Labeling by Exposure to Tritium Gas.[31]—A 0.997-g. sample of 1,2,3,5-tetra-*O*-acetyl-D-ribofuranose[32] (m.p. 83.5–84°) was sealed in a vial with 1.2 curies of carrier-free tritium gas at a pressure of *ca.* 340 mm. After 15 days at room temperature the vial was opened and the tritium gas removed. The resulting material (m.p. 81.5–83°) had a faint odor of acetic acid and assayed 5.9×10^6 c.p.m./μmole (0.0014 atom % tritium). The pentose content of the material was determined to be 98 ± 2% of the theoretical by the use of a standard procedure,[14] except that either aqueous ribose or ethanolic solutions of 1,2,3,5-tetra-*O*-acetyl-D-ribofuranose were used as standards. When the latter was used, and the last traces of ethanol were removed *in vacuo* over phosphorus pentoxide before the addition of further reagents, there was no experimental difference between the intensities of the final colors produced from the two standards.

Preparation of 6-Methylmercapto-9-β-D-ribofuranosylpurine-ribosyl-*t*.—A 452-mg. sample of 1,2,3,5-tetra-*O*-acetyl-D-ribofuranose-*t* (1.42 mmoles) was converted to 2,3,5-tri-*O*-acetyl-D-ribofuranosyl-*t*-chloride.[33] The solution of the product was slightly brownish in contrast to the colorless product usually obtained. The material was condensed with 620 mg. of chloromercuri-6-methylmercaptopurine (1.55 mmoles) in the presence of 500 mg. of Celite, and the reaction mixture was worked up[15] to yield 6-methylmercapto-9-β-D-ribofuranosylpurine-ribosyl-*t*. The product was recrystallized from water and then alcohol to give 38.5 mg. (9%) of material of m.p. 158–161° (reported[34] 163–163.5°). The radioactivity of the material was determined to be 1.4×10^6 c.p.m./μmole (with the use of A_M values of 12,100 at 221 mμ and 18,900 at 289 mμ for water at *p*H 6). The spectrum was identical with that of an authentic sample of 6-methylmercapto-9-β-D-ribofuranosylpurine.

Preparation of 9-β-D-Ribofuranosyladenine-*ribosyl-t*.—Solutions of inert and radioactive 6-methylmercapto-9-β-D-ribofuranosylpurine-*ribosyl-t* were mixed to give material of specific activity 25,900 c.p.m./μmole. This material (205 mg., 0.69 mmole) was heated in a sealed tube with 15 ml. of methanolic ammonia (saturated at 0°) at 100° for 10 hr. The solution was then evaporated *in vacuo*. Chromatography of the residue indicated that the conversion was incomplete. The material was reheated at 125° for 4 hr. with 5 ml. of methanolic ammonia. Chromatographic examination of the residue indicated virtually complete conversion. The solution was treated with charcoal, concentrated to *ca.* 2 ml. and allowed to crystallize. The product was recrystallized twice from ethanol, from water, and then in two crops from water (27.5 and 8.0 mg. respectively) to give 35.5 mg. of product (19%), m.p. (first crop) 235–236.5°, mixed m.p. with adenosine 234–236°. All further studies were done on the first crop of crystals. The spectrum of the material was identical with that of adenosine, and no impurities were visible on paper chromatograms.

Anal.[35] Calcd. for $C_{10}H_{13}N_5O_4$ (267.2): N, 26.21. Found: N, 26.16.

The specific activities of the material after the indicated re-crystallization steps are as follows: third ethanol crystallization, 22,700 c.p.m./μmole; first water crystallization, 22,800 c.p.m./μmole; second water crystallization 22,400 c.p.m./μmole.

Electrophoresis Experiments.—The studies were done by the use of the E. C. Paper Electrophoresis Apparatus, E. C. Apparatus Co., Walnut Lane, Swarthmore, Penna., with Whatman 3 MM paper. The borate buffers were prepared by adjusting the *p*H of saturated aqueous boric acid with 10 *N* sodium hydroxide, except that the *p*H 9.2 borate buffer was a 0.05 *M* solution of sodium tetraborate.

Acknowledgment.—The authors wish to thank Mrs. Dina Van Praag for assistance with the initial phases of this investigation. The receipt of samples of the four 1-β-D-pentofuranosylthymines from Drs. J. J. Fox and J. F. Codington, and the coöperation of Dr. Seymour Rothchild, Technical Director, New England Nuclear Corporation, are gratefully acknowledged. We wish to thank Dr. David I. Magrath of the Australian National University, Canberra, Australia, for the suggestions concerning the theoretical basis of the electrophoretic separations, and Dr. Fox for discussions of the stereoisomerism involved.

(31) The exposure to gaseous tritium was performed by the New England Nuclear Corporation, 575 Albany Street, Boston 18, Mass.

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(34) A. Hampton, unpublished results.

(35) Analysis by J. F. Alicino, Metuchen, N. J. The sample was dried *in vacuo* at 100° for 4 hr. over phosphorus pentoxide.

NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION, CORNELL UNIVERSITY MEDICAL COLLEGE]

The Synthesis of Cytidine-2-C^{14}-ribosyl-*t*[1]

BY JOHN F. CODINGTON, RONALD FECHER, M. HELEN MAGUIRE, R. Y. THOMSON AND GEORGE BOSWORTH BROWN

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The synthesis of cytidine has been adapted to the use of tetra-*O*-acetylribofuranose, and a synthesis from cytosine-2-C^{14} and tetraacetylribofuranose-*t*-has been carried out.

Studies with cytidine[2,3] and with cytidylic acid,[4] labeled with C^{14} in both the cytosine and ribosyl moieties, have demonstrated in the rat that the base–ribose bond remains intact during the incorporation of the cytidine unit into ribonucleic acids. Those studies also have confirmed the earlier deductions[5] that the ribosyl derivative can be converted to the deoxyribosyl derivative without cleavage of the glycosyl bond. In *Escherichia coli*,[2] however, the ribosyl bond is extensively cleaved and the pyrimidine is utilized independently. The use of cytidine labeled with C^{14} and tritium in the aglycone and glycosyl moieties, respectively, would simplify surveys of the type of reaction which takes place in various tissues and species. It should also permit studies of the mechanism by which ribosyl derivatives are "deoxygenated" to deoxyribosyl derivatives.

(1) This investigation was supported in part by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant No. CY-3190), and from the Atomic Energy Commission (Contract No. AT. (30-1), 910).

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The recent synthesis of cytidine by Fox, Yung, Wempen and Doerr[6] utilized 1-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose. That general procedure now has been adapted to the use of tetra-*O*-acetylribofuranose, which is now available labeled with tritium.[7] Cytosine-2-C^{14} was synthesized, with the incorporation of urea-C^{14}, by a combination of the methods of Tarsio and Nicholl[8] and Bendich, Getler and Brown.[9] N-Acetylcytosine-2-C^{14}-mercury[6] was condensed with tri-*O*-acetyl-D-ribofuranosylchloride-*t*, and the product deacetylated to cytidine-2-C^{14}-*ribosyl-t* in 35% yield. The over-all yield in the five-step procedure was 12%.

This product was essentially pure on the basis of its ultraviolet absorption, but when chromatographed in butanol–NH_4OH–H_2O a tritium-containing impurity remained at the origin. This trace impurity represented at least 25% of the total tritium activity. Such an impurity also was present in the tritiated tetraacetylribofuranose and is apparently a highly labeled decomposition product(s).[9a] The impurity could be separated from the cytidine by chromatography over a cellulose column with butanol–NH_4OH–H_2O, and a sample so purified did not show any significant radioactivity outside the cytidine spot in paper chromatograms.

Experimental

Cytosine-2-C^{14}.—Under dry conditions 0.38 g. (0.0165 mole) of sodium was added to 13 ml. of 1-butanol, freshly distilled from sodium. After completion of the reaction the solution was cooled to 15° and 1.70 ml. (1.60 g., 0.0165 mole) of β-ethoxyacrylonitrile[10] was added, then 1.00 g. (0.0167 mole) of urea,[11] and the mixture was quickly heated to reflux, with efficient stirring and protection from moisture. An orange-colored solid soon began separating. After 90 minutes the mixture was cooled to 0° for 2 days. The solid was collected and washed on the filter with a little cold 1-butanol and then ether. It weighed 1.42 g.[12]

In order to assure complete removal of cytosine from the reaction mixture the filtrate was reduced in volume to 10 ml. and extracted four times with a total of 30 ml. of 2 *N* H_2SO_4. The acid extract was added to the solid above, and the solution stirred at room temperature for one hour. It was brought to *p*H 9 with concd. NH_4OH and then to *p*H 7.5 with glacial acetic acid. Upon cooling, a yield of colorless platelets of 0.84 g. (46%) was obtained, m.p. 297–301° (uncor.)(dec. with efferv.). Its spectrum in the ultraviolet was characteristic of cytosine,[13,14] A_M 10.4 × 10^3 at 276 mμ, *p*H 1.

N-Acetylcytosine.—A mixture of 0.76 g. (0.0069 mole) of cytosine, dried *in vacuo* over P_2O_5, and 20 ml. of acetic anhydride was refluxed with vigorous stirring for 4 hours. The reaction mixture was cooled at 3–4° for 3 days. The solid was collected and washed on the filter with cold water and then ethanol. The yield of colorless needles was 0.76 g. An additional 0.04 g. of pure material was obtained from the filtrate. The total yield was 76%, m.p. 326–328° (dec.) and the ultraviolet absorption spectrum at *p*H 1 and 6 indicated the presence of very little, if any, cytosine.

N-Acetylcytosine-mercury.—A suspension of 0.75 g. (0.0049 mole) of N-acetylcytosine in 370 ml. of water was stirred vigorously. All solid entered the solution within one minute after the addition of 4.9 ml. (0.0049 mole) of NaOH, 1.0 *N*. A solution of 1.33 g. (0.0049 mole) of mercuric chloride in 13 ml. of ethanol was immediately added. The solution became turbid. Its *p*H was about 5.

The mixture was warmed on a water-bath at 70°, then cooled to 40°. The *p*H of the mixture was brought to *p*H 7.0, with the addition of 1.0 *N* NaOH. This required 5.9 ml. (0.0059 mole). The suspension was warmed to 70°, then cooled for 15 hours at 2°. The colorless solid was collected, triturated with water on the filter, collected, and washed well with water and ethanol. After drying in air the material weighed 1.78 g. (103%).

1-(2′,3′,5′-Tri-*O*-acetyl-β-D-ribofuranosyl)-4-acetylaminopyrimidone-2.—A solution of 3.47 g. (0.0109 mole) of tetra-*O*-acetyl-D-ribofuranose[15,16] in 250 ml. of dry ether was saturated at 0° with dry HCl gas. It was allowed to stand one week at 3°. The pale yellow solution was taken quickly to near dryness *in vacuo*, avoiding contact with moisture. It was taken up twice in dry benzene, and the volume reduced *in vacuo* to 5–10 ml. to remove all HCl. It was then dissolved in about 50 ml. of benzene and added directly to a refluxing suspension of 1.72 g. (0.0048 mole) of N-acetylcytosine-mercury (dried over P_2O_5 *in vacuo*) in 150 ml. of dry toluene, which previously had been azeotropically distilled to remove all traces of moisture. About 50–60 ml. of solvent was removed immediately, freeing the reaction mixture of benzene and moisture. Within 5–10 minutes most solids had dissolved, but shortly thereafter a gum began to separate. Refluxing was continued for one hour.

About 600 ml. of petroleum ether was added to the cooled mixture, which was allowed to stand several hours at 0°. The liquid was decanted from a yellow gum, which was taken up in chloroform, washed with KI (30%), then water. After drying, the chloroform was removed *in vacuo*, leaving 1.86 g. of a yellow gum. As the material did not readily crystallize, it was converted directly to cytidine-2-C^{14}-*ribosyl-t* sulfate.

Cytidine-2-C^{14}-ribosyl-*t* Sulfate.—The crude material was dissolved in 15 ml. of absolute ethanol and sealed in a glass tube with 60 ml. of absolute ethanol saturated with ammonia gas at 0°. The temperature was maintained at 75° for 16 hours. The solution was taken to dryness *in vacuo* and the residue taken up in ethanol, then reduced to dryness *in vacuo*. This procedure was repeated several times.

The ethanolic solution was treated with charcoal and, after filtering, was acidified with concd. H_2SO_4 (4 drops) until precipitation was complete. After standing several hours, the precipitate was collected. It was crystallized by solution in water and addition of 20 parts of hot ethanol, and cooling. The yield of colorless needles was 0.50 g., 35% based upon N-acetylcytosine.

The product melted at 216–217° (dec. with efferv.) (uncor.), which compared with 214–216° for natural cytidine converted to the sulfate, and with 217–219° for an analytically pure synthetic sample.[17]

It exhibited a typical cytidine spectrum at *p*H 1 on a solution containing 17.4 μg. per ml.; A_M at 280 mμ was found to be 12.8 × 10^3 (Fox, *et al.*,[6] report 13 × 10^3). The radioactivity was determined on infinitely thin films in

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(7) M. P. Gordon, O. M. Intrieri and G. B. Brown, *ibid.*, **80**, 5161 (1958).

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(9) A. Bendich, H. Getler and G. B. Brown, *J. Biol. Chem.*, **177**, 565 (1949).

(9a) K. E. Wilzbach, THIS JOURNAL, **79**, 1013 (1957).

(10) β-Ethoxyacrylonitrile, obtained from Kay–Fries Chemicals, Inc., was distilled twice; b.p. 42° (0.9 mm.). It was stored at 0°, and protected from light and moisture until used. It was colorless, $n^{25}D$ 1.4495,[8] when used.

(11) This consisted of 0.29 g. of urea-C^{14} (Volk Radiochemical Co.), 5.0 millicuries, m.p 130–134°, and 0.71 g. of urea (Mallinckrodt Chemical Works), m.p. 132–135° (cor.). The samples had been mixed and dried in a vacuum desiccator over P_2O_5.

(12) Before acidification this material was found to exhibit an ultraviolet absorption spectrum typical of cytosine in either neutral or basic aqueous solution, and is apparently the sodium salt of cytosine. Bendich, *et al.*,[9] starting from cyanoacetal, reported a solid intermediate with no absorption in the ultraviolet, and felt their material to be the sodium salt of a non-cyclic ureido compound. The different spectral properties of the two intermediates remain unexplained.

(13) D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta*, **9**, 199 (1952).

(14) G. H. Hitchings, G. B. Elion, E. A. Falco and P. B. Russell, *J. Biol. Chem.*, **177**, 357 (1949).

(15) This material contained *ca.* 7.8 × 10^6 c.p.m. per μmole. The tritium was introduced by exposure to tritium gas, as described in the previous paper.[7] The m.p. of the tritiated sample was 76.5–80.5° and that of the untreated material 81.5–83° (cor.).

(16) Exposed at the New England Nuclear Corporation, 575 Albany Street, Boston 18, Mass., to 0.39 atm. of tritium (9.6 curies) for 2 weeks.

(17) Kindly supplied by Dr. J. J. Fox.

an internal Geiger–Müller flow counter (Radiation Counter Laboratories, Inc., Mark 12, Model No. 1, helium–isobutane gas). The total activity (C^{14} and tritium) of dilutions of the above solution, or on dilutions to which 100 parts of carrier cytidine had been added, was $2.2 \pm 0.2 \times 10^6$ c.p.m. per μmole. Cytosine isolated after $HClO_4$ hydrolysis of the sample with carrier added had a C^{14} activity of 3080 c.p.m. per μmole. The ratio of C^{14} to total disintegrations was 1:7. Thus the C^{14} activity of the synthetic material was *ca.* 0.31×10^6 c.p.m. per μmole.

Detection of Impurities.—The above product was chromatographed on paper, descending, with 1-butanol–NH_4OH–H_2O (86:5:9) for 36 hours, with the solvent front running off the paper and the cytidine moving about 3 inches. When scanned with an automatic recording windowless paper scanning device (Radiological Service Co.) radioactivity was found not only in the main peak at the cytidine spot but also in a major, non-ultraviolet absorbing, spot at the origin. With a liquid scintillation spectrometer[18] the C^{14}/H^3 ratio of the cytidine recovered from chromatograms was approximately 1.6. That of the material applied was 1.04, and of the material at the origin was 0.009.

Similar chromatography of the tetraacetylribofuranose in butanol–H_2O (86:14) showed a similar contaminant at the origin, with tetraacetylribofuranose near the solvent front.

(18) A Packard "Tri-Carb" Scintillation Spectrometer Model 314 was used. The readings were taken at voltage tap 7 with the discriminators set at 10 v., 50 v., and 100 v. Relative ratios of C^{14}/H^3 were determined by calibrating the instrument with crotonside-2-C^{14} and adenosine-*t*. The ratios are arbitrary; they are dependent upon the efficiency of the instruments for each isotope at the settings used, and bear no relation to those found by measurement in a Geiger–Müller apparatus. We wish to express appreciation to Dr. Leon Bradlow for helpful advice and willing coöperation.

Column Chromatographic Purification of Cytidine.—A column (33 × 7 cm.) was gravity-packed with a suspension of cellulose powder (Whatman Standard Grade Ashless) in 1-butanol–NH_4OH–H_2O (86:9:5), and washed until the effluent was colorless. A solution of 382 mg. of cytidine sulfate in 15 ml. of butanol–NH_4OH–H_2O (10:2:3.5) was applied to the top of the column. It was developed with butanol–NH_4OH–H_2O (86:9:5) at the rate of 8 ml. per 3 min. The first 300 ml. was discarded, the 300 fractions of 8 ml. each were collected. Fractions 187 to 268, containing the ultraviolet absorbing material, were combined. The residue from a 1-ml. aliquot was dissolved in 0.01 *N* HCl. Its spectrum was identical with that of cytidine, and it was calculated that the recovery, as cytidine, was 264.2 mg. (80%). The main solution was largely evaporated *in vacuo* and finally lyophilized.

This purified cytidine was chromatographed in butanol–NH_4OH–H_2O, in water at *p*H 10, and in 2-propanol–HCl–H_2O. Eluates of 2-cm. segments of the papers were evaporated on planchets and assayed. Not over 1 to 3% of the total activities on the strips were found outside the cytidine areas. In the scintillation spectrometer this cytidine gave C^{14}/H^3 ratios of 1.6 to 1.7, which are of the same order as that obtained upon elution of the original material from a paper chromatogram.

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NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, AND THE STUDY GROUP ON RHEUMATIC DISEASES, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Electrophoretic Behavior of Chondromucoprotein[1]

BY ROBERT C. WARNER AND MAXWELL SCHUBERT

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An electrophoretic study has been made of the chondromucoprotein of bovine nasal cartilage and of its polysaccharide component, chondroitin sulfate. The mobility–*p*H curves over the range 2 to 11, while close, are distinct and have parallel trends with the chondroitin sulfate always a little faster than the chondromucoprotein. At *p*H 12.5 the chondromucoprotein appears to be dissociated completely into two components of which one has the same mobility as chondroitin sulfate while the other is slower. Bringing the *p*H of such a solution to 3.3 does not reverse this dissociation.

The chondromucoprotein of beef nasal cartilage appears to be a compound of protein and chondroitin sulfate in which all the carboxyl and ester sulfate groups of the polysaccharide component occur as free anionic groups. In native cartilage these anionic groups are presumably associated with sodium cations and account for the high sodium content of cartilage. In the preparation which has been described these anionic groups are associated with potassium cations.[2] The nature of the linkage between the protein and the polysaccharide components is not known but it is broken in 20 hr. at 25° in 0.1 *M* NaOH.[3] A study of the *p*H range over which the combination of protein and polysaccharide is stable might be helpful in a study of this linkage. The electrophoretic behavior of the chondromucoprotein has, therefore, been examined over the *p*H range 2 to 12 and compared with that of chondroitin sulfate.

Methods

The electrophoretic experiments were carried out at 0° as previously described.[4] Solutions were prepared by dissolving the dry material, chondromucoprotein or chondroitin sulfate as their potassium salts, in the buffer to be used and dialyzing about 16 hr. against two changes of buffer. Solutions prepared at extreme *p*H's were kept at 0 to 2° during all preparative operations. In the *p*H range from 3 to 5 the solutions were cloudy. Similar patterns were obtained whether or not such solutions were centrifuged to remove small amounts of insoluble material. In all experiments a total concentration of 0.33 g. of polyelectrolyte per 100 ml. was used.

Methods for the preparation of chondromucoprotein and of chondroitin sulfate, both as potassium salts, have been described elsewhere.[2,3]

Results

The patterns obtained with the mucoprotein and with chondroitin sulfate showed in general a single boundary. Some of these are illustrated in Fig. 1

(1) This investigation was supported by research grants H1642 and A-28(C) from the National Heart Institute and the National Institute of Arthritis and Metabolic Diseases, respectively, United States Public Health Service.

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