[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Incorporation of Isotopic Formate into the Thymine of Bone Marrow Deoxyribonucleic Acid *in Vitro*[1]

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Bone marrow was incubated with isotopic formate and the purines and thymine of the nucleic acids isolated. Thymine was found to contain much more formate carbon than the purines. Degradation of the thymine indicated that the 5-methyl group contained almost all of the activity.

Earlier it was shown that the carbon of formate injected *in vivo* was rapidly assimilated into the thymidylic acid of chick and of rat viscera deoxyribonucleic acid (DNA).[2] The present experiments were designed to determine if a similar utilization of formate could be demonstrated *in vitro*. Bone marrow was chosen for this investigation because, as Abrams and Goldinger have demonstrated, there is a relatively rapid apparent synthesis of nucleic acid as shown by radioactive adenine uptake.[3] Although these workers failed to obtain a very active uptake of formate into nucleic acid purines they did not investigate the thymine.[4] We have therefore studied the incorporation of radioactive formate into the purines and pyrimidines of both RNA (ribonucleic acid) and DNA in suspensions of bone marrow from untreated rabbits.

We find that the thymine of the DNA from the marrow is more radioactive than the purines. Degradation procedures show that the activity of the thymine is largely in the 5-methyl group.

The data from the experiments are recorded in Table I. The relative uptake of formate into the DNA adenine indicates a minimum net synthesis of this purine of about 0.027% per day, assuming that positions 2 and 8 are equally labeled. On the other hand, the calculated "renewal rate" of thymine was found to be 1.4% per day. Abrams and Goldinger[3] found values of 1.5–3% for DNA renewal in erythroid marrow as calculated from adenine uptake. It is therefore obvious that the conditions of our experiments permit the utilization of formate for thymine synthesis while the utilization of formate for purine synthesis was greatly retarded. The addition of other possible precursors of the purine molecule (amino imidazole carboxamide or glutamine, HCO_3^-, glycine, alone or in combination; see also ref. 4) did not accelerate the *de novo* synthesis of purines as measured by this method. It is possible that rabbit bone marrow does not possess the capacity to synthesize purines in sufficient quantity to supply its own needs.

TABLE I

SPECIFIC ACTIVITIES OF BONE MARROW RNA AND DNA PURINES AND THYMINE AFTER 3 HOURS INCUBATION WITH C^{14}-LABELED FORMATE[a]

	RNA, d./s./μmole	DNA, d./s./μmole
Adenine	38.9	20.4
Guanine	10.2	12.7
Thymine	..	514.8
Iodoform from acetol portion of thymine	..	441.4[b]

[a] 3.6 μc. (0.45 μmole) of C^{14} potassium formate per milliliter of bone marrow suspension. Counts were made to ±3% accuracy. [b] Calculated from total counts recovered as iodoform. Degradation was carried out after addition of 50 mg. of inactive thymine. The specific activity of the thymine in different experiments showed a wide excursion, the lowest value being one-fourth that shown in the table. Guanine specific activities deviated a maximum of 50% of the recorded value, while adenine varied only 10%.

Experimental

The C^{14} potassium formate used in these experiments was prepared by catalytic reduction of potassium bicarbonate essentially as described by Melville, *et al.*[5]

Bone marrow was obtained from femurs and tibias of young male albino rabbits. Preparation of the suspensions was made according to the method of Marvin[6] except that Chambers solution,[7] 0.03 *M* in phosphate buffer *p*H 7.1, was used instead of Ringer solution. The marrow from the four bones of one rabbit was made up to a volume of 8–12 ml. Glucose was found to increase the incorporation of formate into the suspensions. Incubation was carried out in beakers in a Dubnoff shaker with an atmosphere of air. The total volume was 2.1 ml., of which 1 ml. was bone marrow suspension, 1 ml. Chambers solution containing 10 mg. of glucose, and 0.1 ml. of potassium formate solution. After 3 hours the suspensions were transferred to 12-ml. centrifuge tubes, the volumes made up to 4.7 ml. with water and 0.3 ml. of 100% trichloroacetic acid added. Thereafter, fractionation was carried out by the procedure of Ogur and Rosen.[8] After removal of the acid-soluble fraction, the phospholipid was extracted with alcohol, alcohol–ether (3:1), and ether, RNA was extracted with 1 *N* $HClO_4$ in the cold (16 hr.) and DNA subsequently by heating at 70° for 20 minutes with 0.5 *N* $HClO_4$.

The perchloric acid extracts containing either RNA or DNA from twelve experiments were combined and evaporated to dryness after removal of most of the perchloric acid with KOH. A small volume of 70% $HClO_4$ was added, and hydrolysis carried out on the steam-bath for 1 hour.[9] Samples were diluted, most of the $HClO_4$ removed with an equivalent amount of KOH, and the resulting solutions subjected to ion-exchange chromatography according to the method of Cohn.[10] The bases were separated first by cation exchange. The thymine was rechromatographed on an anion column.

The recovered radioactive thymine was diluted with inactive thymine and degraded by the procedure of Baudisch and Davidson.[11] The resulting acetol was converted to iodoform which therefore contained the carbon atom of the 5-methyl group.

The counting of all samples was done with a thin-window

(1) Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

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Geiger–Müller tube which was standardized with material of known specific activity. Corrections for geometry and self-absorption were made and the results are expressed as disintegrations per second.

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OAK RIDGE, TENNESSEE

[CONTRIBUTION FROM THE WHITMORE LABORATORIES OF THE COLLEGE OF CHEMISTRY AND PHYSICS OF THE PENNSYLVANIA STATE UNIVERSITY]

Synthesis and Properties of Deuterocarbons. Benzene-d_6 and Cyclohexane-d_{12}[1a]

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Samples of benzene-d_6 and cyclohexane-d_{12} of up to 99.3 and 98.8% deuteration, respectively, have been prepared by stepwise equilibration of benzene with sulfuric acid-d_2, followed by reduction with deuterium over nickel catalyst for conversion to the cyclohexane-d_{12}. Density and refractive index of these materials at 20, 40 and 60° have been measured, and molar volumes and refractions calculated. The physical properties are compared with those of the protium isomers. A relation between the density–temperature coefficients and the molecular weights of the isotope pairs is noted, and some possible implications suggested.

Present theories of the liquid state are extremely complex and unfortunately always involve several quantities which cannot be determined or estimated accurately.[2–4] It follows, of course, that prediction of liquid properties (*e.g.*, viscosity) suffer from the same disadvantages, and the calculation of physical properties from first principles for a relatively simple normal paraffin molecule is beyond the ability of present techniques. Quantitative knowledge of the contribution of the various parameters of liquid properties could be of considerable value in bridging this gap.

Comparison of deuterocarbons and their hydrocarbon analogs should be useful in determining the magnitude of the mass parameter for liquid properties. Apparently replacement of hydrogen by deuterium in an organic molecule little affects the outer molecular force fields or the molecular volume. To begin this study, benzene-d_6 and cyclohexane-d_{12} have been prepared and their physical properties determined.

Although the literature on deuterated compounds is voluminous,[5] the only important work relating to the effect of deuterium on liquid properties was reported by Ingold, Wilson and co-workers.[6] These authors studied benzene-d_6 in which 99.1% of the hydrogen was replaced by deuterium.

Experimental

Reagents.—Deuterium oxide and deuterium were obtained from the Stuart Oxygen Company and specified to have a minimum deuterium percentage of 99.5.

The benzene was Phillips Petroleum Company Research Grade 99.93 (±0.03).

The sulfur trioxide was supplied by the General Chemical Company and was stated to be better than 99%.

The nickel catalyst was a kieselguhr-supported, pelleted material containing 60% nickel, supplied by the Universal Oil Products Company. The catalyst was reduced *in situ* before starting the exchange experiments, and again whenever the exchange rate decreased significantly. Reduction was with hydrogen at 200–250° for eight hours.

Reaction of Benzene and Water.—The all-glass apparatus used in studying this reaction consisted of a boiler, a heated catalyst chamber and a condensing system. In operation, the boiler continuously supplied an azeotropic mixture of benzene and water to the catalyst. After passage through the catalyst chamber the vapors were condensed and returned to the boiler. At the completion of an equilibration the water and benzene were separated. The recovered water was purified by distillation, and the benzene by distillation from powdered colcium hydride.[7] Isotopic compositions were calculated from the densities of the products, and were later determined from mass spectra.

Preparation of Benzene-d_6.—The procedure is similar to that of Ingold.[6] The heavy sulfuric acid was prepared by distilling sulfur trioxide at 50–100 mm. into a receiver containing deuterium oxide. Anhydride vapor readily dissolved in the magnetically-stirred heavy water.

Benzene and 51–52 mole per cent. sulfuric acid-d_2 were charged to a 500-cc. Pyrex bottle having a carefully ground glass closure. The mixture was shaken vigorously for ten days at room temperature in a dry nitrogen atmosphere. The layers separated readily on standing and the sulfuric acid was pipetted from the bottle. When desired, an aliquot of the benzene was removed and distilled from powdered calcium hydride in an all glass system. This procedure removed water, sulfuric acid and benzenesulfonic acids in one step. The equilibration was repeated with fresh portions of sulfuric acid-d_2 until a benzene having the desired deuterium content was obtained. The equilibrium distribution of deuterium between acid and benzene at room temperature was found to be nearly statistical. The number of equilibrations required depends on the mole ratio of acid to benzene used and the desired deuterium concentration of the benzene.

Preparation of Cyclohexane and Cyclohexane-d_{12}.[8]—The reduction of the appropriate benzene with hydrogen or deuterium was carried out over kieselguhr-supported nickel at 400–700 p.s.i. and 100–125° in an Aminco apparatus using a stainless steel liner. The cyclohexane was made by hydrogenation of Phillips Research Grade benzene. The

(1) (a) Presented before the XIIth International Congress on Pure and Applied Chemistry, New York, N. Y., September, 1951. Part of investigation by American Petroleum Institute Research Project 42. (b) Department of Chemistry, Lafayette College, Easton, Pa.

(2) S. Glasstone, K. J. Laidler and H. Eyring, "Theory of Rate Processes," McGraw–Hill Book Co., Inc., New York, N. Y., 1941.

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(7) It was established that no exchange of hydrogen occurs between calcium hydride and benzene under such conditions.

(8) The preparation of perdeuterocyclohexane is reported by A. Langseth and B. Bak, *J. Chem. Phys.*, **8**, 1103 (1940), boiling point 77.8–78.0°.