741. *The Preparation of Permanganate-oxidised Ribonucleic Acid and its Hydrolysis with Pancreatic Ribonuclease*

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Treatment of high-molecular-weight yeast ribonucleic acid with potassium permanganate at 57° for 3 hr. at pH 9 oxidised the guanine, cystosine, and uracil residues to ureido-residues. The oxidised nucleic acid was stable at pH 10 at 25°, but was hydrolysed by pancreatic ribonuclease to give adenosine 3′-phosphate, adenosine 2′,3′-(cyclic phosphate), adenine-containing oligonucleotides, and a substance which was probably a ribosylurea phosphate. The significance of the results with regard to the specificity of pancreatic ribonuclease is discussed.

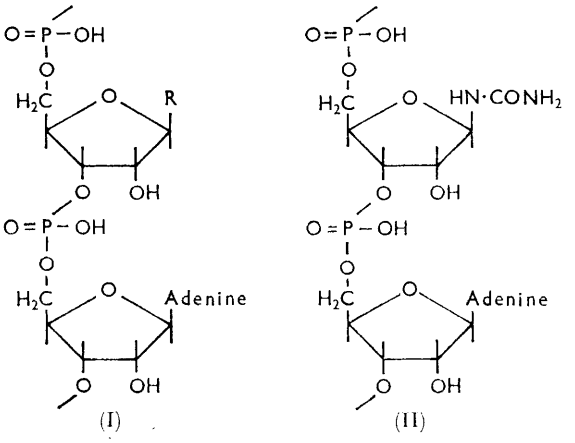
It has already been reported that potassium permanganate at 37° at pH 9 oxidises the guanine, cytosine, and thymine residues of deoxyribonucleic acids to urea residues but leaves the adenine residues unaffected.[1,2] When ribonucleic acid (I) was treated in a similar way, the cytosine and uracil residues were completely oxidised, but the guanine residues were comparatively resistant, about 5% remaining after 18·5 hours' treatment and only about 90% of the adenine residues being not oxidised. The oxidation was studied under a number of conditions, and the results (see Table) showed that, at 57° for 3 hr. with a ribonucleic acid : permanganate ratio of 1 : 1·25 (w/w), a product (II) was obtained which contained 95—97% of the adenine residues of the original ribonucleic acid and no detectable guanine, cytosine, or uracil residues.

Some hydrolysis of the phosphodiester linkages occurred during the oxidation, as shown by the fact that there was only a 77% recovery of non-dialysable material, and that the product had an average chain-length of about ten nucleotide units. Acid hydrolysis of compound (II) gave urea, but no biuret was detected. This result is similar to that obtained with permanganate-oxidised deoxyribonucleic acid [1] and is surprising in view of the fact that biuret residues are produced when substituted cytosines are similarly oxidised.[3] This suggests the structure (II) assigned to the permanganate-oxidised ribonucleic acid and it is confirmed by the carbon, nitrogen, and phosphorus analyses.

It was not possible to degrade compound (II) into adenine-containing oligonucleotides with N-alkali at 100° as with permanganate-oxidised deoxyribonucleic acids,[1,2] because of the alkali lability of polyribonucleotides. At pH 10 at 25°, conditions under which

[1] A. S. Jones, G. W. Ross, S. Takemura, T. W. Thompson, and R. T. Walker, *J.*, 1964, 373.
[2] A. S. Jones and R. T. Walker, *Nature*, 1964, **202**, 24, 1108.
[3] B. Chatamra and A. S. Jones, *J.*, 1963, 811; A. S. Jones and R. T. Walker, *J.*, 1963, 3554.

ribonucleic acid is stable, but at which periodate-oxidised end-groups may be removed,[4] compound (II) was not degraded, thus showing the relative stability of the ureido-residues. At pH 10 at 37°, compounds (I) and (II) were both slowly hydrolysed.



R = guanine, cytosine, or uracil residue

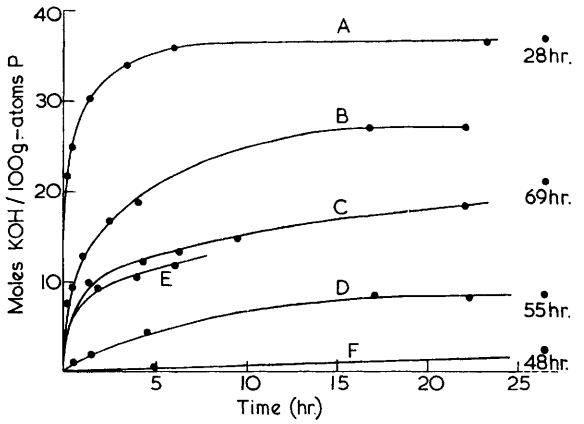
In view of this lack of specific degradation, compound (II) was treated with crystalline bovine pancreatic ribonuclease. This material is not homogeneous [5,6] and contains two enzymes, ribonucleases A and B.[5] It has been claimed that the former catalyses the cleavage of internucleotide bonds in ribonucleic acids between the 3′-pyrimidine nucleoside phosphoryl groups and the 5′-hydroxyl groups of the adjacent purine or pyrimidine nucleoside residues whereas the latter catalyses the cleavage of similar bonds involving 3′-guanosine phosphoryl groups.[7] The crystalline mixture of enzymes liberates only about 1% of the total guanine residues present as guanylic acid, however.[7] Because of this apparent specificity, pancreatic ribonuclease has been used by numerous workers to determine the nucleotide distribution in ribonucleic acids.[8] There have been, however, reports which indicate that the enzyme is capable of splitting interpurine nucleotide linkages. Thus, Levin [9] showed that poly-8-azaguanylic acid was hydrolysed, and Beers [10] showed that polyadenylic acid was rapidly degraded to adenosine monophosphate (tentatively identified as the 3′-phosphate). The latter author urged caution in the interpretation of results of experiments with pancreatic ribonuclease on nucleotide distribution in ribonucleic acids. The action of the crystalline enzyme on internucleotide bonds between 3′-ribofuranosylurea phosphoryl groups and the 5′-hydroxyl group of an adjacent residue is not known (although it has been reported that phosphate esters of ribofuranosylureidopropionic acid 3′-phosphate are not hydrolysed but those of 4,5-dihydrouridine 3′-phosphate are).[11] It was hoped that cleavage of these bonds in compound (II), however, would produce adenine-containing oligonucleotides with a terminal ribofuranosylurea residue.

Hydrolysis of compound (II) did occur when it was treated with the ribonuclease, as

[4] D. M. Brown, M. Fried, and A. R. Todd, *J.*, 1955, 2206.
[5] C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1953, **200**, 493.
[6] G. Taborsky, *J. Biol. Chem.*, 1959, **234**, 2652.
[7] A. A. Hakim, *Arch. Biochem. Biophys.*, 1957, **70**, 591; *Biochim. Biophys. Acta*, 1957, **24**, 445; *J. Biol. Chem.*, 1957, **228**, 459.
[8] B. P. Doctor, C. M. Connelly, G. W. Rushizky, and H. A. Sober, *J. Biol. Chem.*, 1963, **238**, 3985; K. S. McCully and G. L. Cantoni, *ibid.*, 1962, **237**, 3760; R. Lipshitz-Wiesner and E. Chargaff, *Biochim. Biophys. Acta*, 1963, **76**, 372; A. I. Aronson, *ibid.*, 1963, **72**, 176; D. Bell, R. V. Tomlinson, and G. M. Tener, *Biochem. Biophys. Res. Comm.*, 1963, **10**, 304; R. K. Ralph, R. J. Young, and H. G. Khorana, *J. Amer. Chem. Soc.*, 1963, **85**, 2002.
[9] D. H. Levin, *Biochim. Biophys. Acta*, 1962, **61**, 75.
[10] R. F. Beers, *J. Biol. Chem.*, 1960, **235**, 2393.
[11] H. Witzel, *Annalen*, 1960, **635**, 191.

shown by an increase in acidic groups (Figure), but the hydrolysis was slower than with ribonucleic acid. This slower reaction was not due to the presence of traces of manganese ions in compound (II) because there was no increase in the rate of hydrolysis when ethylenediaminetetra-acetic acid was added. The action of ribonuclease on compound (II) was apparently not due to non-enzymic catalysis by the protein, because lysozyme, a similar protein, had very little effect.

The products obtained from the reaction of ribonuclease on compound (II) were fractionated on a column of a strongly basic ion-exchange resin, to give at least five components whose position of elution from the column and ultraviolet absorption spectra indicated that they were adenine-containing oligonucleotides. The major component (30% of the ultraviolet absorption of the total hydrolysate) was adenosine 2′(3′)-phosphate (mixed isomers). Separation of these isomers by paper chromatography showed that the amount



A and B, Ribonucleic acid : pancreatic ribonuclease ratio 1 : 5 and 1 : 50, respectively. C and D, Oxidised ribonucleic acid (II) : pancreatic ribonuclease ratio of 1 : 5 and 1 : 50, respectively. E, As for C but containing 0·01M-ethylenediaminetetra-acetic acid (adjusted to pH 7). F, Oxidised ribonucleic acid (II) treated with lysozyme (enzyme : substrate ratio 1 : 3). All experiments were at 37° and pH 7

of 3′-phosphate present was four times that of the 2′-phosphate. When the total hydrolysate of compound (II) was fractionated by means of paper chromatography in propan-2-ol–ammonia–water, one of the products was adenosine 2′,3′-(cyclic phosphate) (12% of the total ultraviolet absorption). The identity of this product was established by chromatography in five solvent and two two-dimensional solvent systems. In this case no adenosine 2′-phosphate was present, so that its appearance in the products separated on the ion-exchange resin was due to the action on the cyclic phosphate of the acid used for the elution. This was confirmed by treating the hydrolysate of compound (II) with 0·01N-acid when all the cyclic phosphate was converted into adenosine 2′(3′)-phosphate. There was also present a urea-containing compound that had almost the same R_F value in propan-2-ol–ammonia–water as adenosine 2′,3′(cyclic phosphate). This compound also contained phosphorous and therefore was probably a ribosylurea 2′,3′-(cyclic phosphate).

The enzyme was not acting in the manner hoped for, therefore, but was cleaving relatively non-specifically the internucleotide linkages to give adenosine 2′,3′-cyclic phosphate) and adenine-containing oligonucleotides. The lack of complete cleavage was probably due to the inhibitory action of the products on the enzyme.[12] It has already been shown by Beers [10] that adenosine 2′,3′-(cyclic phosphate) is hydrolysed to an adenosine monophosphate by pancreatic ribonuclease, so that it appears that the adenosine 3′-phosphate found in this case in the products of the action of the enzyme on compound (II) was produced by the enzymic hydrolysis of the cyclic phosphate.

The titrimetric method estimated only the second stage of the reaction with ribonuclease, namely the hydrolysis of 2′,3′-(cyclic phosphates), so that the first stage, the cleavage of the internucleotide linkage, may have been more rapid. Although a high

[12] G. Schmidt, "Nucleic Acids," ed. E. Chargaff and J. N. Davidson, Academic Press, New York, 1955, vol. I, p. 570.

enzyme : substrate ratio (1 : 6) was used in most of this work, some hydrolysis of compound (II) occurred at an enzyme : substrate ratio of 1 : 50, and in his work on the hydrolysis of polyadenylic acid Beers used a ratio of 1 : 20. As these ratios were lower than those used by many workers in investigations of nucleotide distribution and end-groups in ribonucleic acids (various ratios from 1 : 10 to 1 : 40 have been used under a variety of conditions [8]) the possibility of the fission of a small number of bonds between 3′-adenosine phosphoryl groups and the 5′-hydroxyl groups of the adjacent nucleosides by the action of pancreatic ribonuclease on ribonucleic acids should be considered.

Experimental

Isolation of Yeast Ribonucleic Acid.—High-molecular-weight yeast ribonucleic acid was isolated essentially by Crestfield, Smith, and Allen's method [13] with the following modifications: (*a*) the extraction solutions and those used in the isolation procedure all contained 0·6 mM Zn^{2+} to inhibit ribonuclease,[14] (*b*) before the final dialysis of the product, residual ribonuclease and other proteins were removed, either by adsorption on bentonite [14] or by Sevag's procedure.[15] A typical batch of ribonucleic acid had the following analysis (after drying at 110° *in vacuo*): N, 14·8; P, 8·4%; ε_P (260 mμ) 8820. The base analysis was carried out by hydrolysis with N-hydrochloric acid at 100° for 1 hr. and the resulting purines and the pyrimidine nucleotides were separated by paper chromatography in methanol–ethanol–10N-hydrochloric acid–water (50 : 25 : 6 : 19) [16] and determined spectrophotometrically, corrections being applied for the 5% dephosphorylation of the pyrimidine nucleotides. The composition was (corrected to moles of base per 100 moles of nucleotides): adenine, 26·7; guanine, 26·0; cytosine, 20·6; uracil, 26·7. Total recovery of bases, 91%.

Permanganate Oxidation of Yeast Ribonucleic Acid.—Yeast ribonucleic acid (100 mg.) in water (20 ml.) was mixed with a solution of potassium permanganate (as shown below) in water (20 ml.). Experiments carried out at pH 9 contained sodium hydrogen carbonate (12·5 mg./ml.) and those at pH 7 were carried out in 0·067M-phosphate. At intervals samples were removed, the excess permanganate destroyed with sodium pyrosulphite and the solutions exhaustively dialysed and freeze-dried. The products were then analysed for purines and pyrimidines as described above. The results shown in the Table give only the optimum reaction times (time of highest adenine and lowest guanine contents) for each series.

Oxidation of ribonucleic acid with potassium permanganate

Expt.	$KMnO_4$: RNA ratio (w/w)	Temp.	Time (hr.)	pH	Recovery of bases (% of base content of original RNA) Adenine	Guanine	Pyrimidines
1 (*a*)	1 : 1	25°	27	9	93—95	10	0·0
(*b*)	1 : 1	37	18·5	9	91	5·0	0·0
(*c*)	1 : 1	57	3	9	91—95	10	0·0
(*d*)	1 : 1	57	4	9	91—95	8·0	0·0
2	1 : 1	57	3	7	91	40	0·0
3	1·25 : 1	57	3	9	95—97	0·0	0·0

Preparation of Permanganate-oxidised Ribonucleic Acid.—A solution of yeast ribonucleic acid (4 g.) in water (800 ml.) was added to a solution of potassium permanganate (5·2 g.) and sodium hydrogen carbonate (26 g.) in water (800 ml.) and heated at 57° for 3 hr. The cooled solution was decolourised by the addition of the minimum volume of sodium pyrosulphite solution and dialysed against running tap-water for 24 hr. The solution of non-diffusible material was then passed down a column (30 × 2 cm. dia.) of ZeoKarb 225 (Na^+) to remove manganese ions and then dialysed against repeated changes of distilled water for 4 days at 0°. The solution was then freeze-dried to give a white product (2·6 g., yield, based on phosphorus content, 77%).

An alternative procedure which avoided the dialysis of large volumes was to precipitate

[13] A. M. Crestfield, K. C. Smith, and F. W. Allen, *J. Biol. Chem.*, 1955, **216**, 185.
[14] T. J. Brownhill, A. S. Jones, and M. Stacey, *Biochem. J.*, 1959, **73**, 434.
[15] M. G. Sevag, *Biochem. Z.*, 1934, **273**, 419.
[16] K. S. Kirby, *Biochim. Biophys. Acta*, 1955, **18**, 575.

the product with ethanol (3 vols.) immediately after the decolourisation with sodium pyrosulphite, redissolve in water (500 ml.), and then continue as before. If the dialysis of the final solution was carried out for 13 days, the yield was only 45%. Base analyses of the products showed that cytosine, uracil, and guanine were absent and that the adenine content was 95—97% of that of the original ribonucleic acid (Found: C, 28·4; N, 13·1; P, 10·8. Calculated for structure (II), in which the adenine content was corrected for complete recovery of the bases in ribonucleic acid: C, 28·4; N, 12·9; P, 10·6%). ε_P (260 mμ), 3440.

Determination of Chain Length.—A solution of permanganate-oxidised ribonucleic acid (II) (15 mg.) in 8mM-ethylenediaminetetra-acetic acid (25 ml.) adjusted to pH 5·6 was treated with prostatic phosphomonoesterase (20 units) as described by Burton and Petersen [17] and the increase in inorganic phosphate measured after 24 hr. at 37° as described by Fiske and Subbarow,[18] except that after the addition of acid the resulting precipitate was centrifuged off and the colour developed in the supernatant liquid. After corrections for the presence of inorganic phosphate in the enzyme preparation had been made it was found that, from the original solution which contained 44·0 μg./ml. of "organic phosphorus," 4·51 μg./ml. of "inorganic phosphorus" was produced by the action of the phosphomonoesterase. Therefore, assuming that there was one phosphate end-group per molecule, the average length was 9·75 nucleotide units.

Acid Hydrolysis.—A solution of permanganate-oxidised ribonucleic acid (II) (10 mg.) in N-sulphuric acid (1 ml.) was heated at 37° for 24 hr. and a similar solution was heated at 100° for 1 hr. The solutions were neutralised with barium carbonate, centrifuged, and portions (0·1 ml.) of the supernatant liquids chromatographed on Whatman No. 1 paper in butan-1-ol–ethanol–water (4 : 1 : 5). Markers of urea (R_F, 0·27) and biuret (R_F, 0·37) were used, also urea and biuret which had been treated with sulphuric acid as described above. The chromatograms were sprayed with the Ehrlich reagent [19] (which could detect 2 μg. of urea and 10 μg. of biuret). The results showed that urea and biuret were not appreciably degraded by the treatment with acid and that in the hydrolysates of compound (II), urea was present but no detectable biuret. Thus, less than 5% of the oxidised residues of ribonucleic acid were present as biuret.

Treatment at pH 10.—Solutions of ribonucleic acid and of compound (II) (4 mg./ml.) were maintained at pH 10 for 19 hr. at 20° and at 37°. They were then neutralised and chromatographed on Whatman No. 3 paper in propan-2-ol–water–ammonia (*d* 0·88) (35 : 15 : 3). Both solutions treated at 20° gave one component and this remained at the origin showing that little breakdown had occurred. The solution of ribonucleic acid treated at 37° gave ten ultraviolet-absorbing components and the solution of compound (II), similarly treated, gave three ultraviolet-absorbing components, thus showing that hydrolysis had occurred in both cases.

The Action of Pancreatic Ribonuclease.—The crystalline pancreatic ribonuclease (Armour Pharmaceutical Co. Ltd.) was shown to be free from phosphodiesterase in that it did not hydrolyse bis-*p*-nitrophenyl phosphate. The action of the enzyme on ribonucleic acid and on compound (II) was determined by titration of the secondary phosphoryl groups which were liberated. The reactions were carried out at pH 7 in glass titration cells (50 ml.) and carbon dioxide-free nitrogen was bubbled through the solutions. A control experiment showed that a solution of 0·1M-sodium chloride did not significantly change in pH when the nitrogen was bubbled through for 3 days.

The substrate (10 mg.) and ribonuclease were dissolved in carbon dioxide-free 0·1M-sodium chloride (10 ml.), adjusted to pH 7 and 37°, and titrated at intervals to pH 7 with carbon dioxide-free 0·0015N-potassium hydroxide until the uptake of alkali ceased. At the end of the reactions, the solutions were analysed for phosphorus. Control solutions which contained the substrate but no enzyme did not change in pH. The results (Figure) show the effect of various concentrations of the ribonuclease and the effect of ethylenediaminetetra-acetic acid on the action of the enzyme on compound (II) is also shown. In addition the action of lysozyme on compound (II) was studied under similar conditions.

Identification of the Products.—A solution of compound (II) (2·5 g.) in water (400 ml.) was mixed with solution of ribonuclease (400 mg.) in 0·02M-acetate buffer (pH 6) (500 ml.), sterilised by filtration through a Seitz asbestos filter and incubated under sterile conditions at 37° for 6

[17] K. Burton and G. B. Petersen, *Biochem. J.*, 1960, **75**, 17.
[18] C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 1925, **66**, 375.
[19] H. J. Hubener, F. Bode, H. J. Mollatt, and M. Wehner, *Z. physiol. Chem.*, 1952, **290**, 136.

days. The solution was then evaporated *in vacuo* at 37° to a small volume and freeze-dried (yield 2·9 g.).

A sample of this solid was dissolved in water (25 ml., optical density of the solution at 260 mμ = 172), the pH adjusted to 9·5, and the solution added to a column (40 × 2 cm. diam.) of Dowex-1 (Cl^-) (2% cross-linked, 100—200 mesh). Elution was carried out with a concave gradient of sodium chloride (0 ⟶ 1M in 2·4 l.) in 0·01M-hydrochloric acid, and 25 ml. portions of the eluate were collected. The fractionation was monitored with a Canalco automatic u.v. column scanner. Five distinct fractions were obtained; the last part of the fifth fraction was eluted with M-sodium chloride in 0·1M-hydrochloric acid. The solutions containing each of the five fractions were pooled and the optical density at 260 mμ measured. The results were as follows:

Fraction	A	B	C	D	E
Position of elution (l. of eluate)	0·850—1·05	1·25—1·50	1·75—2·00	2·05—2·40	2·50—2·75
% of total optical density at 260 mμ	45	6·0	34	5·2	0·6

Fraction A was refractionated on Dowex-1 when two peaks were obtained, a trace component (2% of the fraction) and a major component (67% of the fraction). The major component had the same R_F value in propan-2-ol–water–ammonia (*d* 0·88) (35 : 15 : 3) and the same mobility on paper electrophoresis at pH 5 as adenosine 2′(3′)-phosphate. Paper chromatography of the component in saturated aqueous ammonium sulphate–0·01M-sodium acetate (pH 6·0)–propan-2-ol (79 : 19 : 2) [20] gave two spots corresponding with adenosine 2′-phosphate (19%) and adenosine 3′-phosphate (81%). The base content of this component was found to be 1·08 moles of adenine per g.-atom of phosphorus.

Fraction C was examined by paper chromatography in the propan-2-ol–ammonia–water solvent. It was homogeneous and had a lower R_F value (0·26) than had adenosine 2′(3′)-phosphate. Fractions B, D, and E were not investigated further.

A second portion of the ribonuclease hydrolysate of compound (II) was chromatographed in propan-2-ol–water–ammonia (*d* 0·88) (35 : 15 : 3). Seven urea-containing components (R_F 0·0, 0·08, 0·14, 0·21, 0·29, 0·47, and 0·58) were detected with the Ehrlich spray.[19] The slowest five fractions occurred on the chromatogram in regions which showed high absorption of ultraviolet light. The component of R_F 0·47 was not quite coincident with a u.v.-absorbing component (R_F 0·48) and the component of R_F 0·58 corresponded with urea.

The substances of R_F 0·47—0·48 (fraction A) were isolated from 610 mg. of the ribonuclease hydrolysate of compound (II) by preparative paper chromatography on Whatman No. 3 paper and shown to contain 0·46 mole of adenine per g.-atom of phosphorus. Rechromatography of fraction A in propan-2-ol–water–ammonia and in 1M-ammonium acetate–ethanol (3 : 7) showed that the component of R_F 0·47 decomposed readily to a compound with the same R_F values as those of adenosine 2′(3′)-phosphate. Fraction A contained a component with the same R_F value as adenosine 2′,3′-(cyclic phosphate) in the following solvent systems: (*a*) propane 2-ol–water–ammonia (*d* 0·88) (35 : 15 : 3); (*b*) butan-1-ol–ethanol–water (4 : 1 : 5); (*c*) saturated aqueous ammonium sulphate–0·01M-sodium acetate (pH 6)–propan-2-ol (79 : 19 : 2). The total ribonuclease hydrolysate of compound (II) was examined chromatographically in solvents (*a*) and (*c*), and in (*d*) t-butyl alcohol 0·02M-ammonium formate (pH 3·7) (11 : 9); (*e*) ammonium sulphate (40 g.) in 0·1M-sodium phosphate (pH 7·0) (100 ml.); (*f*) propan-1-ol–water–ammonia (*d* 0·88) (11 : 2 : 7). In all a component corresponding to adenosine 2′,3′-cyclic phosphate) was detected. Solvents (*c*) and (*e*) separated adenosine 2′-phosphate from its 3′-phosphate isomer. No adenosine 2′-phosphate was detected in the ribonuclease hydrolysate of compound (II), but in both cases a component corresponding to adenosine 3′-phosphate was present.

The ribonuclease hydrolysate was subjected to two-dimensional chromatography in solvents (*f*) and (*e*) and in solvents (*d*) and (*a*). In both systems, a component corresponding to adenosine 2′,3′-(cyclic phosphate) was detected. The amount of adenine present as adenosine 2′3′-(cyclic phosphate) was determined spectrophotometrically and found to be 12% of the total adenine of compound (II). It can be concluded, therefore, that fraction A was adenosine 2′,3′-(cyclic phosphate) mixed with a compound(s) which contained urea and phosphate residues and which from its chromatographic properties was probably a ribosylurea 2′,3′-(cyclic phosphate).

[20] R. Markham and J. D. Smith, *Biochem. J.*, 1951, **49**, 401.

The ribonuclease hydrolysate of compound (II) was treated with 0·01N-hydrochloric acid at 25° for 18 hr. and then chromatographed in solvent (*a*). The adenosine 2′,3′-(cyclic phosphate) (R_F 0·47) disappeared and there was an increase in the amount of adenosine 2′,(3′)-phosphate present.

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