was thinned with ethanol for filtration and provided 15.0 g. of product, which melted at 147–150° and gave a single spot, $R_f$ 0.90 in propanol-water (4:1). This material was suitable for synthetic use. For analysis, samples were recrystallized from ethyl acetate and hexane or from acetonitrile and benzene. Although the m.p. was sharp, the reproducibility was poor and samples were obtained with a 1–2 degree m.p. range either about 144° or with a similar range about 160°. Possibly two crystalline modifications were at hand, but this was not the only difficulty since successive m.p. determinations of a given sample varied. In any case constant chromatographic and analytical properties were found. *Anal.* Calcd. for $C_{25}H_{23}O_{12}N_6P$: C, 47.63; H, 4.00; N, 13.33. Found: C, 47.80, 47.69; H, 4.18, 3.98; N, 13.20, 13.40 (samples melting about 144° and 160°, respectively).

**5-Amino-4-Imidazolecarboxamide Ribotide** (VI).—2′,3′-Isopropylideneinosine-5′-di-(*p*-nitrophenyl) phosphate (8.0 g., dried at 100° *in vacuo* prior to use) was added to a suspension of sodium hydride (800 mg. of a 50% mineral oil form) in anhydrous dioxane (320 ml.). Complete solution took place after an hour or so. Chloromethyl methyl ether was distilled under nitrogen and 1.0 ml. was added to the reaction mixture which was left overnight protected from moisture. The dioxane was then removed under reduced pressure and the residue taken up in ethanol (250 ml.). Sodium hydroxide (50 ml. *N*) was added and the solution was refluxed for 1 hour. (Analysis at this point either by a quantitative Bratton–Marshall determination of diazotizable amine or by measuring the optical density ratio of 270/250 mμ of an acidified aliquot was advisable. Successful alkylation and ring opening usually was signalized by a ratio of about 1.3. (In the case of low values, proceeding with the preparation may be inadvisable.)

After removal of the ethanol under reduced pressure, the residue was taken up in water (200 ml.), brought to *p*H 5 with dilute hydrochloric acid, filtered through wet filter paper to remove mineral oil, and extracted repeatedly with ether to remove *p*-nitrophenol. For the enzymatic cleavage of remaining di-ester groups, the aqueous layer, adjusted to *p*H 7, was mixed with 0.1 *M* tris buffer, *p*H 8.8 (200 ml.), $MgCl_2 \cdot 6\,H_2O$ (1.8 g.) and *Crotalus adamanteus* venom (20 mg., Ross Allen's Reptile Institute, Silver Springs, Fla.) followed by incubation at 37° for 1.5 hours. The liberated nitrophenol was removed by ether extraction at *p*H 5 as before. The isopropylidene group was then hydrolyzed off by heating the solution for 1 hour at *p*H 2.7 in a boiling water-bath. The mixture was brought to *p*H 7 for chromatography.

Because of the high ionic content of the reaction mixture at this point due to the sequential operations without purification, demonstration of the presence of aminoimidazolecarboxamide ribotide by paper electrophoresis or chromatography on an ion-exchange column at first did not succeed. A conventional Norite adsorption and elution helped isolation of the nucleotide from inorganic materials prior to column chromatography. The Norite step could be avoided by application to an ion-exchange column in high dilution. For this purpose three-fifths of the reaction mixture was diluted to 1.5 liters and applied to a column of Dowex-1-bromide (200–400 mesh, 2.9 × 32 cm.). The column was washed with water until the eluate was transparent to ultraviolet light, then with 0.008 *N* HBr. The ribotide emerged soon after introduction of the acid. It was identified by its ultraviolet absorption spectrum and diazotizable amino-group; from the extinction coefficient,[3] ε 12,600, the yield in the neutralized, pooled eluate was calculated as 11–16% for a number of runs. It was concentrated under reduced pressure. The column purification serves to separate the imidazole ribotide from inosinic acid arising from unreacted starting material as well as from nucleosides arising from hydrolysis of the 5′-phosphate. The identity of the nucleotide was further confirmed by its serving as a substrate for the enzymatic synthesis of the N-succino-derivative on a preparative scale through the use of yeast adenylosuccinase.[8]

---

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, WASHINGTON UNIVERSITY, ST. LOUIS 30, MO.]

# The Base-catalyzed Alcoholysis of Ribonucleic Acids. A Method for the Determination of End Groups[1,2]

BY DAVID LIPKIN, J. S. DIXON AND P. T. TALBERT

RECEIVED JUNE 12, 1961

A technique is described for the degradation of ribonucleic acids by methoxide ion-catalyzed methanolysis in methanol-formamide mixtures as the solvent. The products of this reaction were separated, identified and quantitatively determined by means of a combination of anion exchange and paper chromatography, spectrophotometry, and chemical and enzymic degradations. The effects of varying reaction conditions on the course of the methanolysis are discussed. The methanolysis reaction was shown to be applicable to the identification and quantitative determination of 2′(3′)-nucleotide end-groups in two ribonucleic acid samples. Evidence was obtained, furthermore, which rules out the possibility of appreciable numbers of phosphate triester branch points in these samples. The relative amounts of isomeric of 2′- and 3′-nucleotides formed in the alkaline hydrolysis of ribonucleic acids, monomethyl esters of the nucleotides and nucleoside-2′:3′-phosphoric acids were determined. The relative amounts of the monomethyl esters of isomeric 2′- and 3′-nucleotides formed in the methanolysis of the two ribonucleic acid samples also are given.

The various methods which have been devised for the determination of end-groups in polyribonucleotides may be divided into two classes. In the first class are those which permit the quantitative determination, but not identification, of chain ends. The methods in the second class may be used both for the identification and quantitative determination of such ends. The alkalimetric titration of nucleic acids[3] and a study of their dye-binding properties[4] are two methods of the first type. Also, the pyrimidine ends in ribonucleic acids (RNA) have been estimated by comparing the hydroxide ion consumed during the course of an exhaustive ribonuclease (RNAase) digestion with the total number of singly esterified pyrimidine nucleotide phosphate groups present in a total digest.[5] In another procedure which falls in this first category, the polynucleotide is treated with prostate phosphomonoesterase. The amount of

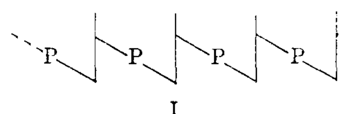
(1) Based on the Ph.D. dissertations of Jonathan S. Dixon, Washington University, 1953, and Preston T. Talbert, Washington University, 1955.

(2) This investigation was supported in part by the United States Atomic Energy Commission. Partial support also was provided by research grant C-3870 from the National Cancer Institute, Public Health Service, and by research grant NSF-G 12451 from the National Science Foundation.

(3) D. O. Jordan in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Academic Press, Inc., New York, N. Y., 1955, Vol. I, pp. 480–482.

(4) L. F. Cavalieri, S. E. Kerr and A. Angelos, *J. Am. Chem. Soc.*, **73**, 2567 (1951); L. F. Cavalieri, A. Angelos and M. E. Balis, *ibid.*, **73**, 4902 (1951).

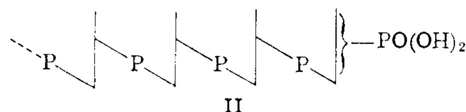
(5) E. Volkin and W. E. Cohn, *J. Biol. Chem.*, **205**, 767 (1953).



I

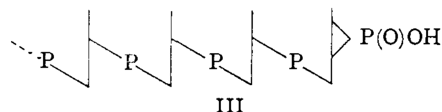
resulting nucleoside end-groups (type I) then is determined either by periodate titration or by alkaline hydrolysis of the sample followed by precipitation of the nucleosides.[6] The number of nucleoside ends also may be determined by oxidation of RNA with periodate followed by reaction of the resulting aldehyde groups with $^{35}$S-labeled thiosemicarbazide.[7] Prostate phosphomonoesterase has been used in still another way. The RNA from tobacco mosaic virus labeled with $^{32}$P was treated with the enzyme. The radioactive inorganic phosphate liberated by this treatment was separated by means of paper electrophoresis and its activity was determined relative to the total radioactivity in the RNA.[8]

Several of the methods in the second group also make use of a phosphomonoesterase as the initial step in the identification and determination of end-groups. The subsequent steps which have been used are varied. One procedure follows the enzymic removal of the monoesterified phosphoric acid with hydrolysis in alkali and paper chromatography of the resulting nucleosides.[9,10] In another approach, the product obtained by periodate oxidation of a prostate phosphomonoesterase-treated polynucleotide was subjected to hydrolysis at *p*H 10[11,12] or to the action of primary amines at *p*H 6–8.[13,14] Chromatography or electrophoresis then was used to identify and determine the heterocyclic base characteristic of the terminal group. This latter procedure involving periodate oxidation has been adapted also to the determination of nucleotide sequences. Chain ends of type II



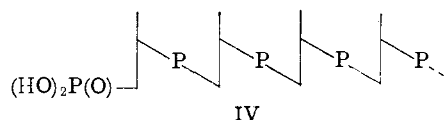

II

are characterized by the last-mentioned group of methods. It is worth noting, however, that the use of this method does not yield information concerning the position of the monoesterified phosphoric acid on the terminal nucleoside. Instead of the prostate enzyme, bone phosphomonoesterase together with alkaline hydrolysis and snake venom diesterase has been used to obtain the nucleotide sequence in a trinucleotide.[9] It also has been shown that RNAase digestion may be used to determine purine nucleotide end-groups which are linked to the chain through pyrimidine nucleotides. Using this technique, it has been demonstrated that such end-groups occur as both monoesterified 3′-phosphates (type II) and 2′:3′-cyclic phosphates[9b] (type III).




III

Alkaline hydrolysis, but without the use of enzymes, also has yielded a considerable amount of information concerning the terminal ends in polynucleotides. Using this technique,[15] in conjunction with paper electrophoresis and paper chromatography, it has been demonstrated that in polynucleotides from two plant viruses there are present type I ends as well as chain terminations of type IV.




IV

Type I ends in "soluble" RNA[13,16] and pyrimidine terminal groups present in the "limit" polynucleotides from an RNAase digest of RNA also were estimated by the use of simple alkaline hydrolysis. The purine nucleotides produced in the degradation of the "limit" polynucleotides were obtained as both 2′- and 3′-isomers, while only the 3′-isomers of the terminal pyrimidine nucleotides were obtained.[5] Alkaline hydrolysis has been applied in still another way. Hydroxide ion-catalyzed hydrolysis in $H_2{}^{18}O$, followed by a determination of the $^{18}$O content of the phosphate group in the resulting mononucleotides, has led to the identification and estimation of type II end-groups in a sample of yeast RNA.[17]

Another approach to the determination of phosphate end-groups (types II and IV) in nucleic acids involved the selective reaction of trialkylammonium salts of monoesters of orthophosphoric acid with methanol in the presence of dicyclohexylcarbodiimide. A combination of chemical and enzymic degradations then was used to permit identification and determination of the end-groups.[18]

Examples are reported in the literature of the alcoholysis of triesters of orthophosphoric acid.[19–22] On the other hand, it has been demonstrated that

(6) G. Schmidt, *et al.*, *J. Biol. Chem.*, **192**, 715 (1951).

(7) R. Dulbecco and J. D. Smith, *Biochem. Biophys. Acta*, **39**, 358 (1960).

(8) M. P. Gordon, B. Singer and H. Fraenkel-Conrat, *J. Biol. Chem.*, **235**, 1014 (1960).

(9) (a) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 558 (1952); (b) **52**, 565 (1952).

(10) R. B. Merrifield and D. W. Wooley, *J. Biol. Chem.*, **197**, 521 (1952).

(11) P. R. Whitfeld and R. Markham, *Nature*, **171**, 1151 (1953); P. R. Whitfeld, *Biochem. J.*, **58**, 390 (1954).

(12) D. M. Brown, M. Fried and A. R. Todd, *Chemistry & Industry*, 352 (1953); D. M. Brown, M. Fried and A. R. Todd, *J. Chem. Soc.*, 2206 (1955).

(13) C.-T. Yu and P. C. Zamecnik, *Biochem. Biophys. Acta*, **45**, 148 (1960).

(14) M. Ogur and J. D. Small, *J. Biol. Chem.*, **235**, PC 60 (1960); J. X. Khym and W. E. Cohn, *ibid.*, **236**, PC 9 (1961).

(15) R. Markham, R. E. F. Mathews and J. D. Smith, *Nature*, **173**, 537 (1954); K. K. Reddi and C. A. Knight, *ibid.*, **180**, 374 (1957); R. E. F. Mathews and J. D. Smith, *ibid.*, **180**, 375 (1957).

(16) L. I. Hecht, P. C. Zamecnik, M. L. Stephenson and J. F. Scott, *J. Biol. Chem.*, **233**, 954 (1958); D. B. Dunn, *Biochim. Biophys. Acta*, **34**, 286 (1959).

(17) D. Lipkin, P. T. Talbert and M. Cohn, *J. Am. Chem. Soc.*, **76**, 2871 (1954).

(18) H. G. Khorana, *ibid.*, **81**, 4657 (1959).

(19) A. Morel, *Compt. rend.*, **128**, 507 (1899).

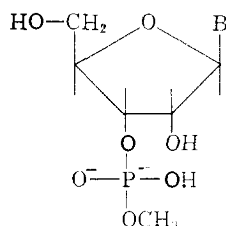
(20) (a) M. I. Smith, E. W. Engel and E. F. Stohlman, *Natl. Inst. Health Bull.*, **160**, 1 (1932); (b) M. I. Smith and E. F. Stohlman, *Weekly Public Health Reports*, **48**, 734 (1933); (c) M. I. Smith and E. F. Stohlman, *J. Pharmacol. Exper. Therap.*, **51**, 217 (1934).

(21) A. D. F. Toy, *J. Am. Chem. Soc.*, **66**, 499 (1944).

(22) W. H. C. Rueggeberg and J. Chernack, *ibid.*, **70**, 1802 (1948).

diesters or monoesters do not alcoholyze at an appreciable rate in alkaline solution.[20b] On the basis of these observations, it was believed that deoxyribonucleic acids (DNA) would not undergo base-catalyzed methanolysis; RNA, however, even though its principal elements of structure are diesterified orthophosphates, should be alcoholyzed rapidly in basic solution because of the cyclization mechanism by which the degradation in alkaline solution proceeds.[17,23–25] Preliminary reports have supported this conclusion.[25,26]

In basic media, phosphorus–oxygen rather than carbon–oxygen bonds are broken in the degradation of an orthophosphate ester.[17,19,20,22,27] It seemed apparent to us, therefore, that if methanol is used as the reaction medium and methoxide ion as the catalyst, for example, whenever a phosphorus-oxygen bond is broken a methoxyl group will become attached to phosphorus. Thus the principal products which will be obtained by methoxide ion catalyzed methanolysis of RNA containing 3′(2′),5′-internucleotide linkages will be monomethyl esters of the mononucleotides (B represents a purine-9 or pyrimidine-1 group), rather than the




ordinary mononucleotides as such. The monomethyl esters will be, of course, a mixture of 2′- and 3′-isomers. Monoester end-groups of type II, on the contrary, will be recovered as ordinary unmethylated mononucleotides, since ions such as $PO_4^{-3}$, $RPO_4^{-2}$ or $R_2PO_4^{-}$ are not esterified by alcohols. It should be emphasized further that these mononucleotide end-groups will be recovered with the phosphate ester group unisomerized.[28] In other words, if the RNA has a 3′-phosphate end-group the recovered mononucleotide representing this end-group will be a 3′-nucleotide, rather than a mixture of 2′- and 3′-isomers. Type IV 5′-monoester end-groups will be converted to monomethyl esters of 2′(and 3′),5′-diphosphorylnucleosides, while the nucleoside ends of such chains will be recovered as nucleosides, just as in hydrolysis.[13,15,16] End-groups bearing a 2′:3′-cyclic phosphate will not be detected by means of methanolysis, since they too will be converted to monomethyl esters of nucleotides.[29]

It is interesting to speculate on the fate of phosphate triester branch points which may be present in the RNA when the polymer is subjected to methanolysis. In the case of all possible types of triester branch points but one, the methanolysis products should be one mole of trimethyl orthophosphate and at least one mole of nucleoside. The improbable type of triester branch which consists of orthophosphate esterified at all three positions to the 5′-positions of nucleosides should yield on methanolysis one mole of trimethyl orthophosphate, but no nucleoside. The occurrence in a methanolysis digest, therefore, of larger amounts of nucleoside than in a hydrolyzate of the same RNA should be indicative of triester branch points in the polymer.[30]

Base- rather than acid-catalyzed alcoholysis was chosen for several reasons. First, acid might bring about splitting of the purine glycosidic bonds.[31] Second, the isomerization of a phosphate on the 2′- or 3′-position of a nucleoside is catalyzed by acids,[28,32] but not by bases.[28] Finally, in acid solution phosphate esters which are not fully esterified may become further esterified or lose ester groups. Methanol was the alcohol of choice for the degradation since it can be handled readily in a vacuum system and, even more important, it is the least sterically hindered of the alcohols. With methanol as the alcohol, the base of choice was obviously methoxide ion. Formamide, a non-aqueous solvent of high dielectric constant, was added to the reaction mixture in order to allow the alcoholysis to be carried out as a homogeneous, rather than heterogeneous, reaction.

Since the occurrence of ordinary mononucleotides in a methanolysis digest of an RNA is to be used as a measure of 3′(2′)-phosphate end-groups in the RNA, it is necessary to avoid even traces of moisture in the reaction mixture in order to prevent adventitious hydrolysis. This is achieved primarily by the use of standard high vacuum techniques, but an additional precaution which may be taken is the addition of an internal desiccant, such as aluminum methoxide or *t*-butoxide, to the reaction mixture before the methanolysis is carried out.

## Experimental

Materials.—Methanol was dried over activated alumina. It then was distilled on the vacuum line (see below) into a storage bulb containing freshly flamed activated alumina and was stored under vacuum. In recent experiments the methanol was kept over aluminum methoxide. *t*-Butyl alcohol was purified and dried by distillation from sodium metal. It then was stored in a bulb on the vacuum line over aluminum *t*-butoxide. Acetic acid was distilled and the distillate was stored under vacuum in a bulb containing freshly activated Drierite. Formamide was purified by distillation *in vacuo* from calcium oxide.[33]

Sodium metal was melted *in vacuo* and evacuated glass tubes, 2 mm. i.d., were filled with the molten metal. After the metal solidified, the glass tubes containing it were stored with their open ends under mineral oil.

The preparation of the charcoal (Darco G-60) used for adsorbing nucleosides and nucleotides from anion exchange column eluates has been described.[17] The two anion exchange resins used in these experiments, Dowex 1 × 2 and

(23) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).
(24) D. M. Brown and A. R. Todd, *ibid.*, 2040 (1953).
(25) D. Lipkin and P. T. Talbert, *Chemistry & Industry*, 143 (1955).
(26) D. Lipkin and J. S. Dixon, *Science*, **116**, 525 (1952).
(27) E. Blumenthal and I. B. M. Herbert, *Trans. Faraday Soc.*, **41**, 611 (1945); P. W. C. Barnard, *et al.*, *Chemistry & Industry*, 760 (1955); W. W. Butcher and F. H. Westheimer, *J. Am. Chem. Soc.*, **77**, 2420 (1955).
(28) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952).
(29) C. A. Dekker and H. G. Khorana, *J. Am. Chem. Soc.*, **76**, 3522 (1954).
(30) One obvious method of detecting triester branch points would be to combine the methanolysis of RNA using $^{14}CH_3OH$ with gas chromatography for determining any radioactive trimethyl orthophosphate which might be formed.
(31) D. G. Doherty, Abstracts Am. Chem. Soc., 118th Meeting, p. 56c, Chicago, Ill., Sept., 1950.
(32) W. E. Cohn, *J. Am. Chem. Soc.*, **72**, 2811 (1950).
(33) D. Lipkin and G. C. McElheny, *ibid.*, **72**, 2287 (1950).

1 × 10 formates (200–400 mesh), also were purified by a procedure which has been used previously.[17]

Crystalline bovine ribonuclease (Armour Laboratories, Chicago, Ill.) was used without further purification.

The sample of yeast RNA used in this work was the same one which had been used previously.[17] It was shown by dialysis to contain no free heterocyclic bases, nucleosides or mononucleotides. The other RNA which was studied was a sample of calf liver RNA prepared by the procedure of Volkin and Carter.[34,35] The composition of these RNA samples, which was obtained by alkaline hydrolysis (0.5 *N* NaOH, 37°, 17 hr.), followed by anion exchange chromatography on Dowex 1 formate,[17,36] is given in Table I.

TABLE I

THE GROSS COMPOSITION OF RIBONUCLEIC ACIDS AS DETERMINED BY HYDROLYSIS AND BY METHANOLYSIS

| Component | Yeast RNA Hydrolysis[a] | Yeast RNA Methanolysis[b] | Calf liver RNA Hydrolysis[a] | Calf liver RNA Methanolysis[b] |
|---|---|---|---|---|
| Nucleosides plus bases | 1.5%[c] | 2.1%[c] | 0.46%[c] | 0.87%[c] |
| Cytidine nucleotides | 19.4 | 20.4 | 29.0 | 31.7 |
| Adenosine nucleotides | 24.0 | 24.2 | 16.0 | 16.6 |
| Uridine nucleotides | 24.0 | 22.8 | 17.3 | 14.2 |
| Guanosine nucleotides | 31.0 | 30.8 | 37.3 | 36.7 |

[a] 0.5 *N* NaOH, 37°, 17 hr. [b] 1 *N* $CH_3ONa$, 65°, 70 min. [c] The mole per cent. of each component is calculated from the appropriate extinction coefficient, the total optical density units in the fractions corresponding to the given component and the total optical density units *eluted* in an anion exchange chromatogram of a digest of the RNA sample.

**Spectrophotometry.**—A Beckman model DU spectrophotometer was used for all measurements of ultraviolet absorption. The spectrophotometric constants of nucleic acid derivatives used throughout this work, except for the 280/260 mμ ratios of the 2′:3′-cyclic phosphates,[37] are those given by Volkin and Cohn.[38] The 280/260 mμ ratio of the guanosine cyclic phosphate in acid solution was found to be the same (0.68) as that of guanylic acid. The average molar extinction coefficient of the mixtures of nucleosides and heterocyclic bases encountered in this work was assumed to be 10,000.

**Paper Chromatography.**—Whatman No. 1 filter paper was used for ascending paper chromatography. The solvent systems used, which all were made up on a volume basis, were: saturated aqueous ammonium sulfate–isopropyl alcohol–water, 79:2:19 (solvent I)[39]; isopropyl alcohol–water, 70:30, with an ammonia atmosphere in the developing tank (solvent II)[40]; isoamyl alcohol layered on 5% aqueous disodium hydrogen phosphate (solvent III)[41]; *n*-butyl alcohol–acetic acid–water, 80:20:20 (solvent IV)[42]; and acetone–*n*-butyl alcohol–water, 80:10:10 (solvent V).[42]

Ultraviolet-absorbing materials on the paper chromatograms were located by visual examination in ultraviolet light (254 mμ). Paper chromatography was used as an aid in identification either by comparing the mobility of a substance with that of an authentic sample or by comparing the $R_f$ of a substance with $R_f$ values given in the literature[43] if an authentic sample was not available. A sodium metaperiodate spray was used to detect compounds with vicinal glycol groups on the paper chromatograms.[44] The presence of phosphorus in spots on the chromatograms was demonstrated by the use of a molybdate spray.[45]

Quantitative determinations of the components of mixtures which were separated by paper chromatography were made by eluting individually the various bands obtained in chromatography and then spectrophotometrically assaying the separate eluates at 260 mμ. The percentage composition of mixtures determined in this manner represents the number of optical density units corresponding to a given substance divided by the total optical density units of all of the bands on the paper.

**Methanolysis Procedure.**—In carrying out the degradation of nucleic acids by means of methanolysis, a high vacuum system (ultimate vacuum $< 10^{-5}$ mm.) and standard high vacuum techniques were used.

The reaction vessel consisted of a glass tube 12–14 mm. o.d. and approximately 17 cm. long which was sealed off at one end. It was joined at the other end through a heavy-walled constriction to a standard taper ground glass joint. Just below the constriction there was attached a side arm 12–14 mm. o.d. The side arm was slanted somewhat from the horizontal and then was bent parallel to the upper part of the main tube. The side arm led to a break-off seal and from there to another standard taper ground glass joint. The reaction vessel was calibrated roughly for volume contained. The adapter by means of which the reaction vessel was attached to the vacuum line consisted essentially of a straight tube 22 mm. o.d. and *ca.* 15 cm. long. At the upper and lower ends were attached standard taper ground glass joints. About midway on the body of the tube, two other standard taper ground glass joints were sealed on in order to accommodate two dumpers. These two ground joints were sealed on at an angle of *ca.* 45° to the body of the adapter and 180° apart. Below these latter joints a tube 8–9 mm. o.d. was ring-sealed coaxially into the main body of the adapter in order to allow solutions in the dumpers to fall clear of the bottom ground joint on the adapter into the reaction vessel.

The reaction mixture was prepared as follows: A 200-mg. sample of nucleic acid was placed in the bottom of the reaction tube together with a small, glass-enclosed iron slug. The tube was attached to the vacuum line and the sample was pumped on for 24 hr. During this operation the side arms on the adapter were closed by means of glass stoppers. After the sample was dry, as evidenced by a drop in pressure in the system, the adapter and reaction vessel were raised to atmospheric pressure by admitting dry air to the system. In one of the dumpers, which contained freshly flamed activated alumina, was placed 5 ml. of formamide. This dumper was inserted in one of the side arms on the adapter. The other dumper, into which was introduced a total of approximately 7 cm. of capillary tubing containing sodium metal (0.22 g., 0.0096 mole), was inserted in the other arm of the adapter. The system was evacuated and then 4 ml. of the formamide was transferred into the reaction vessel by a partial rotation of the dumper containing it. Methanol from a storage bulb on the vacuum line next was distilled into the dumper containing the sodium capillary. After the sodium metal dissolved, the solution of sodium methoxide also was poured into the reaction tube. The contents of the tube was frozen and the system was evacuated. Methanol was distilled into, or out of, the reaction vessel until the total volume of the reaction mixture at room temperature was 8 ml. This resulted in a solution 1 *N* in methoxide ion. For those experiments in which the methoxide ion concentration was other than 1 *N*, an appropriate length of capillary containing sodium metal was used. The reaction tube was sealed off at its heavy-walled constriction with its contents in a frozen state.

The reaction mixture was melted and, in most of our experiments, it was heated at 65° for 70 min. During this interval it was shaken occasionally. At the end of the heating period, a few drops of a concentrated solution of

(34) E. Volkin and C. E. Carter, *J. Am. Chem. Soc.*, **73**, 1516 (1951).

(35) We wish to thank Dr. Waldo E. Cohn, Oak Ridge National Laboratories, for making available to us a generous sample of this RNA.

(36) W. E. Cohn, *J. Am. Chem. Soc.*, **72**, 1471 (1950).

(37) D. M. Brown, C. A. Dekker and A. R. Todd, *J. Chem. Soc.*, 2715 (1952).

(38) E. Volkin and W. E. Cohn in D. Glick, "Methods of Biochemical Analysis," Interscience Publishers, Inc., New York, N. Y., 1954, Vol. I, p. 287.

(39) J. D. Smith and R. Markham, *Biochem. J.*, **49**, 401 (1951).

(40) R. Markham and J. D. Smith, *ibid.*, **52**, 552 (1952).

(41) C. E. Carter, *J. Am. Chem. Soc.*, **72**, 1466 (1950).

(42) G. D. Dorough and D. L. Seaton, *ibid.*, **76**, 2873 (1954).

(43) R. J. Block, E. L. Durrum and G. Zweig, "Paper Chromatography and Paper Electrophoresis," Academic Press, Inc., New York, N. Y., 1958, p. 181.

(44) J. S. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, 3162 (1950).

(45) C. A. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

dibromothymolsulfonphthalein in methanol was placed in the side arm of the reaction vessel. A glass-enclosed iron slug was carefully introduced on top of the break-off seal and the tube was attached to the vacuum system by means of the ground joint on the side arm. After freezing the reaction mixture once again, the break-off seal was shattered and the system was degassed. Sufficient glacial acetic acid then was distilled into the reaction tube from a storage bulb to neutralize the digest. Following the neutralization of the methoxide, the system was evacuated once again to remove readily volatile materials from the methanolysis mixture. The sample, 3–4 ml. in volume, now was ready for anion exchange chromatography.

The degradation of RNA by means of *t*-butoxide ion was carried out by a modification of the above procedure. Instead of preparing the alkoxide solution on the vacuum line, a 1 *N* solution of potassium *t*-butoxide in *t*-butyl alcohol was prepared in a flask and an aliquot of this was pipetted into one of the dumpers.

When aluminum methoxide or *t*-butoxide was used as an internal desiccant, the compound was added to the dumper containing the sodium metal. After the digestion was completed and the sample was neutralized with acetic acid and evaporated *in vacuo*, water was added. The aluminum ions were removed from this solution by adding dilute sodium hydroxide to *p*H 11–12 and then bubbling in carbon dioxide until the *p*H fell to 6.5. The dense aluminum hydroxide which formed was removed by centrifugation and it was redissolved in acid. The precipitation of aluminum hydroxide was repeated. These operations for the removal of aluminum ions were all carried out at 0°. The resulting two supernatant solutions, which contained 99% of the ultraviolet-absorbing material in the digest, was subjected to anion exchange chromatography.

**Anion Exchange Chromatography of Alcoholysis Reaction Mixtures.**—Chromatography was carried out in columns of 4.5 sq. cm. cross section which contained a 15 cm. bed of anion exchange resin. Dowex 1 × 2 formate gave better separations, in general, than the 1 × 10 resin. Formate columns were preferable to chloride columns, since with the latter hydrolysis of nucleotide esters took place during elution. The flow rate through the columns was approximately 3 ml. per min. An automatic fraction collector was used to collect fractions of either 52 or 65 ml. in volume. The elution was followed by determining the optical density of each fraction at both 260 and 280 mμ. Fractions corresponding to a given chromatographic peak were combined and stored at −20° until needed for further experiments.

The sample to be chromatographed was diluted to 100 ml. with water. An aliquot was brought to *p*H 2 and it was assayed by measuring the optical density at 260 mμ. The remainder of the sample was adjusted to *p*H 9–10 by adding concentrated ammonium hydroxide and it then was adsorbed on the anion exchange column. The sample was followed on the column by approximately 400 ml. of water, after which elution was carried out with formic acid-sodium formate buffer solutions. Typical chromatograms of yeast RNA and calf liver RNA methanolysis digests are reproduced in Figs. 1 and 2, respectively. It was demonstrated (see below) that each of the peaks in the chromatograms does not necessarily correspond to a single substance.

The total recovery of 260 mμ-absorbing material in a chromatogram, based on the 260 mμ absorption of the solution charged to the column, was 85–90%. These recoveries are in the same range as those obtained in the hydrolysis of the RNA samples (Table I).[34] An experiment in which the methanolysis was carried out at 65° for 4 hr. instead of 70 min. gave essentially the same recovery. This seemingly non-quantitative recovery undoubtedly is not due to handling losses. It can be explained instead by one or more of the following factors. First, the presence in RNA digests of ultraviolet-absorbing substances, which may not even be purine- or pyrimidine-containing compounds, that are not eluted from the chromatography columns. Second, the solution of a digest which is to be adsorbed on a column is assayed at *p*H 2, while the effluent fractions from the column are assayed at the *p*H's corresponding to the various eluting solutions which are used. Finally, in calculating recoveries, differences in molecular weight or extinction coefficient of the various components are not taken into account. It is worth noting, further, that throughout this paper percentage compositions determined by means of anion exchange chromatography are based on the total optical density units (O.D. × volume) ***eluted*** from the column.

In one experiment, a sample of the yeast RNA was subjected to methanolysis with 1 *N* methoxide except that the anhydrous formamide was omitted. Even after 8 hr. at 65°, the sample was not completely dissolved. On anion exchange chromatography only 32% of the optical density units put on the column was recovered in the column effluent. The composition of the recovered material, based on total material in the effluent as 100 mole %, is: nucleosides and bases, 5.0; cytidine nucleotides, 16.2; adenosine nucleotides, 24.5; uridine nucleotides, 15.5; and guanosine nucleotides, 38.9 mole %. The corresponding data for the same sample on complete digestion under standard conditions (86% recovery) is given in Table I, column 2.

A sample of calf thymus DNA was subjected to methanolysis under standard conditions, except that the reaction mixture was heated for 8 days. On chromatography only 15% of the sample was recovered under elution conditions which lead to the removal of mononucleotides from the column.

Digests of RNA obtained by *t*-butanolysis were treated in the same way as methanolysis digests and then were adsorbed on an anion exchange column. The following eluting solutions were used successively to displace the stated materials from the column: nucleosides and bases, 0.3 l. of water; cytidine nucleotides, 5.7 l. of 0.01 *N* formic acid; adenosine nucleotides, 3.4 l. of 0.070 *N* formic acid 0.007 *N* in sodium formate; U-2′:3′-P,[46] 1.5 l. of 0.0017 *N* formic acid 0.030 *N* in sodium formate; uridine nucleotides, 1.0 l. of 0.0030 *N* formic acid 0.050 *N* in sodium formate plus 1.0 l. of 0.0045 *N* formic acid 0.103 *N* in sodium formate; G-2′-P, 2.3 l. of the last-mentioned eluting solution; and, finally, G-3′-P and G-2′:3′-P, 2.0 l. of 0.0045 *N* formic acid 0.176 *N* in sodium formate. Chromatography of these *t*-butanolysis digests was carried out in a cold room kept at 3° in order to prevent hydrolysis of the cyclic phosphates. As a further precaution, when 0.01 *N* formic acid or 0.070 *N* formic acid–0.007 *N* sodium formate was used as the eluting agent, the samples of column effluent were run directly into a saturated sodium formate solution (5 ml.) in order to bring the *p*H of the medium above 4.5.

Substances corresponding to the various peaks in the anion exchange chromatograms were recovered for further experiments from appropriate portions of the column effluent as follows: The solution at 0° was treated with charcoal (0.4–1.6 g.) until the O.D. of the supernatant at 260 mμ was less than 0.015. The mixture was centrifuged and the supernatant was discarded. The charcoal was washed once with water and it was again separated by centrifugation. This supernatant seldom showed any trace of 260 mμ-absorbing material. The washed charcoal was next shaken with 30 ml. of a 10% (v./v.) aqueous pyridine solution and the mixture was allowed to stand at 3° for 20 hr. After this time the mixture was centrifuged. The supernatant was poured off and stored at 3° while the treatment of the charcoal with aqueous pyridine was repeated. The two aqueous pyridine supernatant solutions obtained in this way were combined and evaporated *in vacuo* at 5–10° to a volume of 20 ml. In some cases solid materials were recovered by lyophilization of this solution at −40 to −20°. The recovery of ultraviolet-absorbing material from the column eluates by this procedure was 85–90%.

**Paper Chromatography of Fractions from Anion Exchange Chromatography.**—The material from peaks 1 and 2 of the yeast RNA (Fig. 1) was identified by means of paper chromatography in solvent I and also in solvents IV and V used successively in the same direction.[42] It consisted of: adenosine, 0.3; cytidine, 0.9; guanosine, 0.2; uridine, 0.5; adenine, 0.1; and guanine, 0.1 mole%. The corresponding peaks from hydrolyzates had the same composition except that only 0.2 mole % adenosine and 0.3 mole % uridine were present. The identity of the nucleosides was confirmed by the fact that they gave a positive periodate spray re-

(46) The following abbreviations are used: uridine-2′-phosphoric acid, U-2′-P; uridine-3′-phosphoric acid, U-3′-P; uridine-2′:3′-phosphoric acid, U-2′:3′-P. The corresponding nucleotides derived from adenosine, cytidine and guanosine will be represented by analogous symbols but with A, C or G, respectively, substituted for the U preceding the numbers.

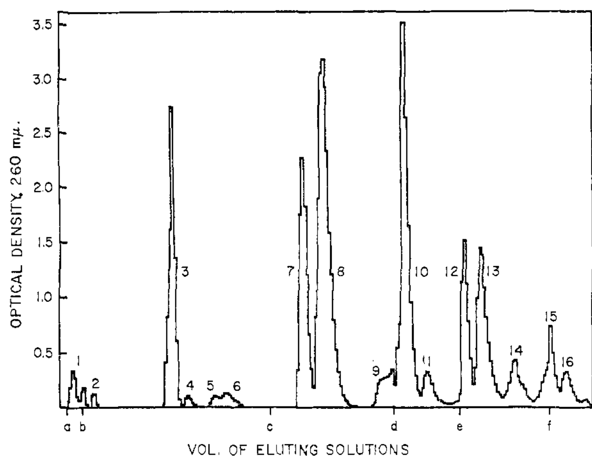



Fig. 1.—Column chromatogram (Dowex 1 × 2 formate) of methanolysis digest (1 $N$ $CH_3O^-$, 65°, 70 min.) of yeast RNA. Eluting solutions: a, 0.4 l. of $H_2O$; b, 2.5 l. of 0.01 $N$ HCOOH; c, 2.9 l. of 0.070 $N$ HCOOH 0.007 $N$ in HCOONa; d, 1.5 l. of 0.0017 N HCOOH 0.030N in HCO·ONA; e, 3.4 l. of 0.0042 $N$ HCOOH 0.075 $N$ in HCOONa; f, 1.7 l. of 0.10 $N$ HCOOH 0.10 $N$ in HCOONa.

action.[44,47] It was demonstrated by means of dialysis and chromatography that neither RNA sample was contaminated with any of these low molecular weight compounds or mononucleotides.

Paper chromatography in solvents I, II and III was used to obtain additional information concerning the composition and identity of the other peaks in anion exchange chromatograms of both methanolysis and $t$-butanolysis reaction mixtures. $R_f$ values of the monomethyl esters of nucleotides and 2′:3′-cyclic phosphates encountered in this work are summarized in Table II. These values are in agreement with data previously reported.[24,48-51] Chromatography in solvent II was particularly useful for quantitatively determining the composition of those peaks which were a mixture of nucleotide and the corresponding cyclic phosphate and monomethyl esters derived from the nucleotide.

TABLE II

PAPER CHROMATOGRAPHY OF 2′:3′-CYCLIC PHOSPHATES AND METHYLPHOSPHATES OF NUCLEOSIDES

| Compound[46] | $R_f$, solv. I | $R_f$, solv. II | $R_f$, solv. III |
|---|---|---|---|
| C-2′-P methyl ester | 0.63 | 0.45 | .. |
| C-3′-P methyl ester | .56 | .45 | .. |
| C-2′:3′-P | .48 | .38 | 0.78 |
| A-2′-P methyl ester | .25 | .44 | .. |
| A-3′-P methyl ester | .16 | .43 | .. |
| A-2′:3′-P | .12 | .41 | 0.52 |
| U-2′-P methyl ester | .57 | .40 | .. |
| U-3′-P methyl ester | .57 | .40 | .. |
| U-2′:3′-P | .51 | .34 | 0.76 |
| G-2′-P methyl ester | .43 | .26 | .. |
| G-3′-P methyl ester | .30 | .21 | .. |
| G-2′:3′-P | .28 | .18 | 0.67 |

**Further Identification of Alcoholysis Products.**—Additional evidence that some of the peaks in the anion exchange chromatograms are phosphate diesters of nucleosides, either cyclic phosphate or monomethyl esters, was obtained by

(47) No other periodate-oxidizable products were obtained in the methanolysis of the RNA samples.

(48) D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, 2708 (1952).

(49) D. M. Brown, D. I. Magrath and A. R. Todd, *ibid.*, 4396 (1955).

(50) L. A. Heppel and P. R. Whitfeld, *Biochem. J.*, **60**, 1 (1955).

(51) G. M. Tener and H. G. Khorana, *J. Am. Chem. Soc.*, **77**, 5349 (1955).

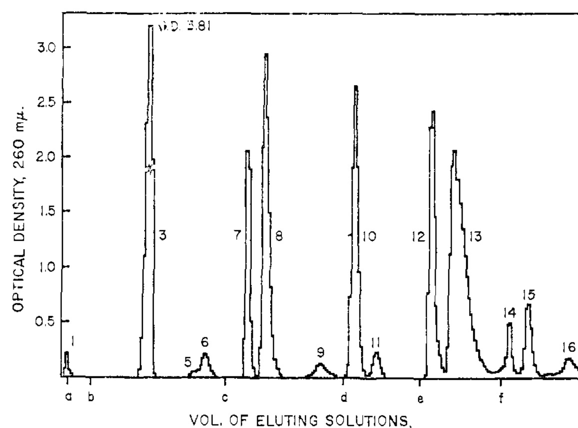



Fig. 2.—Column chromatogram (Dowex 1 × 2 formate) of methanolysis digest (1 $N$ $CH_3O^-$, 65°, 70 min.) of calf liver RNA. Eluting solutions: a, 0.4 l.; b, 2.8 l.; c, 3.9 l.; d, 2.3 l.; e, 2.4 l.; f, 2.9 l. The composition of these solutions is given in the legend of Fig. 1.

the use of alkaline hydrolysis. Material corresponding to peak 3, Fig. 1, was analyzed both before and after hydrolysis (0.5 $N$ NaOH, 30°, 20 hr.). This demonstrated that the material in peak 3 was converted to a mixture of C-2′-P and C-3′-P. In a like manner it was shown that peaks 7–16, inclusive, in addition to peak 3, correspond to phosphate diesters.[24,28,48]

Since it was suspected that the material in peak 3, Fig. 1, was a mixture of monomethyl esters of both C-2′-P and C-3′-P, attempts were made to separate it by means of paper chromatography, cellulose column chromatography and anion exchange chromatography. Because of the fact that these separations were unsuccessful, use was made of the specificity of RNAase activity[24] to determine its composition.[49,52] A 5-mg. sample of the material from peak 3, together with 0.4 mg. of RNAase, was made up in water to a volume of 1 ml. The $p$H of the solution was adjusted to $p$H 8–8.5 with ammonium hydroxide. A blank was made up in the same way except that the RNAase was omitted. Both solutions were incubated at 37° for 12 hr. Samples from both the digest and the blank were subjected to paper chromatography in solvent II. The blank gave only one ultraviolet-absorbing spot whose $R_f$ (0.45) was the same as that of the starting material. The RNAase digest showed two ultraviolet-absorbing spots. One, methyl cytidylate, had the same $R_f$ (0.45) as the starting material. The other had an $R_f$ of 0.10, corresponding to that of an ordinary nucleotide. Anion exchange chromatography of the RNAase digest gave two peaks. One of these corresponded in position to a methyl ester of cytidylic acid (43%) and the other to C-3′-P (57%). These data demonstrate that the material in peak 3 is a mixture of the monomethyl esters of C-2′-P and C-3′-P in a ratio of 43:57. A similar experiment with the material corresponding to peak 10 showed that it was a mixture of monomethyl esters of U-2′-P and U-3′-P in a ratio of 38:62.

Peaks in the original anion exchange chromatograms (methanolysis and $t$-butanolysis) which were suspected of being C-2′:3′-P or U-2′:3′-P were subjected to degradation by means of RNAase.[37] The products, which were examined by means of both paper and anion exchange chromatography, were the corresponding 3′-nucleotides only. As expected, the 280/260 mμ ratio changed from 1.59 to 2.04 in the C-2′:3′-P case and from 0.20 to 0.33 in the U-2′:3′-P experiment.

The digests obtained in the above experiments were analyzed by means of chromatography with a micro-column (0.07 cm.$^2$ × 15 cm.) containing Dowex 1 × 10 formate. The eluting solutions used were: cytidine nucleotides, 0.01 $N$ formic acid; adenosine nucleotides, 0.070 $N$ formic acid 0.007 $N$ in sodium formate; uridine nucleotides, 0.003 $N$ formic acid 0.050 $N$ in sodium formate; and guanosine nucleotides, 0.0042 $N$ formic acid 0.176 $N$ in sodium formate.

(52) D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, 1442 (1954).

**Methanolysis of Nucleoside-3′-phosphoric Acids.**—A sample of A-3′-P (280/260 m$\mu$ at $p$H 2, 0.21) was subjected to the usual procedure using 1 $N$ methoxide. The reaction product was analyzed by anion exchange chromatography using the micro-column and eluting solutions described in the preceding section. Only one peak appeared in the chromatogram, corresponding in position to unchanged A-3′-P (280/260 m$\mu$ at $p$H 2, 0.21). Methanolyses and chromatographic analyses were carried out also with C-3′-P, U-3′-P and G-3′-P. In all three cases a single peak was observed in each chromatogram in the same position as the starting material. Furthermore, the 280/260 m$\mu$ ratio in each case was the same for the starting material and the peak in the chromatogram of the methanolysis mixture.

**Methanolysis of Dimethyl Adenosine-2′-phosphate.**—A sample of A-2′-P (175 mg.) was dissolved in a mixture of dimethylformamide (125 ml.) and methanol (50 ml.). An ether solution of diazomethane was added to the A-2′-P solution[24,49,50] and the mixture was shaken at frequent intervals over a period of several hours. At the end of this time it was concentrated *in vacuo* to a small volume. Paper chromatography of a sample in solvent II gave two ultraviolet-absorbing spots ($R_f$'s 0.5 and 0.85), neither of which was oxidizable by periodate. Anion exchange chromatography (Dowex 1 × 10 formate) of another small portion of the concentrated solution indicated that one-third of the reaction product was probably dimethyl adenylate, since this percentage of the total material put on the column could be eluted with water. Further confirmation of this supposition was obtained by hydrolyzing another small portion of the reaction product (0.5 $N$ NaOH, 37°, 17 hr.). Anion exchange chromatography showed that only 3% of the ultraviolet-absorbing material in the hydrolyzate could be eluted with water, indicating that 30% of the original preparation was the dimethyl ester of adenylic acid.

The concentrate containing the reaction product was evaporated to dryness and powdered under dry acetone. The powder was further dried on the high vacuum line for several days and it then was subjected to methanolysis under the usual conditions. Chromatography on a Dowex 1 × 2 formate column revealed that 26% of the product was eluted from the column with water. The material in the water eluate was recovered by the charcoal procedure and the pyridine–water eluate was recovered by the charcoal procedure to a small volume. After alkaline hydrolysis (0.5 $N$ NaOH, 37°, 17 hr.) of a portion of this solution, it could not be adsorbed on an anion exchange column. This indicated that a dephosphorylation occurred during the methanolysis. Paper chromatography of other samples in solvents I and II showed that adenosine was the product of the methanolysis of the dimethyl ester. The ultraviolet-absorbing spots were oxidizable by periodate.

## Discussion

The methoxide ion-catalyzed methanolysis of RNA in formamide–methanol mixtures as the solvent leads to a complex mixture of products. The reaction mixture was first separated by anion exchange chromatography. The fractions obtained by this procedure were then examined further. Table III summarizes the information obtained on the composition of the methanolysis digests of both samples of RNA.

Identification of each of the ultraviolet-absorbing components was based on one or more of the following criteria. Firstly, the position of a peak in an anion exchange chromatogram. Relative positions of peaks were compared,[24,37,52] but even more important was the comparison of peak positions in a methanolysis chromatogram with peak positions in chromatograms of RNA samples which were degraded either by alkaline hydrolysis or by $t$-butanolysis. Secondly, ultraviolet absorption spectra at $p$H's of 2, 7 and 12, as well as 280/260 m$\mu$ ratios. Thirdly, paper chromatographic behavior with one or more solvent systems. Periodate and phosphate spray tests were used in conjunction with the paper chromatography. Fourthly, alkaline degradation followed by identification of the products. Finally, the degradation of phosphate diesters of pyrimidine nucleosides, either cyclic phosphate or monomethyl esters, by means of RNAase. It is worth noting that the only basis for the specific assignment of 2′ and 3′ designations to the methyl esters of the adenylic and guanylic acids (peaks 7, 8, 12 and 13, Figs. 1 and 2) is by analogy with the relative positions of A-2′-P and A-3′-P, and G-2′-P and G-3′-P, in anion exchange chromatograms.

Table III

Composition of Peaks in Anion Exchange Chromatograms of Methanolysis Digests of Ribonucleic Acids

| Peak No. | Yeast RNA[46] | Calf liver RNA[46] |
|---|---|---|
| 1 | Nucleosides | Nucleosides and bases |
| 2 | Nucleosides and bases | Nucleosides and bases |
| 3 | Methyl esters of:<br>C-2′-P, 43%<br>C-3′-P, 57% | Methyl esters of:<br>C-2′-P, 43%<br>C-3′-P, 57% |
| 4 | C-2′-P | .......... |
| 5 | C-3′-P | C-3′-P |
| 6 | C-2′:3′-P | C-2′:3′-P |
| 7 | Methyl ester of A-2′-P | Methyl ester of A-2′-P |
| 8 | A-2′-P, 9%<br>Methyl ester of A-3′-P, 91% | Methyl ester of A-3′-P |
| 9 | A-3′-P, 70%<br>A-2′:3′-P, 30% | A-3′-P, 33%<br>A-2′:3′-P, 67% |
| 10 | Methyl esters of:<br>U-2′-P, 38%<br>U-3′-P, 62% | Methyl esters of:<br>U-2′-P, 38%<br>U-3′-P, 62% |
| 11 | U-2′:3′-P | U-2′:3′-P |
| 12 | U-2′-P, U-3′-P } 20%<br>Methyl ester of G-2′-P, 80% | U-3′-P, 5%<br>Methyl ester of G-2′-P, 95% |
| 13 | Methyl ester of G-3′-P | Methyl ester of G-3′-P |
| 14 | G-2′-P, 55%<br>G-2′:3′-P, 45% | G-2′:3′-P |
| 15 | G-2′:3′-P | G-2′:3′-P |
| 16 | G-3′-P, 70%<br>G-2′:3′-P, 30% | G-3′-P, 50%<br>G-2′:3′-P, 50% |

Combining the data in Table III with the data obtained by anion exchange chromatography of the appropriate methanolysis reaction mixtures, the percentages of the nucleotide end-groups in the two RNA samples were calculated (Table IV). The reciprocal of the sum of these percentages for each RNA sample, multiplied by 100, gives the

Table IV

Type II End-groups in Ribonucleic Acids

| End-group[46] | Mole %[a] from Yeast RNA | Mole %[a] from Calf liver RNA |
|---|---|---|
| C-2′-P | 0.76 | .. |
| C-3′-P | 0.88 | 0.61[a] |
| A-2′-P | 1.42 | .. |
| A-3′-P | 1.33 | 0.31 |
| U-2′-P | 1.23 | .. |
| U-3′-P | 2.25 | 0.68 |
| G-2′-P | 1.47 | .. |
| G-3′-P | 2.14 | 0.60 |

[a] See footnote $c$, Table I.

number of nucleotide units per total number of type II end-groups in the sample. The average number of nucleotides per end-group in the yeast RNA sample was found to be 8.7. This value is in reasonable agreement with the intrinsically less accurate value of 6.6 nucleotides per end-group found[17,53] for the same sample by means of hydroxide ion-catalyzed hydrolysis in $H_2{}^{18}O$. The calf liver RNA was found to have an average of 45 nucleotides per end-group of Type II. In connection with these calculations of nucleotides per end-group there are several points worthy of emphasis. Firstly, the nucleosides and bases found in the RNA digests are not considered as originating from end-groups; evidence is presented later that these are artifacts in large part. Secondly, no correction is made in these calculations for the fact that recovery of ultraviolet-absorbing material from either the anion exchange columns or the charcoal used to recover material from column effluents is not quite quantitative (see Experimental section). Thirdly, as shown by means of appropriate blank experiments, no conversion of type II end-groups to methyl esters takes place under the methanolysis conditions which are used. There is, therefore, no loss of end-groups by this path. Finally, the calculations do not take into account the possibility of 2′:3′-cyclic phosphate end-groups in either RNA sample.

It is not surprising to find that the yeast RNA, which was a purified commercial sample, has fewer nucleotides per end-group than the calf liver RNA. It should be noted, furthermore, that the nucleotide end-groups in the yeast RNA are present as both 2′- and 3′-isomers, while these end-groups are present as only the 3′-isomers in the calf liver RNA. The fact that only 3′- ends were found in the calf liver RNA constitutes evidence that adventitious hydrolysis did not take place during the methanolysis of the RNA.

A comparison of the composition of the methanolysis reaction products, in terms of phosphorylated derivatives of the four heterocyclic bases and the amount of free nucleosides and bases, as degradation conditions are varied is of interest. Increasing the reaction time from 70 min. to 240 min. in the methanolysis of yeast RNA with 1.0 *N* methoxide ion caused an increase of *ca.* 1.5 mole % in the amount of phosphorylated uridines and a corresponding decrease in the amount of phosphorylated cytidines.[54] At the same time the amount of free nucleosides plus bases is increased from 2.1 to 3.1 mole %. Similar variations in the composition of the reaction products are observed in comparing hydroxide ion-catalyzed hydrolysis with 1 *N* methoxide ion-catalyzed methanolysis, under standard conditions, for each of the two RNA samples (see, for example, Table I). Deamination of phosphorylated cytidines to uridines in the hydrolysis of both RNA samples is apparent when such a comparison is made. Furthermore, the amount of nucleosides plus bases is less in the hydrolyzates than in the methanolysis digests. On the other hand, the amount of free nucleosides and bases is found to be the same when one compares hydrolysis (0.5 *N* NaOH, 37°, 17 hr.) and methanolysis with 0.25 *N* methoxide ion (65°, 70 min.) That this agreement is fortuitous, however, is indicated by the results obtained in a study of the composition of the reaction products as the methoxide ion concentration was varied approximately tenfold. The yeast RNA was subjected to methanolyses in which the methoxide ion concentration was varied from 0.25–2 *N*, while the calf liver RNA was subjected to methanolyses in which the methoxide ion concentration was varied from 0.1–1 *N*. In neither case was there a variation in the amounts of phosphorylated uridines and cytidines with increasing methoxide ion concentration. On the contrary, the free nucleosides plus bases increased from 1.6 to 2.9 mole % in the case of the yeast RNA and from 0.26 to 0.87 mole % in the case of the calf liver RNA. It is apparent from these latter data on the calf liver RNA and the data in column 3, Table I, that sufficiently mild conditions of methanolysis yield a smaller amount of nucleosides and bases than does hydrolysis.

Although data obtained by various authors has been interpreted as indicating the presence in RNA of one triester branch point per four phosphorus atoms,[3] chemical evidence has been presented which indicates that the occurrence of phosphotriester groups in RNA is unlikely.[49] As we have shown, methoxide ion-catalyzed methanolysis converts the dimethyl ester of A-2′-P to adenosine, unlike the alkaline hydrolysis of such esters.[49] On the basis of this observation and the expectations set forth earlier in this paper, a comparison of the nucleoside content of the reaction products from the hydrolysis and methanolysis of samples of the same RNA should give an estimate of the number of triester branch points in the RNA sample, with the one exception noted previously. Since the amount of nucleosides formed in the methanolysis of RNA is a function of reaction conditions, it becomes obvious that no quantitative conclusions can be drawn from our data regarding the number of triester branch points in the RNA samples. On the other hand, our data definitely rule out the possibility of a high degree of branching such as would be indicated by one phosphotriester group per four phosphorus atoms.

Another interesting effect of increasing the methoxide ion concentration in methanolysis was observed. Methoxide ion and 2′:3′-cyclic phosphate on the one hand are in equilibrium with a monomethyl ester of a nucleotide on the other. Increasing methoxide ion concentration, therefore, should increase the amount of methyl esters in the methanolysis reaction products and decrease the amount of cyclic phosphates. This conclusion is borne out by the data. A comparison was made for the yeast RNA of the variation with methoxide ion concentration of anion exchange chromatographic peaks which correspond to pure methyl esters or cyclic phosphates (peaks 3, 6, 7, 10, 11 and 15, Fig. 1). It was found that as the methoxide ion concentration was varied from 0.25 *N* to 2 *N*,

(53) D. M. Brown and A. R. Todd, *Ann. Rev. Biochem.*, **24**, 311 (1955).

(54) These terms are meant to include the total amount of ordinary nucleotides, monomethyl esters of nucleotides and 2′:3′-cyclic phosphate esters of nucleosides corresponding to a given base.

TABLE V

RELATIVE AMOUNTS OF 2'- AND 3'-ISOMERS FORMED IN HYDROLYSIS AND METHANOLYSIS OF MONOESTERS OF NUCLEOTIDES

| | Hydrolysis[b] | | | Methanolysis[b] | |
|---|---|---|---|---|---|
| Product[a] | RNA | Monomethyl esters | 2':3'-Cyclic esters | Yeast RNA | Calf liver RNA |
| C-2'-P[46] | 43 | 46 | 45 | 43 | 43 |
| C-3'-P | 57 | 54 | 55 | 57 | 57 |
| A-2'-P | 43 | 46 | .. | 31 | 33 |
| A-3'-P | 57 | 54 | .. | 69 | 67 |
| U-2'-P | 42 | 40 | 40 | 38 | 38 |
| U-3'-P | 58 | 60 | 60 | 62 | 62 |
| G-2'-P | 44 | 47 | 45 | 31 | 35 |
| G-3'-P | 56 | 53 | 55 | 69 | 65 |

[a] The hydrolysis products are the nucleotides as such. The methanolysis products are the monomethyl esters of the nucleotides. [b] See footnotes *a* and *b*, Table I.

each of the methyl ester peaks increased by several per cent. while each of the peaks corresponding to the cyclic esters decreased by a corresponding amount. The same effect was observed with the calf liver RNA (peaks 3, 6, 7, 8, 10 and 11, Fig. 2).

Since the base-catalyzed hydrolysis of monoesters of 2'- or 3'-nucleotides involves 2':3'-cyclic phosphates as intermediates,[17,23,40] the ratio of 2'- to 3'-isomers formed on hydrolysis of a monoester of a given nucleotide should be independent of the particular ester which is being hydrolyzed. The data given in Table V illustrate this point for hydroxide ion-catalyzed hydrolysis (0.5 *N* NaOH, 30–37°, 17–20 hr.) of RNA, monomethyl esters of nucleoside-2'(3')-phosphoric acids and nucleoside-2':3'-phosphoric acids. The ratios of 2'- to 3'-isomers also are given in this table for the monomethyl esters of the nucleotides which are formed in the methanolysis of RNA (65°, 1 *N* methoxide ion). These ratios vary significantly from the corresponding ratios in hydrolysis. This is not surprising. The isomer ratio obtained in hydrolysis is determined by the relative rates of breaking the $C_2'$ and $C_3'$ P–O bonds in a 2':3'-cyclic phosphate. On the contrary, the isomer ratios of the methyl esters obtained in methanolysis reflect not a rate phenomenon, but the relative thermodynamic stabilities of the two isomers in a given pair.

Preliminary experiments carried out recently[55] indicate that methoxide ion-catalyzed methanolysis is applicable to the determination and identification of RNA end-groups of type II in whole turnip yellow mosaic virus nucleoprotein. It will be of interest to determine the fate of the protein in this degradation.

(55) These experiments were done by R. Markham, M. W. Rees and D. Lipkin (Senior Visiting Fellow, Office for European Economic Coöperation, 1960) in the laboratories of the Virus Research Unit, Agricultural Research Council, Cambridge, England.

---

[CONTRIBUTION FROM DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, NEW YORK]

# The Synthesis of 1-(Hemi-homocystine)-oxytocin and A Study of Some of its Pharmacological Properties[1]

BY DEREK JARVIS,[2] MIKLOS BODANSZKY AND VINCENT DU VIGNEAUD

RECEIVED JUNE 19, 1961

1-(Hemi-homocystine)-oxytocin, an analog of oxytocin in which the half-cystine residue of the hormone which bears the free amino group is replaced by a half-homocystine residue, has been synthesized and tested for some of the pharmacological properties characteristic of oxytocin. This analog, which possesses a 21-membered disulfide ring in contrast to oxytocin, which contains a 20-membered ring, did not exhibit avian depressor or rat pressor activity. It possessed a very slight but definite oxytocic activity (0.75 unit per mg.). The mixed disulfide of L-cysteine and L-homocysteine has been synthesized, and its chromatographic behavior in the Beckman–Spinco amino acid analyzer has been shown to be the same as that of the mixed disulfide produced by acid hydrolysis of 1-(hemi-homocystine)-oxytocin.

The complete elucidation of the structures of oxytocin[3] and the vasopressins[4] demonstrated for the first time the occurrence of a twenty-membered disulfide ring in nature. Subsequently, a cyclic disulfide of the same size was found in insulin.[5] The question arises as to whether the size of the cyclic moiety is critical for the possession of biological activity. An indication that this might be true in the case of oxytocin was afforded by the pharmacological behavior of synthetic 4-isoglutamine oxytocin[6] which was found to be completely devoid of oxytocic and avian depressor activities. This substitution of isoglutamine for glutamine in oxytocin leads to an increase in the size of the disulfide ring from twenty members, as in oxytocin, to twenty-two members. However, concomitant with this, the carboxamide grouping, which in oxytocin is separated from the ring by two methylene units, is attached directly to the ring in the isomeric octapeptide. 5-Isoasparagine oxytocin,[7] an analog of oxytocin in which isoasparagine replaces asparagine, was also inactive. In this instance the cyclic moiety has

(1) This work was supported in part by a grant from the National Heart Institute, U. S. Public Health Service, Grant No. H-1675.

(2) N.A.T.O. Research Fellow.

(3) V. du Vigneaud, C. Ressler and S. Trippett, *J. Biol. Chem.*, **205**, 949 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954); H. Tuppy, *Biochim. et Biophys. Acta*, **11**, 449 (1953); H. Tuppy and H. Michl, *Monatsh.*, **84**, 1011 (1953).

(4) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, *J. Am. Chem. Soc.*, **75**, 4880 (1953); E. A. Popenoe and V. du Vigneaud, *J. Biol. Chem.*, **206**, 353 (1954); V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 4751 (1954); R. Acher and J. Chauvet, *Biochim. et. Biophys. Acta*, **12**, 487 (1953).

(5) F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **58**, vi (1954); A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *ibid.*, **60**, 541 (1955).

(6) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **79**, 4511 (1957).

(7) W. B. Lutz, C. Ressler, D. E. Nettleton, Jr., and V. du Vigneaud, *ibid.*, **81**, 167 (1959).