 43 | 67 | 76 | 96 | 120 |
| Dephosphorylation, % (B.D.H.) ..... | 61 | 80 | 90 | 99 | — | — | — |
| „ „ (Boehringer) | — | — | — | — | 89 | 94 | 100 |

*Action of Bone Phosphomonoesterase on Yeast Nucleic Acid.*—Yeast nucleic acid (7·4 mg.), dissolved in a little dilute sodium carbonate, phosphomonoesterase solution (5 c.c.), borate buffer at $p_H$ 8·6 (5 c.c.), and M/10-magnesium sulphate (0·5 c.c.) were mixed, diluted to 20 c.c. with water (toluene), incubated at 37° and $p_H$ 8·6, and samples (4 c.c.) were taken.

| | | |
|---|---|---|
| Time (hrs.) ..... | 5 | 23 |
| Dephosphorylation, % (B.D.H.) ..... | 6·5 | 6·5 |
| „ „ (Merck) ..... | 0 | 0 |

*Dephosphorylation of Yeast Nucleic Acid.*—(i) *By mixed phosphomonoesterase and the venom of Russell's viper or the water moccasin.* The conditions of the following experiment were typical, and some characteristic examples of the numerous experiments carried out are tabulated below.

| | Percentage dephosphorylation. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | B.D.H. | | | | | Boehringer. | | Merck. | | |
| Time, hrs. | A. | B. | C.* | D. | E. | F. | G. | H. | J. | K. |
| 24 | 67 | 73 | 60 | 75 | 73 | 64 | 65 | 66 | 66 | 68 |
| 48 | 71 | 78 | 73 | 82 | 75 | 67 | 65 | 70 | 70 | 72 |
| 72 | 75 | — | 77 | 75 | 83 | 73 | — | 78 | — | — |
| 96 | 75 | 75 | — | — | — | — | — | — | — | — |
| 120 | — | — | — | — | — | — | 74 | — | 75 | — |
| 144 | 77 | — | — | — | — | 77 | — | — | — | — |

* At room temperature.

Yeast nucleic acid (14·9 mg.) in a little sodium carbonate, phosphomonoesterase solution (10 c.c.), venom (20 mg.), borate buffer at $p_H$ 8·6 (10 c.c.) and M/10-magnesium sulphate (1 c.c.) were mixed, diluted to 50 c.c. with water (toluene), incubated at 37° or kept at room temperature, and samples (4 c.c.) were taken.

Similar results were obtained when Russell's viper venom was allowed to act alone, effecting 35% dephosphorylation, and the products of this fission were then mixed with phosphomonoesterase.

(ii) *By venoms of* Naja naja *or Wagler's pit viper.* B.D.H. Yeast nucleic acid (14·0 mg.) and venom (20 mg.) were treated exactly as in (i), but magnesium sulphate was omitted and incubation was at 37°. At * a further amount of enzyme (phosphorus-free) was added.

| | Percentage dephosphorylation. | | | |
|---|---|---|---|---|
| | Naja naja. | | | Wagler's pit viper. |
| Time, hrs. | A. | B. | C. | D. |
| 4 | 50 | 56 | 50 | 58 |
| 6 | — | 57 | — | 67 |
| 24 | 60 | 69 | 60 | 75 |
| 48 | 63 | — | 63 | 75 |
| 72 | 67 | — | 67 | — |
| 120 | — | — | 70 * | — |
| 200 | — | — | 70 | — |

(iii) *By mixed Albers' kidney phosphatase and Russell's viper venom.* B.D.H. Yeast nucleic acid (10·0 mg.), kidney phosphatase preparation (30 mg.; Albers and Albers, *Z. physiol. Chem.*, 1935, **232**, 165, 171), Russell's viper venom (20 mg.), borate buffer at $p_H$ 8·6 (5 c.c.), and M/10-magnesium sulphate (0·5 c.c.) were mixed, diluted with water to 20 c.c. (toluene), incubated at 37° and $p_H$ 8·6, and samples (2 c.c.) were taken. At 47 hours, further quantities of both enzymes were added.

| Time (hrs.) | 4 | 6 | 23 | 47 | 72 |
|---|---|---|---|---|---|
| Dephosphorylation, % | 38 | 56 | 70 | 70 | 70 |

(iv) *By the venom of Russell's viper or the water moccasin.* Yeast nucleic acid (7·0 mg.), venom (10 mg.) and borate buffer at $p_H$ 8·6 (5 c.c.) were diluted to 10 c.c., the further details being as in (iii).

| | Percentage dephosphorylation. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | B.D.H.; Russell's viper. | | | | B.D.H.; water moccasin | | | Merck; Russell's viper. |
| Time, hrs. | A. | B. | C. | D. | E. | F. | G. | H. |
| 4 | 18 | 18 | 19 | 30 | 26 | 29 | 35 | 34 |
| 6 | 26 | 24 | 23 | 33 | 36 | 36 | 37 | — |
| 24 | 35 | 33 | 32 | 35 | 45 | 36 | 37 | 42 |

*Failure of Change of Buffer to affect Enzymic Dephosphorylation of Yeast Nucleic Acid.*—B.D.H. Yeast nucleic acid (8·0 mg.), bone phosphomonoesterase solution (5 c.c.), Russell's viper venom (10 mg.), M/10-magnesium sulphate (0·5 c.c.), and either Sørensen's glycine buffer (10 c.c.) or Clark and Lubs's borate buffer (10 c.c.), in each case at $p_H$ 8·6, were mixed, diluted to 20 c.c. with water (toluene), incubated at 37° and $p_H$ 8·6, and samples (4 c.c.) taken.

| | Percentage dephosphorylation. | | | |
|---|---|---|---|---|
| | 1st Experiment: | | 2nd Experiment: | |
| Time, hrs. | borate. | glycine. | borate. | glycine. |
| 4 | 30 | 32 | 36 | 35 |
| 24 | 60 | 57 | 73 | 73 |
| 48 | 87 | 80 | 78 | 86 |
| 72 | — | — | 88 | 86 |

*Alkaline Fission of Adenosine-3- and -5-phosphates.*—No inorganic phosphate was liberated when solutions of adenosine-3- (8·5 mg.) or -5-phosphate (7·8 mg.) in 1% sodium hydroxide solution (5 c.c.) were kept at room temperature for 18 hours or heated at 100° for 45 minutes. When the nucleosides and nucleotides were then precipitated with mercuric sulphate, no organically combined phosphate (ribose phosphate) was found in the filtrate.

Solutions of adenosine-3- (6·4 mg.) and -5-phosphate (6·6 mg.) in 5% sodium hydroxide (6 c.c.) were heated at 100°, and samples withdrawn for estimation of inorganic phosphate.

| | | | | |
|---|---|---|---|---|
| Time, hrs. | | 0·75 | 1·5 | 2·25 |
| Percentage dephosphorylation | 3-Phosphate | 0 | 19 | 29 |
| | 5-Phosphate | <12 | 29 | 41 |

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