193. *The Constitution of Yeast Ribonucleic Acid. Part II. Guanine-uridylic Acid.*

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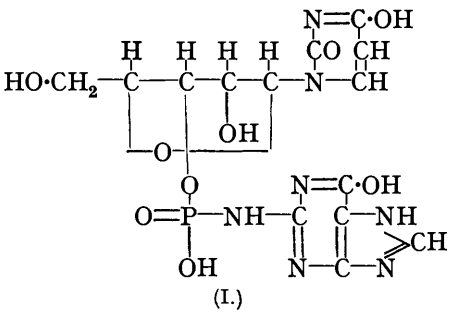
From the products of the aqueous hydrolysis of yeast ribonucleic acid Bredereck and Richter isolated guanine-uridylic acid, whereas Levene and Tipson were unable to prepare this compound and denied its existence. It is now shown that samples of ribonucleic acid supplied by certain firms yield guanine-uridylic acid, whereas those supplied by others do not. This implies the existence of two types of nucleic acid, possibly interconvertible, and throws doubt on the conclusion of Bredereck that guanine-uridylic acid is a secondary product of the procedure used in its preparation. Evidence bearing on the structure of guanine-uridylic acid is discussed, and the results of similar experiments on nucleic acids are considered. It is demonstrated that the group in ribonucleic acids shown by Gulland and Jackson to be resistant to enzymic fission is not that which unites the components of guanine-uridylic acid.

GUANINE-URIDYLIC acid (I) was isolated by Bredereck and Richter (*Ber.*, 1936, **69**, 1129) from the products of the warm aqueous hydrolysis of yeast ribonucleic acid, supplied by the firm of Boehringer in Germany. It was described as monobasic, amorphous, readily hydrolysed to guanine and uridylic acid, but apparently not deaminated by nitrous acid, although quantitative estimations of amino-nitrogen were not recorded. Its existence was held to indicate the probable presence of hitherto unsuspected P–N linkages in nucleic acids.



(I.)

Subsequently, however, Bredereck, Köthnig, and Lehmann (*Ber.*, 1938, **71**, 2613) concluded that guanine-uridylic acid may not be a structure inherent in the molecule of ribonucleic acid, but is more probably a secondary product of its hydrolysis. It was also deduced that the amino-groups of the purines and pyrimidines in ribonucleic acid are not substituted but are present as $-NH_2$; that the earlier failure to deaminate guanine-uridylic acid was to be attributed to too low a temperature and too short a duration of experiment; and that the union between guanine and uridylic acid was to be sought in an ester linkage between the phosphoryl radical and the hydroxyl group of guanine (Bredereck, "Nucleinsauren, Fortschritte der Chemie organischer Naturstoffe," 1938, I, 154, Springer, Vienna).

Tipson and Levene (*J. Biol. Chem.*, 1939, **127**, 105) criticised Bredereck and Richter for failing to obtain a substance of the alleged composition (I) in crystalline condition. Using a yeast ribonucleic acid of presumably American origin, they followed the directions of the German authors for the preparation of guanine-uridylic acid, and obtained substances having approximately the theoretical analytical compositions required for (I). The properties of these substances, however, showed clearly that they were mixtures of guanosine and purine nucleotides; no traces of pyrimidine nucleotides were found. These authors therefore concluded that no sufficient evidence has as yet been furnished in support of the view that the individual nucleotides are linked through P–N linkages in ribonucleic acid.

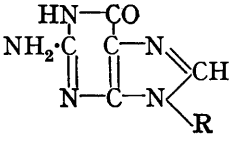
In view of these discrepant results it seems desirable to record our experiments on this question. Six attempts were made to prepare guanine-uridylic acid from yeast ribonucleic acid supplied by British Drug Houses Ltd., following the directions of Bredereck and Richter. In five of these, one specimen of the acid being used, mixtures of guanosine and guanylic acid in the ratio 30 : 70 were the invariable products; these mixtures had approximately the analytical composition required by guanine-uridylic acid, and their components were readily separated. The sixth attempt, made with another specimen of B.D.H. yeast

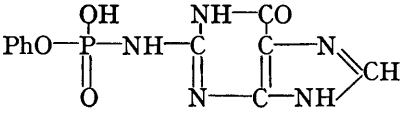
nucleic acid, resulted in a mixture consisting of guanylic and adenylic acids, guanosine and adenosine. In these experiments neither guanine-uridylic acid nor any pyrimidine nucleotide was found. These results, which were obtained over a year ago, agree closely with the observations of Tipson and Levene, and had we reported our findings at that date we should undoubtedly have written substantially the same account and come to the same conclusion as these authors.

It seemed improbable, however, that Bredereck and his collaborators could have been so deceived by their results as the acceptance of these conclusions would have implied. Accordingly we investigated the isolation of guanine-uridylic acid from samples of yeast ribonucleic acid supplied by the firms of Boehringer and Merck, and had no difficulty in preparing guanine-uridylic acid by the method of the German authors. It was a hygroscopic, amorphous, monobasic acid, which readily underwent hydrolysis at alkaline reactions to guanine and uridylic acid with consequent liberation of a second acidic group, as shown by forward and backward titrations. Moreover, purification of guanine-uridylic acid was effected through the lead salt, prepared by means of neutral lead acetate; this in itself is a proof of the state of combination, and not admixture, of the two components.

There are two alternative constitutions for guanine-uridylic acid; it may contain a P–NH linkage, or an ester linkage between the phosphoryl radical and the lactim form of the CO·NH linkage of guanine as now advocated by Bredereck. The choice between these cannot yet be made with certainty, but the balance of evidence seems to be in favour of the P–NH linkage of structure (I).

First, van Slyke estimations of amino-nitrogen showed that guanine-uridylic acid (I), guanylic acid, guanosine, guanine (II) and *phenylphosphorylguanine* (III) underwent deamination at 0° and 20°. At 0° guanine-uridylic acid and phenylphosphorylguanine reacted more slowly than the others (Table I), but at 20° there was no difference in the rates of deamination of guanine-uridylic acid and the guanine derivatives (II) with free amino-groups.

(II.) 

 (III.)

R = H in guanine.
= ribose in guanosine.
= ribose-3-phosphate in guanylic acid.

PhO·PO(OH)·NHPh (IV.)
PhO·PO(OH)·NH_2 (V.)
PhO·PO(OH)·NH·CPh:NH (VI.)

These results are consistent with the presence of the phospho-amide group in guanine-uridylic acid; this linkage is attacked readily by nitrous acid, the original isolation of guanine in poor yield by Bredereck after attempted deamination of guanine-uridylic acid being the result of incomplete reaction.*

Secondly, electrometric titrations with alkali of phenylphosphoanilide (IV), phenylphosphorylbenzamidine (VI), phenylphosphorylguanine (III), and guanine-uridylic acid (I) tend to support the P–N linkage. These results, and other electrometric titrations, will form the subject of a future publication.

On the other hand, comparison of the stabilities towards alkali of guanine-uridylic acid and analogous compounds provides the only evidence which at present can be invoked to support the alternative lactim-phospho-ester structure. Back-titration with acid of guanine-uridylic acid after it had been kept for 24 hours at p_H12 showed that under these conditions alkaline hydrolysis had liberated one additional acidic group, and it will be shown below that this hydrolysis occurs even at p_H 8·6. The phospho-amide linkage is usually regarded, however, as being relatively stable, and this is confirmed by our experience that phenyl-

* Tipson and Levene (*J. Biol. Chem.*, 1939, **127**, 105) state " In disagreement with these authors " (Bredereck and Richter) " our substances gave in van Slyke's apparatus an amount of nitrogen equivalent to about 20% of the total nitrogen." Bredereck and Richter do not record any experiments with van Slyke's apparatus as imputed by Tipson and Levene. Had they done so, they would not have gained the impression that guanine-uridylic acid is stable to nitrous acid.

phosphoanilide (IV), phenylphosphoamide (V), phenylphosphorylbenzamidine (VI) and phenylphosphorylguanine (III) are stable at p_H 8·6 under conditions in which guanine-uridylic acid is labile. In fact, 1% sodium hydroxide solution at 20° effects fission of phenylphosphorylguanine into phenol and phosphorylguanine more readily than it attacks the P–N linkage. Nevertheless, the value of this evidence may not be great, since these results have been obtained with derivatives of phenylphosphoric acid, and it is not known to what extent comparisons between aromatic and aliphatic esters of phosphoric acid are permissible; it seems that massing of aromatic radicals in the ester groups increases the stability towards alkaline fission, since Kipping and Challenger (J., 1911, **99**, 626) record that the amide, *l*-menthylamide, and *p*-toluidide of phenyl-β-naphthylphosphoric acid are all very resistant towards alkali. Suitable compounds of glycerophosphoric acid, an example which would yield derivatives more analogous with guanine-uridylic acid, have so far defied preparation. Analogues of the lactim-phospho-ester structure are unknown.

Whatever may be the nature of the linkage between the guanine and uridylic acid radicals in guanine-uridylic acid, the preparation of this substance from Boehringer and Merck but not from British Drug Houses ribonucleic acid suggests that the first two nucleic acids differ from the last as regards the internal linkages of their components, quite apart from any question of relative molecular size. The existence of structural differences in these samples of nucleic acids is confirmed by an observation that an enzyme preparation from beef bone extract absorbed on kaolin liberated, as inorganic phosphate, 100% of the phosphorus of British Drug Houses ribonucleic acid, whereas the same preparation set free only 75% of the phosphorus of Boehringer and Merck nucleic acids in identical conditions. These enzyme results are being investigated further.

These structural differences may account for variations in the ease of hydrolysis by alkali of various samples of yeast nucleic acid recorded without explanation by Steudel (*Z. physiol. Chem.*, 1930, **188**, 203) and by Makino (*ibid.*, 1935, **236**, 201). It is hoped to obtain an answer to the question whether the differences between these samples of ribonucleic acid are inherent in the acids themselves or are brought about by manipulation during extraction. As a working hypothesis it seems safe to assume that one of the differences between these samples of ribonucleic acids is the presence of a higher proportion of the guanine-uridylic acid complex *in situ* in the Boehringer and Merck acids than in the B.D.H. acid, in which the complex is either absent or present in small amount. As corollaries it is possible that the type of nucleic acid which produces guanine-uridylic acid may be convertible by hydrolysis into the other type, or *vice versa*, and that different specimens of yeast ribonucleic acid may be mixtures of these types in varying proportions.

Quantitative estimations of amino-nitrogen in various nucleotides and nucleic acids were carried out by the van Slyke method. At room temperature (Table I) guanosine, cytidine and adenosine yielded more than the theoretical amounts of nitrogen, a fact which is well recognised (Wilson, *J. Biol. Chem.*, 1923, **56**, 183; Richardson, *Proc. Roy. Soc.*, 1934, *B*, **115**, 142). In similar conditions, B.D.H. yeast and thymus nucleic acids also yielded amounts of nitrogen in excess of those required for three amino-groups per tetranucleotide. When, however, the two sets of results were considered conjointly and allowance was made for the excess of nitrogen liberated by a purine or pyrimidine radical, it was clear that the results for yeast and thymus nucleic acid were in close agreement with those required for the presence of three amino-groups. Similar experiments were also carried out at 4—5° with adenylic, guanylic and cytidylic acids; adenosine, guanosine and guanine; yeast and thymus nucleic acids. At this lower temperature the curves representing the deamination of yeast and thymus nucleic acids suggested that possibly one only of the three potential amino-groups was undergoing deamination, whereas the individual nucleotides and nucleosides were being fully deaminated. These facts, coupled with the results now recorded for the behaviour of guanine-uridylic acid in the van Slyke apparatus, might be regarded as evidence that yeast and thymus nucleic acids each contain one free amino-group in the tetranucleotide, and that two amino-groups are present as phospho-amides. Nevertheless, we prefer to record these results without drawing that conclusion, and to regard them at present as an indication that much greater care must be exercised in the interpretation of deamination experiments than has hitherto been the

case. It may be possible at a later date to correlate these facts with other experiments; but the marked dependence of the results of deaminations on temperature, and the danger of comparing experiments on suspensions (nucleic acids) with those on solutions (nucleotides and nucleosides), make it unsafe to place too implicit reliance on interpretations of structure based solely on deaminations with nitrous acid.

In view of what has been recorded above, we do not accept the conclusion of Bredereck, Köthnig, and Lehmann that guanine-uridylic acid is necessarily a secondary product; it remains an open question whether the complex is inherently present in the natural nucleic acid, or is produced during the preparation of the nucleic acid, or is formed in the aqueous hydrolysis which results in the isolation of guanine-uridylic acid. It may be mentioned that fruitless attempts were made to produce such secondary compounds by warming, and boiling, aqueous solutions containing either guanine or adenine and different nucleotides, thus simulating the conditions for the fission of ribonucleic acid which precede the isolation of guanine-uridylic acid.

Attention was drawn by Gulland (J., 1938, 1722) to the possibility that the group in B.D.H., Boehringer, and Merck ribonucleic acids which resisted enzymic fission and caused dephosphorylation to cease at 75% might be the linkage joining the components of guanine-uridylic acid. Dephosphorylation of guanine-uridylic acid occurred with sheep bone phosphomonoesterase, although not with Russell's viper or water moccasin venoms, but further investigation showed that hydrolytic fission in absence of enzyme took place at p_H 8·6 at 37°, the conditions of the enzyme experiments, with consequent deposition of guanine. The degrees of hydrolytic fission and enzymic dephosphorylation were of the same order, confirming that the effect of phosphomonoesterase was merely the dephosphorylation of uridylic acid set free by mild alkaline hydrolysis. Dephosphyloration by a mixture of phosphomonoesterase and either of the snake venoms was much more rapid, however, indicating that the venoms contain a phosphoamidase capable of splitting guanine-uridylic acid into its components so that the resulting uridylic acid suffers dephosphorylation by phosphomonoesterase. The existence of this phosphoamidase has been confirmed by its hydrolytic action on phenylphosphoamide (V) and phenylphosphorylbenzamidine (VI).

It seems clear, therefore, that the linkage of the guanine-uridylic acid complex is not that which defies enzymic fission when mixtures of phosphomonoesterase and either of the snake venoms act on yeast ribonucleic acids (Part I; Gulland and Jackson, J., 1938, 1492).

Experimental.

Attempted Preparation of Guanine-uridylic Acid from B.D.H. Yeast Ribonucleic Acid.—I. [With R. Falconer.] B.D.H. yeast nucleic acid (100 g. in batches of 10 g.) was added during 1 hour to water (600 c.c.) stirred and heated on a boiling water-bath. The solution was then boiled under reflux for 2·5 hours, allowed to cool to room temperature, and left for 1 hour in an ice-bath. The deposited solid was collected, washed with ice-cold water, mixed thoroughly with water (70 c.c.), and added to water (55 c.c.) at 90°. The mixture was warmed to 80° to effect solution, cooled to 50°, and filtered through a warm water-jacketed Büchner funnel. The filtrate was placed in ice for 1 hour, and the precipitate collected, washed with ice-water, mixed thoroughly with water (15 c.c.), and added to boiling water (15 c.c.). The mixture was warmed to effect solution, cooled to 50°, filtered through a warm water-jacketed funnel, and the filtrate cooled in ice for 1 hour. The precipitate was collected, washed with ice-water, and purified by being dissolved four successive times in warm water and caused to separate by cooling in ice. Finally the colourless amorphous material (A) was washed with alcohol and ether and dried at 100° in a vacuum over phosphoric oxide (Found in material from four different experiments: N, 21·1, 22·0, 22·0, 21·1; P, 5·8, 6·3, 6·3, 6·4%. See below for percentages required for guanine-uridylic acid).

Separation into guanylic acid and guanosine. (a) *Through the lead salts.* 20% Neutral lead acetate solution was added to the material A (0·40 g.) in warm water (10 c.c.) until there was no further precipitate. The lead salt was collected, washed thoroughly with water, decomposed with hydrogen sulphide in water, and the lead sulphide again treated with hydrogen sulphide in hot water. The filtrates were combined, freed from hydrogen sulphide by a current of air, concentrated in a vacuum at 40° to small bulk, and poured into alcohol (100 c.c.). The colour-

less amorphous precipitate of guanylic acid (0·25 g.) was centrifuged and washed with alcohol and ether; it decomposed at 179—181° (Found for material dried at 100° in a vacuum over phosphoric oxide: N, 19·1; P, 8·4. Calc. for $C_{10}H_{14}O_8N_5P$: N, 19·3; P, 8·5%), and when hydrolysed by hot dilute hydrochloric acid yielded needles of guanine hydrochloride, furfural, and phosphoric acid.

The filtrate from the neutral lead acetate treatment (above) was made alkaline with ammonia, and lead acetate and ammonia were added until there was no further precipitate. The lead salt was collected and decomposed thoroughly with hydrogen sulphide as described above and the filtrates were concentrated at 40° under reduced pressure until crystals began to form. Guanosine was collected when cold and recrystallised from water, forming needles, m. p. 238° (decomp.) (Found for material dried at 100° in a vacuum over phosphoric oxide: N, 25·0. Calc. for $C_{10}H_{13}O_5N_5$: N, 24·7%). It was free from phosphorus, gave a murexide test, and when hydrolysed by hot dilute hydrochloric acid yielded guanine hydrochloride and furfural.

(b) *Through picrate and brucine salt.* The picrate, decomp. 190—195°, which separated when a saturated aqueous solution of picric acid was added to an aqueous solution of material A was collected, washed, and decomposed by being shaken with dilute sulphuric acid and ether. The sulphuric acid was removed as barium sulphate from the aqueous layer, rhodizonic acid being used as an indicator, and the resulting filtrate was concentrated under reduced pressure to small volume. Guanosine separated from the cold solution (Found for dried material: N, 25·1. Calc. for $C_{10}H_{13}O_5N_5$: N, 24·7%); it gave murexide and pentose tests, and when hydrolysed by hot 5% sulphuric acid, followed by neutralisation with ammonia, yielded guanine (Found: N, 46·2. Calc. for $C_5H_5ON_5$: N, 46·4%).

When the faintly alkaline solution obtained by dissolving brucine in an aqueous solution of the material A was kept in the refrigerator, brucine guanylate, m. p. 200° (decomp.), slowly separated in needles.

Ratio of guanylic acid and guanosine in mixture A. (a) *Titration.* 0·07121 G. in 25 c.c. of distilled water required 13·96 c.c. of N/50-sodium hydroxide for neutralisation to phenolphthalein, and 0·0495 g. in 25 c.c. of distilled water required 4·90 c.c. of N/50-sodium hydroxide for neutralisation to bromocresol-green. Whence it follows that material A contained 70·0% of guanylic acid; the calculated values given below are based on a mixture of 70% of guanylic acid and 30% of guanosine. Jones and Richards (*J. Biol. Chem.*, 1914, **17**, 71) obtained a 50 : 50 mixture of guanylic acid and guanosine.

(b) *Acid hydrolysis.* Material A (200 mg.) was heated for 4 hours on a water-bath with 5% sulphuric acid (5 c.c.). The hot solution was neutralised with ammonia and cooled, and after some hours the precipitated guanine (73 mg.) was centrifuged, washed, and dried (calc., 90 mg.).

(c) *Alkaline hydrolysis.* Material A (100 mg.) was heated for 4 hours with 4% aqueous sodium hydroxide (3 c.c.), and the solution neutralised with acetic acid and left overnight in the refrigerator. The guanine which separated (42·7 mg.) was centrifuged, washed with alcohol and ether, and dried (calc., 45·0 mg.).

(d) *Amino-nitrogen estimation.* 1·04 C.c. of a solution of material A (0·1112 g.) in water (6 c.c.) were shaken in a micro-van Slyke apparatus for 1 hour at 4—5°, a blank experiment being made for the correction (Found: Amino-N, 4·0. Calc., 4·2%).

Dephosphorylation by enzymes [with E. M. Jackson]. After incubation for 6 hours 83% of the total phosphorus had been liberated as inorganic phosphate in a mixture (20 c.c.) of material A (7·6 mg.), p_H 8·6 borate buffer (5 c.c.), N/10-magnesium sulphate (0·5 c.c.), phosphomonoesterase solution (4 c.c., free from diesterase) (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 590), and water. This hydrolysis is thus much more rapid than that of guanine-uridylic acid in similar circumstances (see below).

Material A did not undergo dephosphorylation by the 5-nucleotidase of Russell's viper venom at p_H 8·6 (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 597) and hence the phosphoryl group of the guanylic acid does not esterify the hydroxyl at C_5 of the ribose radical.

II. [With G. I. Hobday.] *Separation into nucleotides and nucleosides.* B.D.H. yeast nucleic acid (100 g.) was added steadily during 30 minutes to water (600 c.c.) heated on a water-bath and stirred mechanically; this acid did not dissolve so readily as Boehringer and Merck yeast nucleic acids. Heating and stirring were continued and after 1·75 hours the acid had dissolved except for a small residue of gum. The solution was decanted and boiled under reflux for 2·5 hours in an oil-bath at 115°. The subsequent treatment followed exactly that described below in the case of the Boehringer and Merck nucleic acids, the final purified product weighing 0·1 g. This material was divided into nucleotide and nucleoside fractions by succes-

sive precipitation with neutral lead acetate and with lead acetate and ammonia respectively; the examination of these fractions is described below.

The lead salts of the nucleoside fraction were washed, suspended in water, and decomposed with hydrogen sulphide, the filtrate from the lead sulphide being concentrated to 10 c.c. and left overnight in the refrigerator. The colourless crystals (10 mg.) which had separated (filtrate, see below) gave positive murexide and pentose tests, but failed to give the diazo-reaction for guanine or to reduce Fehling's solution unless previously hydrolysed by hot hydrochloric acid. The substance was thus guanosine (Found: N, 24·1. Calc. for $C_{10}H_{13}O_5N_5$: N, 24·7%).

The filtrate (above) was mixed with aqueous picric acid solution, and the precipitate recrystallised from water. Adenosine picrate separated in yellow plates, m. p. 184°, alone or mixed with an authentic specimen.

The lead salts of the nucleotide fraction were washed, suspended in water, and decomposed with hydrogen sulphide, the filtrate from lead sulphide being evaporated to dryness under reduced pressure below 40°. Industrial methylated spirit (30 c.c.) was added to a solution of the residue (75 mg.) in water (20 c.c.) and the solid which slowly separated was centrifuged (solution, see below) and dissolved in water, and the solution concentrated in an evacuated desiccator. Guanylic acid separated as an amorphous solid (Found: N, 19·5; P, 8·6. Calc. for $C_{10}H_{14}O_8N_5P$: N, 19·3; P, 8·5%), which gave tests for combined guanine, pentose and combined phosphate.

The solution (above) was evaporated to dryness under reduced pressure below 40°, and the residue dissolved in water (10 c.c.) and mixed with industrial methylated spirit (20 c.c.). The solid was discarded, and the solution again evaporated to dryness. When the residue was recrystallised from water, adenylic acid separated in colourless needles, m. p. 193° (Found: N, 19·9; P, 8·8. Calc. for $C_{10}H_{14}O_7N_5P$: N, 20·2; P, 8·9%), which gave no murexide reaction, contained pentose and combined phosphate, and yielded a brucine salt, m. p. 173°, in colourless needles.

Ratio of nucleotides and nucleosides. Approximately 75% of the nucleotide was guanylic acid, and approximately 90% of the nucleoside fraction was guanosine. The ratio total nucleotide/total nucleoside was about 80/20.

Preparation of Guanine-uridylic Acid from Boehringer Yeast Ribonucleic Acid [with G. I. Hobday].—(*a*) A suspension of Boehringer yeast nucleic acid (100 g.) in water (600 c.c.) was heated on the water-bath. The solution was decanted from a small amount of sticky solid, boiled under reflux in an oil-bath at 115° for 2·5 hours, allowed to cool, and placed in ice for 1 hour. The yellow precipitate was collected, washed three times with ice-water, suspended in water (70 c.c.), and added to water (55 c.c.) at 90°. The mixture was heated to 80°, allowed to cool to 50°, and filtered, the filtrate being kept for 1 hour in ice. The precipitated solid was collected, washed with ice-water, suspended in water (15 c.c.), and added to boiling water (15 c.c.). This mixture was kept warm until practically complete solution had occurred; it was then allowed to cool to 55° and filtered through a warm water-jacketed funnel. The guanine-uridylic acid which separated when the filtrate was cooled for 1 hour in ice was purified by three successive solutions in hot water, followed by filtration through a jacketed funnel, and cooling in ice, the final material (1·5 g.) being washed with alcohol and ether (Found for material dried at 100° in a vacuum over phosphoric oxide: N, 22·2; P, 6·6. Calc. for $C_{14}H_{16}O_9N_7P$: N, 21·4; P, 6·8%). It was a pale buff, amorphous solid, moderately readily soluble in water, especially when slightly warm. An aqueous solution yielded no brucine salt and, in the case of most preparations, no picrate, although occasionally guanine picrate separated slowly from concentrated solutions, either as the result of hydrolysis or because small amounts of uncombined guanine were present (see purification as the lead salt, below). Hydrolysis with boiling 3·8% sulphuric acid yielded guanine sulphate and uridylic acid, identified as the brucine salt, and alkaline hydrolysis also gave guanine.

(*b*) Boehringer yeast nucleic acid (100 g.) was added steadily during 30 minutes to water (600 c.c.), vigorously stirred and heated on a water-bath. The stirring was continued, and after 1 hour all solid had dissolved, with the exception of a little gum. The solution was decanted and heated under reflux for 2·5 hours in an oil-bath at 115°. The subsequent treatment followed that just described, the final yield of guanine-uridylic acid (0·3 g.) often being contaminated by traces of guanine.

Because of the presence of small quantities of impurities in guanine-uridylic acid prepared as above, it has been found necessary to supplement the purification by conversion into the lead salt, and the details of a typical experiment are recorded below. Guanine-uridylic acid (0·7 g.) was dissolved as completely as possible in 30 c.c. of warm water. The solution was

filtered from a small insoluble residue (identified as guanine), cooled sufficiently to avoid precipitation, and mixed with 20% neutral lead acetate solution until no further precipitate of the lead salt of nucleotide separated. This was then centrifuged, and the liquid mixed with ammonia and lead acetate until no further precipitate of lead salt of the nucleoside and purine formed.

The nucleotide lead salt was decomposed by hydrogen sulphide in warm water, and the resulting solution concentrated to small volume under reduced pressure at 35° and then in a vacuum desiccator. Two fractions of guanine-uridylic acid were obtained as pale buff, hygroscopic, amorphous powders, which were collected (M.L. see below) and ground with alcohol [Found for material dried at 110° in a vacuum : (for one of the fractions) C, 36·1; H, 4·5; N, 21·3; P, 6·7; (for the other) N, 21·3; P, 6·9. Calc. for $C_{14}H_{16}O_9N_7P$: C, 36·7; H, 3·5; N, 21·4; P, 6·8%]. When 0·1262 g. in water (50 c.c.) was titrated with 0·0855N-sodium hydroxide, with phenol-red as indicator, 3·30 c.c. were required (calc., 3·23 c.c.). A third fraction was obtained as a colourless powder by adding several volumes of alcohol to the mother-liquors, but this probably contained small amounts of other nucleotides, possibly uridylic acid (Found : N, 19·5; P, 7·1%). In normal preparations, the third fraction was neglected.

The nucleoside lead salt fraction was decomposed with hydrogen sulphide in the usual way, and on concentrating the solution under reduced pressure, three fractions were obtained : (*a*) guanine, (*b*) a mixture of guanosine, guanine, and a trace of nucleotide, (*c*) guanosine. The combined weights of these fractions amounted to only 10—15% of those of the nucleotide fractions.

Preparation of Guanine-uridylic Acid from Merck Ribonucleic Acid [with G. I. Hobday].—The procedure was exactly that described in the case of the Boehringer acid; the guanine-uridylic acid formed a pale buff, amorphous powder (Found for material dried at 110° in a vacuum : N, 21·2; P, 6·7%).

Estimations of Amino-nitrogen [with R. Falconer and G. I. Hobday].—These were made in a micro-van Slyke apparatus, either used normally at room temperature, or so arranged that the reaction chamber was immersed continuously in a bath of ice and water, both when at rest and when being shaken. Water was the solvent in the case of guanine-uridylic acid, the nucleotides, nucleosides and phenylphosphorylguanine; the nucleic acids were dissolved in the minimum amount of very dilute alkali solution and added immediately to the mixture of nitrite and acetic acid in the reaction chamber, the nucleic acids being thus precipitated in a very fine state of division; guanine was dissolved in a little 2% hydrochloric acid (Wilson, *J. Biol. Chem.*, **1923, 56,** 183). Some results are given in Table I, but in practice the experiments were prolonged to 140 minutes and curves were constructed on the basis of estimations made at 20-minute intervals.

TABLE I.

van Slyke Estimations.

In each case the lower figure is the percentage deamination at 30 minutes, the higher at 100 minutes. The values for nucleic acids are calculated for three amino-groups per tetranucleotide.

	0°.	4—5°.	20°.
Phenylphosphorylguanine	3, 12	—	20, 44
Guanine-uridylic acid	5, 33	—	110, 130
Guanine	35, 67	—	104, 116
Guanosine	24, 58	40, 72	100, 114
Adenosine	—	43, 84	94, 108
Guanylic acid	22, 55	75, 110	108, 124
Adenylic acid	—	56, 90	84, 110
Cytidylic acid	—	30, 62	64, 110
Thymonucleic acid	—	12, 24	68, 98
Yeast ribonucleic acid	—	18, 32	74, 105

Fission of Guanine-uridylic Acid in Enzyme Experiments [with E. M. Jackson].—(a) *In presence of sheep bone phosphomonoesterase.* A mixture of guanine-uridylic acid (11·5 mg.), p_H 8·6 borate buffer (10 c.c.), M/10-magnesium sulphate (1 c.c.), and purified enzyme solution (Gulland and Jackson, *Biochem. J.*, 1938, **32,** 590) (10 c.c.) was diluted to 50 c.c. at p_H 8·6 with water (toluene) and incubated at 37°, samples (5 c.c.) being withdrawn at appropriate intervals for estimation by the Bell–Briggs colorimetric method of the percentage of total phosphorus liberated as phosphate :

Time in hours	3	4	5	22	29	46	70	140
Expt. I	—	—	26	—	—	66	68	80
Expt. II	—	17	—	25	29	42	—	—
Expt. III	16	—	18	23	33	55	—	—

(b) *At p_H 8·6 without enzyme.* No inorganic phosphate was liberated when a solution of guanine-uridylic acid at p_H 8·6 was maintained at 37°, this being in accordance with expectation, since uridylic acid is stable under those conditions.

Solutions of guanine-uridylic acid (37·0 mg. and 39·1 mg.) in water (20 c.c.) at p_H 8·6 were maintained at 37° for 17 and 20 hours respectively, the precipitated guanine being collected and weighed. The degree of hydrolysis was in approximate agreement with that which occurred in the presence of phosphomonoesterase during the same period.

(c) *In presence of mixed sheep bone phosphomonoesterase and Agkistrodon piscivorus venom.* The venoms of the water moccasin and Russell's viper are enzymically closely similar (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 597) and when acting alone do not liberate inorganic phosphate from guanine-uridylic acid at p_H 8·6, since they do not contain the general phosphomonoesterase capable of attacking the phosphoryl radical of uridylic acid.

A mixture of guanine-uridylic acid (11·5 mg.), p_H 8·6 borate buffer (10 c.c.), M/10-magnesium sulphate (1 c.c.), snake venom (20 mg.), and purified phosphomonoesterase solution (10 c.c.) was diluted with water to 50 c.c. at p_H 8·6 (toluene) and incubated at 37°, samples (5 c.c.) being taken at appropriate intervals for the estimation of inorganic phosphate, shown below as the percentage of total phosphorus:

Time in hours	3	4	5	22	29	46	92
Expt. I	—	—	—	—	—	79	91
Expt. II	—	36	—	50	52	79	—
Expt. III	13	—	21	49	54	72	—

Comparison of the greater amounts of phosphate liberated in these experiments with the lesser amounts in those with phosphomonoesterase alone (for example, at 22 and 46 hours) indicated clearly that these snake venoms contain a phosphoamidase capable of hydrolysing guanine-uridylic acid into its components and then liberating the phosphoryl radical.

(d) *Action of mixed phosphomonoesterase of sheep bone and phosphoamidase of snake venom on other phosphoamides.* Purified phosphomonoesterase of sheep bone failed to liberate phosphate from phenylphosphoamide, phenylphosphoanilide, and phenylphosphorylbenzamidine in p_H 8·6 borate buffer in presence of magnesium sulphate, and therefore did not contain a phosphoamidase capable of hydrolysing the P–N linkage of these substances.

Mixtures of the substrates (10—15 mg.), p_H 8·6 borate buffer (10 c.c.), M/10-magnesium sulphate (1 c.c.), phosphomonoesterase solution (10 c.c.), and Russell's viper or water moccasin venom (20 mg.) were diluted with water to 50 c.c. at p_H 8·6 (toluene), and incubated at 37°, samples (5 c.c.) being withdrawn for estimation of phosphate, stated below as percentages of total phosphorus:

Time in hours	3	5	22	28	46	52	94	118
			Phenylphosphoamide.					
Russell's viper	12	17	42	—	—	—	—	—
Water moccasin	—	—	12	13	19	—	25	33
	—	—	13	14	17	21	31	—
			Phenylphosphorylbenzamidine.					
Russell's viper	—	16	46	51	59	—	—	—
	—	12	41	—	62	—	—	—
Water moccasin	11	—	27	41	47	—	50	64
	—	14	31	37	40	46	54	58

Phenylphosphoanilide yielded no phosphate when acted on by a mixture of sheep bone phosphomonoesterase and water moccasin venom under the conditions cited above. Crude beef bone phosphatase prepared according to the Martland–Robinson method (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 590) rapidly dephosphorylated phenylphosphoamide. It is clear that purified phosphomonoesterase preparations do not contain a phosphoamidase, but that this enzyme (or enzymes) is present in the snake venoms and shows considerable selectivity in its hydrolytic effect on the P–N linkage.

Phenylphosphoamide [with G. I. Hobday].—The barium salt, prepared by hydrolysis of diphenylphosphoamide with baryta (Ephraim, *Ber.*, 1911, **44**, 633), crystallised in rhombic plates [Found for material dried at 110° in a vacuum: P, 12·9; Ba, 28·1. Calc. for $(C_6H_7O_3NP)_2Ba$: P, 12·9; Ba, 28·4%]. For enzyme experiments the barium salt was transformed into the sodium salt by treatment with the requisite amount of sodium sulphate solution.

Phenylphosphoanilide. The phenyl ester of anilinophosphoryl chloride, prepared by the action of aniline (2 mols.) on monophenyl phosphoryl dichloride in dry ether (Michaelis, *Annalen*, 1902, **326**, 224), crystallised from benzene in colourless needles, m. p. 136°, which were hydrolysed with cold sodium hydroxide solution. The anilide crystallised from alchol–ether in plates, m. p. 134° (Found : P, 12·3. Calc. for $C_{12}H_{12}O_3NP$: P, 12·4%).

Monophenylphosphorylbenzamidine. Monophenyl phosphoryl dichloride was prepared by a modification of the method of Jacobsen (*Ber.*, 1875, **8**, 1521) by boiling a mixture of anhydrous phenol (1 mol.) and redistilled phosphorus oxychloride (1·25 mols.) under reflux in an oil-bath for 10 hours. The mixture was distilled from the flask and collected between 240° and 250°, the distillate being fractionated under reduced pressure. Phenyl phosphoryl dichloride, free from phosphorus oxychloride and hydrogen chloride, was collected at 121—122°/11 mm.

A mixture of benzamidine hydrochloride (1 g.) and monophenyl phosphoryl dichloride (1·7 g.; 1·25 mols.) was heated in an oil-bath at 160° for 12 hours, moisture being excluded. The evolution of hydrogen chloride had then ceased, and the excess of phenyl phosphoryl dichloride was removed by distillation under reduced pressure. The viscous residue solidified when cold, and after being repeatedly triturated with cold water, was dissolved in a slight excess of dilute sodium hydroxide solution, and the excess neutralised with hydrochloric acid. The solution (100 c.c.) was concentrated under reduced pressure to about 25 c.c.; the *sodium* salt of monophenylphosphorylbenzamidine then began to crystallise, separation being complete on cooling. When recrystallised from water, it formed colourless needles (Found for material dried at 110° : N, 9·2; P, 10·2. $C_{13}H_{12}O_3N_2PNa$ requires N, 9·4; P, 10·4%), which were hydrolysed by hot acid to give benzamidine, isolated as the picrate, m. p. 227°. Addition of hydrochloric acid to a dilute solution of the sodium salt did not precipitate the free acid, which was rather soluble in water; in the case of more concentrated solutions, however, the acid separated as a colourless precipitate, which did not dissolve in excess of mineral acid. The free acid resinified fairly readily, the product being insoluble in cold sodium hydroxide solution.

Phenylphosphorylguanine. Dried guanine (0·5 g.) and phenyl phosphoryl dichloride (1 g.; 1 mol.) were heated at 140° for 15 hours; hydrogen chloride was slowly evolved and the mixture turned reddish-brown. The mixture was washed repeatedly with ice-water, dissolved in ice-cold, dilute aqueous sodium hydroxide, the dark red solution acidified with hydrochloric acid, and the precipitated solid collected by centrifugation and washed with water. It was dissolved in dilute aqueous sodium carbonate, and the filtered solution acidified with hydrochloric acid. The brownish-red amorphous precipitate of *phenylphosphorylguanine* was centrifuged, washed with water and alcohol, and dried (yield, 50%) (Found for material dried at 110° : N, 22·6; P, 10·0. $C_{11}H_{10}O_4N_5P$ requires N, 22·8; P, 10·1%). It gave the murexide and other tests for guanine. It was not appreciably soluble in water, dilute mineral acids or organic solvents, but dissolved readily in dilute aqueous alkali to form a red solution. The addition of barium chloride to an aqueous solution of the sodium salt precipitated the barium salt as a grey powder.

Hydrolytic fission was complete in 2 hours at 100° with either 3% sodium hydroxide solution or 4% sulphuric acid, as shown by estimations of free phenol (97·5 and 90·0% respectively) and inorganic phosphate (100% and 89·2% respectively). Under milder conditions of alkaline hydrolysis, however, it was evident that hydrolysis of the phenyl ester group occurred in preference to that of the phospho-amide. 1% and 3% Sodium hydroxide solution at room temperature had similar effects, and estimations of free phenol and inorganic phosphate at intervals showed that at 20 hours 70% of the total phenol had been set free, this value rising to only 75% at 80 hours; the amount of phosphate was then less than 5% of that theoretically possible. Since phenylphosphoric acid is stable under these conditions, it is clear that the main product of reaction is phosphorylguanine, the phenyl ester linkage being less stable than the phosphoamide.

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