[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Synthesis of Uridine 5'-Diphosphate[1]

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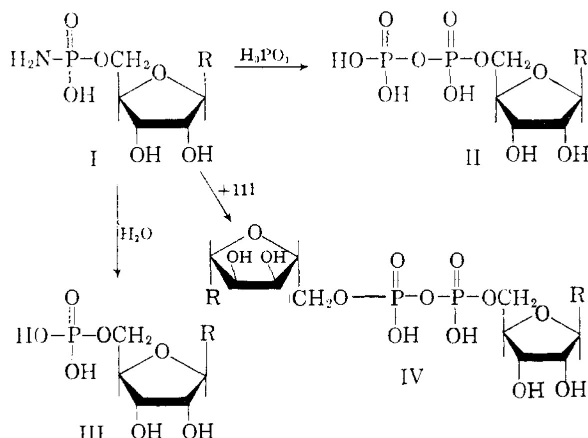
The synthesis of uridine 5'-diphosphate (UDP) from uridine 5'-phosphoramidate and phosphoric acid is described. The preparation of P^{32}-labeled uridine 5'-phosphate and its conversion to radioactive UDP is reported.

The discovery that phosphoramidates could be used for the formation of pyrophosphate bonds opened a new and promising approach to the synthesis of a variety of difficulty accessible nucleotides.[2] Among these are the important ribonucleoside 5'-diphosphates which serve as substrates for the enzyme polynucleotide phosphorylase.[3] To illustrate the amidate method, adenosine 5'-diphosphate (II, R = adenine) was prepared by a simple, one-step reaction between adenosine 5'-phosphoramidate (I, R = adenine) and phosphoric acid.[2,4] Finally, the development of an efficient procedure for the preparation of the nucleoside phosphoramidates[5] added greatly to the attractiveness of the amidate method and prompted us to extend our studies to the preparation of other naturally occurring nucleoside 5'-diphosphates. This paper describes the synthesis of uridine 5'-diphosphate (II, R = uracil, UDP).



Preliminary experiments established that the dicyclohexylgua idinium salt of uridine 5'-phosphoramidate (I, R = uracil, $UMP-NH_2$) reacted smoothly with 85% phosphoric acid to form UDP. In addition, uridine 5'- monophosphate (III, R = uracil, UMP) and a trace of P^1,P^2-diuridine 5'-pyrophosphate (IV, R = uracil) were formed.

Examination of the product distribution after various reaction times indicated that in 1 hr. all of the $UMP-NH_2$ had reacted to form the products described above. The rapidity of the reaction under these strongly acidic conditions compared to the very slow reaction of $UMP-NH_2$ with glucose 1-phosphate in *pyridine*[6] emphasizes the catalytic effect of hydrogen ions.[7] Furthermore, after all of the $UMP-NH_2$ had reacted (1 hr.) the ratio of UDP to UMP remained approximately 2:1 during 5 hr. of continued incubation. Thus, the UMP must arise by hydrolysis of $UMP-NH_2$ rather than by hydrolysis of UDP. It also seems likely that P^1, P^2-diuridine 5'-pyrophosphate is formed by a reaction between $UMP-NH_2$ and UMP (I → IV) rather than by a reaction between two molecules of $UMP-NH_2$ followed by hydrolysis.

One advantage of the amidate method is the relative simplicity of the reaction mixtures. Thus, separation of UDP from the other products was easily effected by ion-exchange chromatography. However, the problem of separating UDP from chloride ions in the effluent is worthy of comment. Precipitation of the nucleotide as its barium salt was not completely satisfactory because it was often difficult to wash out the excess chloride ions. The use of calcium chloride as an eluent and subsequent isolation of UDP as its calcium salt[8] proved difficult on a preparative scale because of the extremely hygroscopic nature of calcium chloride. By far the most satisfactory procedure, in our hands, was elution with lithium chloride followed by evaporation of the UDP fraction to a dry powder and removal of lithium chloride by extraction with dry methanol. In this manner, pure UDP was isolated almost quantitatively from the column effluent. The yield of UDP was 50% starting with 1 gram of $UMP-NH_2$.

In order to test our procedure for the preparation of radioactive nucleoside diphosphates as well as to provide labelled UDP for experiments with polynucleotide phosphorylase,[9] the synthesis of UDP labeled with radioactive phosphorus (P^{32}) in the stable phosphate position was investigated. The first step required the synthesis of UMP^{32}.

Phosphorylation of 2'-3'-O-isopropylidine uridine (acetone uridine) with a mixture of phosphoric acid and phosphorus pentoxide[10] is ideally suited for the preparation of UMP^{32} because the only radioactive material required is the readily available $H_3P^{32}O_4$. Thus, when acetone uridine was phosphorylated with a mixture of $H_3P^{32}O_4$ and

(1) This work was supported by a grant from the National Science Foundation (NSF-G4864). A preliminary account of the work was presented at the 133rd meeting of the American Chemical Society, April, 1958.

(2) R. W. Chambers and H. G. Khorana, *Chem. Ind.*, 1022 (1956).

(3) M. Grunberg-Manago, P. J. Ortiz and S. Ochoa, *Biochim. et Biophys. Acta*, **20**, 269 (1956).

(4) R. W. Chambers and H. G. Khorana, THIS JOURNAL, **80**, 3749 (1958).

(5) R. W. Chambers and J. G. Moffatt, *ibid.*, **80**, 3752 (1958).

(6) J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 3756 (1958).

(7) In unpublished experiments of C. A. Dekker and R. W. Chambers the effect of hydrogen ion concentration on the rate of hydrolysis of phosphoamidic acid was studied. In 0.05 *M* phthalate buffers there was no detectable difference in the rate at *p*H 4.8 and 3.8. A significant increase in rate was observed at *p*H 2.8 and a further increase occurred at *p*H 2.12. At *p*H 1 (0.1 *N* HCl) a very marked increase in rate was found.

(8) J. Baddiley, J. G. Buchanan and R. Letters, *J. Chem. Soc.*, 1000 (1958).

(9) P. J. Ortiz and S. Ochoa, *J. Biol. Chem.*, in press.

(10) R. H. Hall and H. G. Khorana, THIS JOURNAL, **77**, 1871 (1955).

P_2O_5 (specific activity 1.36 × 10^4 c.p.m./mole P), UMP^{32} with a specific activity of 1.37 × 10^4 c.p.m./μmole was obtained. In order to obtain a higher specific activity, another run with a mixture of $H_3P^{32}O_4$ and $P_2{}^{32}O_5$ (specific activity of the mixture = 4.51 × 10^4 c.p.m./μmole P) was made and UMP^{32} having a specific activity of 4.20 × 10^4 c.p.m./μmole was obtained.

The only other point in the UMP^{32} synthesis which deserves comment concerns the separation of the UMP from H_3PO_4. Several different procedures, including ether–alcohol precipitation,[10] fractionation of barium salts[11] and charcoal adsorption[12] have been used previously for this purpose. However, for the small scale (1 mmole) runs employed in this work, separation on an ion-exchange column proved advantageous. At low *p*H values where the secondary phosphate groups are protonated, UMP and H_3PO_4 have essentially the same charge and consequently they are eluted almost simultaneously. At higher *p*H values, however, advantage can be taken of the difference between the secondary dissociations of UMP and H_3PO_4.[13] Thus, a clean separation of these compounds can be effected by elution of H_3PO_4 from a chloride ion-exchange column with 0.025 *M* ammonium chloride followed by elution with 0.015 *M* hydrochloric acid to remove the UMP. One distinct advantage of this procedure is that the separation can be effected with no loss of the nucleotide, a feature not shared by the other methods.[14]

Pure UMP^{32} prepared in this manner was converted to UMP^{32}-NH_2 with dicylcohexylcarbodiimide and ammonia and isolated as its dicyclohexylguanidinium salt.[5] This crystalline product was reacted with phosphoric acid as described above to give UDP labeled in the stable phosphate position. In order to minimize the loss of radioactivity by decay, a rapid extraction procedure was developed for the isolation of UDP. In this procedure advantage was taken of the fact that UDP is a stronger acid than either UMP or H_3PO_4. Thus, when the reaction mixture (in *o*-chlorophenol and chloroform) was extracted with water, most of the UDP remained in the organic phase as its dicyclohexylguanidinium salt, while the UMP and H_3PO_4 were extracted into the water. After three such extractions the UDP was displaced into water by the addition of a little lithium chloride.[15] The UDP was isolated as its barium salt and converted to its sodium salt. The yield of UDP^{32}, based on UMP^{32}-NH_2, was about 30% and the specific activity was 4.21 × 10^4 c.p.m./μmole. The somewhat lower yield of UDP by this extraction procedure is partially offset by the saving in time and labor compared to the more efficient ion-exchange purification.

This work demonstrates further the utility of nucleoside 5'-phosphoramidates for the preparation of important unsymmetrical nucleoside pyrophosphates. The synthesis of cytidine 5'-phosphoramidate, guanosine 5'-phosphoramidate, their corresponding diphosphates and some studies on the synthesis of nucleoside 5'-triphosphates by the amidate procedure will be reported in subsequent papers.

Experimental

Analytical Methods.—Paper chromatography was carried out as described previously.[4] Paper electrophoresis was performed on strips (12 cm. × 57 cm.) of formic acid washed Whatmann 3MM paper[16] using an apparatus purchased from the E-C Apparatus Co., Swarthmore, Pennsylvania. The paper was saturated with 0.02 *M* citrate buffer, *p*H 4.0, and subjected to a 1,000 volt potential for 2 hr.

Phosphorus determinations and measurement of ultraviolet absorption were performed as described previously.[4]

Radioactivity measurements were made on liquid samples (1-ml. aliquots in a 1 inch diameter planchet) using an end window Geiger–Mueller tube and a Tracerlab binary scaler. All counts were made in duplicate for 5 minutes and, unless stated otherwise, are corrected for background and decay.

Preparation of P^{32}-Labeled 1,3-Dicyclohexylguanidinium Uridine 5'-Phosphoramidate.—2',3'-Isopropylidene uridine, prepared by the method of Levene and Tipson,[17] was dried overnight at 100° *in vacuo* over phosphorus pentoxide using a special drying vessel.[18] Phosphorylation of the acetone uridine and isolation of UMP were carried out by a modification of the procedure of Hall and Khorana.[10] The reaction vessel was shielded with a glass safety shield (Fisher Scientific Co.) and handling was carried out with a remote handling device (Atomic Accessories, Inc., Bellrose, New York).

Radioactive phosphorus pentoxide[19] (2.84 g. containing 7.1 mc. of P^{32}) was placed in a 15 ml. conical, 3-necked flask[20] fitted with a mercury seal stirrer, a silica gel drying tube and a glass stopper. Radioactive phosphoric acid (3.99 g., approximately 2.4 ml.)[21] was delivered with a pipet directly onto the phosphorus pentoxide with stirring. The mixture became quite hot and the phosphorus pentoxide dissolved slowly. While the mixture was still warm (approximately 60°) about 0.5 ml. was removed and discarded.[22] The specific activity of the remaining phosphorylation mixture was 4.5 × 10^4 c.p.m./μmole P. The drying vessel[18] containing 284 mg. (1 mmole) of dry acetone uridine was fitted into the reaction flask so that the delivery tube was close to the stirrer and directly over the sirupy phosphorylation mixture. The reaction vessel was placed in a thermostated water-bath at 60° and the acetone uridine added, with stirring, by tapping the side of the drying vessel. The mixture was stirred slowly for 2 hr. Then 6 ml. of water was added and the mixture was heated in a boiling water-bath (with stirring) for 30 minutes.

The aqueous solution was transferred with a pipet to a 40-ml. polyethylene centrifuge tube and 2 drops of 1% phenolphthalein indicator was added. The tube was cooled in ice and the solution was stirred mechanically while lithium hydroxide (about 6 g. in 30 ml. of water) was added until the indicator turned pink. The lithium phosphate was removed by centrifugation and washed 3 × 5 ml. with ice-cold water containing one drop of 4 *N* lithium hydroxide. The total volume of the original supernatant

(11) A. M. Michelson, *J. Chem. Soc.*, 1927 (1958).

(12) H. G. Khorana, unpublished results.

(13) W. E. Cohn in "The Nucleic Acids," Vol. I, E. Chargaff and J. N. Davidson, editors, Academic Press, Inc., New York, N. Y., 1955, p. 225.

(14) Because of the large volume of ammonium chloride solution required to remove the H_3PO_4, the procedure becomes impractical on a large scale.

(15) Dicyclohexylguanidine hydrochloride remains in the organic phase and the nucleotide extracts as its lithium salt.

(16) H. E. Wade and D. M. Morgan, *Biochem. J.*, **60**, 264 (1955).

(17) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934).

(18) This vessel was made from a 25-ml. volumetric pipet by cutting off one of the tubes at the bulb and sealing the bulb. The tube on the other end was about 4 inches long and fitted with a rubber stopper which fitted a 19/22 ₸ joint.

(19) Prepared by Nuclear-Chicago, Chicago, Illinois.

(20) Metroware, Metro Industries, Middle Village, Long Island, New York.

(21) This was prepared by mixing 0.23 g. of radioactive 93.4% phosphoric acid containing 6.7 mc. of P^{32} (Nuclear-Chicago) with 2.23 ml. (3.76 g.) of non-radioactive 85% phosphoric acid.

(22) This aliquot was removed in order to give approximately the same volume of polyphosphoric acid as had been used on previous runs (1.42 g. of P_2O_5 + 1.07 ml. of 85% H_3PO_4). Even more of the mixture could have been discarded and this probably would have been desirable since it would have reduced the amount of lithium phosphate which had to be removed.

plus the washes was 67.5 ml., TOD^{260} = 9,100[23] (91% recovery based on acetone uridine). This solution was percolated onto a column of Dowex-1-chloride resin (1.5 cm. diameter × 9 cm. long, 200-400 mesh, 8% cross-linking). The ultraviolet absorbing material emerging from the column was followed with an absorption meter.[24]

After the anionic material had been absorbed by the column, inorganic phosphate and a small amount of ultraviolet absorbing material (1,094 ml., TOD^{260} = 309) was eluted with 0.025 *M* ammonium chloride. The column was washed with water to remove the ammonium chloride[25] (46 ml., TOD^{260} = 16). UMP was then eluted with 0.015 *M* hydrochloric acid (188 ml., TOD^{260} = 5,890, 59% yield based on acetone uridine). The specific activity of the UMP was 4.2 × 10^4 c.p.m./μmole.[26] The remaining nucleotides were eluted from the column with 2 *N* hydrochloric acid (194 ml., TOD^{260} = 3,100). Total OD recovered from the column was 9,309 (102%).

The UMP fraction was concentrated to a sirup under reduced pressure. The hydrochloric acid which remained was removed by dissolving the sirup in ethanol and evaporation of the alcohol. After several repetitions of this procedure the odor of hydrogen chloride was no longer detectable and the sirup was stored in a desiccator at room temperature over phosphorus pentoxide and sodium hydroxide *in vacuo* overnight.

The sirupy UMP was dissolved in 1.25 ml. of 2 *M* ammonium hydroxide plus 0.83 ml. of formamide. Dicyclohexylcarbodiimide (0.51 g.) dissolved in 3.2 ml. of *t*-butyl alcohol was added to the UMP solution and the heterogeneous solution was heated in a stoppered flask in an oven at 80°. The mixture was swirled occasionally and after about 2 hr. the solution became homogeneous and some dicyclohexylurea began to crystallize. After 7 hr. the mixture was cooled to room temperature and some water was added. The *t*-butyl alcohol was removed by evaporation at reduced pressure leaving a slightly cloudy solution containing a considerable quantity of solid material. The liquid was transferred to a separatory funnel and the solid was washed well with water. The washes were combined with the main fraction and extracted once with ether. The aqueous phase was concentrated under reduced pressure and the last traces of water were removed *in vacuo* (oil pump and Dry Ice trap). Dry acetone[27] was added to the formamide solution until turbidity could just be detected and the solution was set aside overnight at room temperature. Large crystals of 1,3-dicyclohexylguanidinium uridine 5′-phosphoramidate[5] which adhered tightly to the wall of the flask (50-ml. round bottom) formed. The mother liquor was carefully removed and the crystals, still attached to the flask wall, were washed with dry acetone. The crystals were then transferred to a small beaker and triturated with dry acetone, breaking up the crystal mass in the process. The crystalline product was collected by filtration and dried over phosphorus pentoxide *in vacuo* at room temperature. The yield was 267 mg. of material which was homogeneous by chromatography in isopropyl alcohol–ammonia–water (7:1:2). A radioautogram of the chromatogram gave a single spot corresponding to the ultraviolet absorbing spot which was identical in R_f value with the authentic UMP-NH_2[5] run simultaneously.

Preparation of P^{32}-Labeled Uridine 5′-Diphosphate.—1,3-Dicyclohexylguanidinium uridine 5′-phosphoramidate (described above) was dried over phosphorus pentoxide at 100° *in vacuo* for 16 hr. The dry material was dissolved in 10 ml. of *o*-chlorophenol and the solution was cooled in an ice-bath. Phosphoric acid (0.54 ml. of 85%) was added and the heterogeneous mixture was stirred rapidly at 0° for 1 hr. The mixture was transferred to a separatory funnel with the aid of 10.7 ml. of chloroform and 16 ml. of ice-cold water (these volumes are fairly critical). After equilibration of the phases, the aqueous layer was drawn off and discarded. Two more extractions with 16 ml. of ice-cold water were made and then a small amount (about 40 mg.) of lithium chloride was added to the organic layer which was then extracted twice with 13 ml. of ice-cold water. The water layer was neutralized to *p*H 7 with 4 *N* lithium hydroxide and concentrated to dryness at reduced pressure and a bath temperature less than 35°. The residue was taken up in water. A small amount of insoluble solid was removed by filtration and washed with water. Barium acetate (0.2 ml. of 2 *M*) was added to the clear filtrate (total volume 4.6 ml.) and precipitation of the barium salt completed by addition of an equal volume of 95% ethanol. The barium salt was allowed to settle in the cold for 30 minutes and then collected by centrifugation. The product was washed twice with 50% ethanol to remove the excess chloride.

The barium UDP was suspended in 1 ml. of water and stirred with about 1 ml. of Amberlite IR-120-hydrogen form resin.[28] The slightly turbid solution was transferred to another centrifuge tube. The resin was washed with several small portions of water and the washes added to the original solution. The resin treatment was repeated. The combined solution (*p*H 3) were adjusted to *p*H 6.7 with sodium hydroxide and centrifuged to remove a small amount of insoluble white solid and some resin particles. The clear solution (3.0 ml., 99.6 μmoles) had a specific activity of 4.21 × 10^4 and contained 3 μmoles of inorganic phosphate. The yield of UDP^{32} based on acetone uridine was 10%. Electrophoresis showed only a single ultraviolet absorbing spot corresponding to authentic UDP. The preparation of labeled polymer with this material is described elsewhere.[9]

Preparation of Unlabeled Uridine 5′-Diphosphate on a Gram Scale.—1,3-Dicyclohexylguanidinium uridine 5′-phosphoramidate containing 1 mole of formamide of crystallization was prepared as described previously.[5] One gram of this material was dissolved in 20 ml. of *o*-chlorophenol contained in a 250-ml. centrifuge bottle fitted with a mercury seal stirrer. The solution was cooled in ice and 2 ml. of 85% phosphoric acid was added. The heterogeneous mixture was stirred for 1 hr. at 0°. Petroleum ether (40 ml., b.p. 30-60°) was added and the solution mixed well. The lower layer was washed 2 × 20 ml. with dry ether, centrifuging each time to clarify the ether layer. The resulting oil was dissolved in 62 ml. of 1 *N* ammonium hydroxide[29] (TOD^{260} = 14,950 at *p*H 2.8; 97% recovery of the OD based on UMP-NH_2).

The nucleotide solution was allowed to run onto a column of Dowex-1-chloride resin (4 cm. diameter × 6.5 cm. long), and elution was carried out at a flow rate of about 8 ml./minute in the following manner: After absorption of the nucleotide fraction, the column was washed with water until the optical density fell to less than 0.1 (220 ml., TOD^{260} = 365).[30] Elution with 0.003 *N* HCl + 0.04 *M* LiCl removed two peaks (586 ml., TOD^{260} = 171 and 2,056 ml., TOD^{260} = 5140).[31] Inorganic phosphate came off with the second peak of ultraviolet absorbing material and was removed completely before starting the next eluent. Elution with 0.003 *N* HCl + 0.1 *M* LiCl removed two peaks (480 ml., TOD^{260} = 62 and 867 ml., TOD^{260} = 7,980).[32] The first peak was identified as P^1,P^2-diuridine 5′-pyrophosphate. The second was UDP and represented 52% of the TOD based on the UMP-NH_2 used.

After careful neutralization of the combined UDP fraction[32] to *p*H 7.0 with lithium hydroxide, it was concentrated

(23) Optical density (measured at *p*H 2 and 260 mμ in a Beckman DU spectrophotometer) times effluent volume equals total optical density (TOD^{260}).

(24) Gilson Medical Electronics, Madison, Wisconsin.

(25) Washed until effluent gave a negative Nessler's test. A. P. Vanselow, *Ind. Eng. Chem., Anal. Ed.*, **12**, 516 (1940).

(26) Based on ultraviolet measurements and using 10^4 as the ϵ_{max} for UMP.

(27) Dried by refluxing several hours over anhydrous potassium carbonate and distilling.

(28) Rohm and Haas Co., Washington Square, Philadelphia 5, Penna. The resin was cycled three times through the sodium and hydrogen forms. The final sodium form was washed well with water just before use until the optical density was zero.

(29) The temperature in all the washing steps was kept as near 0° as possible. After dissolving in ammonium hydroxide, the *p*H should be about 8 or slightly higher and there should be very little insoluble solid. If the *p*H is too low, considerable insoluble, non-ultraviolet absorbing material is usually present.

(30) Most of the material in this peak was dicyclohexylguanidine hydrochloride.[5]

(31) The nucleotide material in these peaks was isolated by charcoal adsorption (see E. Cabib, L. F. Leloir and C. E. Cardini, *J. Biol. Chem.*, **203**, 1055 (1953)). The material in both peaks was identified by chromatography and electrophoresis as UMP. The double peak is attributed to the presence of inorganic phosphate on the column. In fact, similar double peak effects can be produced with mixtures of orthophosphate and pure UMP.

(32) The fraction containing the second peak was collected in three portions using a receiver which was chilled in ice. Each portion was neutralized to *p*H 7 with lithium hydroxide.

to a small volume under reduced pressure (bath temperature <35°) using a flash evaporator. When evaporation became very slow, absolute alcohol was added to aid removal of water by co-distillation. The last traces of water were removed using an oil pump fitted with a Dry Ice trap and the solid residue was dried over phosphorus pentoxide *in vacuo* at 4°.

The dry solid was triturated with absolute methanol (about 15 ml.) and removed by centrifugation. This procedure was repeated once more. The residue was transferred to a sintered glass funnel with the aid of absolute methanol and washed with this solvent until the chloride ion in the filtrate was negative (silver nitrate test). The total volume of the methanol washes was 36 ml., and it contained only 248 OD units (3% loss). The product was washed with acetone and then anhydrous ether to give a dry, amorphous, white powder—421 mg., uridine:labile P:total P; 1.00:1.00:1.97. This material was homogeneous by electrophoresis and a 50-mg. sample was recovered in 96% yield after rechromatography on a 1 × 6 cm. column of Dowex-1-chloride (single peak using elution similar to that just described). The material was active both in the P^{32} exchange assay and in polymer formation with polynucleotide phosphorylase.[3] The lithium was slightly inhibitory in the exchange assay and reduced the rate of polymer formation about 9%.

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Electrophoretic and Ultracentrifugal Components of Human Salivary Secretions

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Electrophoretic and ultracentrifugal analyses were made on samples of individual human parotid and submaxillary secretions. Each type of secretion contained about 10 to 12 electrophoretic components, although some were minor and often did not appear in many individual secretions. Three or four components were evident in the ultracentrifuge patterns. One sample of sublingual secretion was analyzed in the ultracentrifuge and showed six components with a major 15.6S component. All secretions seemed much different from blood serum. The presence of several materials behaving as cations at *p*H 8.5 was noted. The parotid secretion also contained small amounts of several fast-moving components. The parotid and submaxillary secretions showed many similarities but were quite different from the single sample of sublingual secretion studied.

Introduction

Prior to the completion of the present work, Tiselius electrophoretic and ultracentrifugal analyses of the individual salivary secretions had not been carried out, and only two earlier studies were available for whole saliva.[2-4] Subsequently, electrophoretic data for parotid secretions have been reported by Zipkin, Adamik and Saroff[5] and by Drevon and Donikian[6] and for whole salivas and parotid secretions by Geller and Rovelstad[7] in abstract form. Amylase was identified in the electrophoretic and ultracentrifugal patterns of parotid secretion in an earlier report from this Laboratory.[8]

Paper electrophoresis has been applied to whole saliva by Kinersly and Leite[9,10] and to parotid saliva by Drevon and Donikian.[6] However, the resolution is very poor despite extensive attempts to improve it.

The present paper describes the results of Tiselius electrophoretic analyses of human parotid and submaxillary secretions at *p*H 6.0, 7.0 and 8.5 in Miller–Golder[11] buffers, ionic strength, 0.1. It also presents ultracentrifugal analyses of human parotid and submaxillary secretions and one sample of sublingual secretion. No data on whole saliva were obtained because of the complexity of the component secretions and because of the rapid changes that occurred in the viscosity and the ability to form a mucin clot.

Experimental

Human parotid and submaxillary secretions were collected separately and concentrated by dialysis to about one-fourth of their original volume by dialysis against polyvinylpyrrolidone as described earlier.[8] The solutions were then dialyzed against the buffer for 65 to 70 hr. The secretions were kept at 0 to 4° during all of these stages. The buffers were Veronal or phosphate containing considerable sodium chloride, ionic strength 0.1.[11]

Most of the electrophoretic measurements were made in a 6-cc. cell, but some were made in a 2-cc. cell using the Perkin–Elmer Model 38 Tiselius Electrophoresis Apparatus. The ultracentrifugal analyses were performed in the Spinco Model E Ultracentrifuge at about 26–32° and usually at 59,780 r.p.m. A 12 mm., 4° cell was used.

Results

Electrophoretic Components.—Satisfactory electrophoretic analyses of human parotid and submaxillary secretions could be made by the use of the Miller–Golder buffers at *p*H 6, 7 and 8.5 at ionic strength, 0.1. It was necessary to concentrate the secretions to one-third or one-fourth of their original volume and to use the 6-cc. cell. The

(1) Public Health Service predoctorate research fellow of the National Institutes of Health, 1950–1956. The work has also been supported by other NIH grants (A-216-C; A-1303-C). It represents a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of Alabama, 1956.

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(3) A. J. Reid, Master's Thesis, University of Alabama, Tuscaloosa, 1952.

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(7) J. H. Geller and G. H. Rovelstad, *J. Dent. Res.*, **37**, 27 (1958).

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(9) T. Kinersly, *Yale J. Biol. & Med.*, **26**, 211 (1953).

(10) T. Kinersly and H. B. Leite, *ibid.*, **29**, 496 (1957).

(11) G. L. Miller and R. H. Golder, *Arch. Biochem.*, **29**, 420 (1950).