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

The Identification of Phosphorylated Metabolites of 9-β-D-Ribofuranosylpurine[1]

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9-β-D-Ribofuranosylpurine-5′-phosphate and 5′-diphosphate have been obtained from the livers of rats previously injected with the corresponding nucleoside. The possible implications of this finding are discussed.

Previous studies of the metabolism of 9-β-D-ribofuranosylpurine-8-C^{14} (I-8-C^{14}) in the rat have shown that the purine moiety of this nucleoside is converted to polynucleotide adenine and guanine and is catabolized to urinary allantoin. The presence of three derivatives of purine in the perchloric acid extracts of rat liver was demonstrated.[4] The pattern of their elution from a column of Dowex-1 (formate)[5] suggested the possibility that these materials are, respectively, the 5′-mono-, di- and triphosphoric acid derivatives of 9-β-D-ribofuranosylpurine. A comparison has now been made of synthetic 9-β-D-ribofuranosylpurine-5′-phosphate (II)[6] and the major component of the first peak (the postulated monophosphate). The purine-containing material in the second peak (the postulated diphosphate) also has been partially characterized.

Results and Discussion

In these experiments it was desirable to obtain the largest possible amount of the metabolites without death of the animals from the toxicity of the 9-β-D-ribofuranosylpurine. Single intraperitoneal injections at the level of 100 mg./kg. were found to be suitable for these studies.[7] Material with an 8-C^{14} label was used to facilitate isolation procedures.

The cold perchloric acid soluble fraction from the livers of the injected animals was chromatographed by the use of a column of Dowex-1 (formate). Only 6% of the radioactivity placed on the column was removed by the water wash (in which purine and Compound I would appear). This compares favorably with the amount of radioactivity not retained by the resin (5%) when the animals were given much smaller injections of 9-β-D-ribofuranosylpurine-8-C^{14}.[4] Since previous experience had indicated that the purine-containing compounds undergo decomposition on anion exchange resins,[8] the present fractionations were done at 4°. Three main peaks of radioactivity were again found in the positions[4] where nucleoside-5′-mono-, di- and triphosphoric acids are normally eluted. The radioactive material in the first peak was shown to be identical with synthetic II by means of the following criteria: (a) migration in four paper chromatographic systems with concomitant positive tests for the *cis*-glycol[9] and the phosphate moieties,[10] (b) ultraviolet absorption spectra in acid, neutral and alkaline solutions, (c) electrophoretic mobility and (d) in two solvent systems the radioactivity, determined by radioautography, migrated with II. The relative specific activity of the II obtained was, within experimental error, identical with that of the administered nucleoside, and the probable absence of an endogenous pool of purine nucleoside or nucleotides is indicated.

The purine-containing compound in the second peak was admixed with other ultraviolet absorbing materials. Electrophoresis permitted the separation of a material which migrated at the rate characteristic of a diphosphate. This material gave a positive test for *cis*-glycol groups, gave spectra in acid, neutral and alkaline solutions similar to those of I, and its relative specific activity was approximately identical with that of the administered compound. These properties indicate that 9-β-D-ribofuranosylpurine-5′-diphosphate was the major radioactive component of the second peak.

High salt concentration made work with the third peak difficult; however, the above results strengthen the possibility that it was 9-β-D-ribofuranosylpurine-5′-triphosphate.

It has been postulated that the extreme toxicity of 9-β-D-ribofuranosylpurine is due to formation of analogs of normal nucleoside phosphates.[4] Thus, the 9-β-D-ribofuranosylpurine-5′-phosphate and the corresponding di- and triphosphates may interfere with some of the myriad metabolic reactions of the corresponding normal metabolites, adenosine-5′-phosphate (AMP), diphosphate (ADP) and triphosphate (ATP).[11]

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(4) M. P. Gordon and G. B. Brown, *J. Biol. Chem.*, **220**, 927 (1956).

(5) R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *ibid.*, **209**, 23 (1954).

(6) D. I. Magrath and G. B. Brown, THIS JOURNAL, **79**, 3252 (1957).

(7) A. P. Truant and H. E. D'Amato, *Fed. Proc.*, **14**, 391 (1955), report a subcutaneous LD_{50} of 220 mg./kg. for this compound in rats. Death occurred 6 to 48 hr. after injection.

(8) In previous work with these materials, poor recovery of the total radioactivity was obtained,[4] and attempts to purify purine-containing components by rechromatography on the same resin resulted in complete loss of the material. In view of the decomposition of the synthetic II on Dowex-2 (formate),[6] we believe the previous poor recoveries of radioactivity were due to a decomposition of the purine nucleotides by the anion exchange resin with a loss of the isotopically labeled carbon atom 8.

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

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

(11) It is significant that the toxicity of I toward tissues in culture is most effectively reversed by AMP and ATP; J. J. Biesele, M. C. Slautterback and M. Margolis, *Cancer*, **8**, 87 (1955); II is as inhibitory as I, and the alkali degradation products of either are inhibitory only at 100-fold greater concentrations; I and II show equal toxicity in Swiss mice, and the alkali degradation products of I show no toxicity at 30-fold greater doses; Dr. D. C. Clarke, private communication. The fact that 5′-deoxy-9-β-D-ribofuranosylpurine also did not lead to toxicity (H. M. Kissman, B. R. Baker and M. J. Weiss, N. Y. Section, Meeting in Min., March, 1957) can be correlated with the impossibility of the formation of a 5′-phosphate from it.

Experimental

Radioactivity determinations were carried out on infinitely thin samples plated on 10 cm.^{2} aluminum planchets with an internal Geiger–Müller flow counter (Radiation Counter Laboratories, Mark 12, model 1, helium–isobutane gas), probable error ±5%, except when otherwise specified. Ultraviolet absorption spectra were determined with the Beckman model DU spectrophotometer with matched 1-cm. quartz cells.

Toxicity Studies.—Male Sherman strain rats (obtained from Rockland Farms, New City, N. Y.) weighing 200 to 300 g. were used in these studies. Animals given intraperitoneal injections of 9-β-D-ribofuranosylpurine (I) at levels of 200, 150 and 100 mg./kg. died after 3, 5 and 20 hours, respectively.

Formation of Phosphorylated Derivatives of 9-β-D-Ribofuranosylpurine.—Eleven male Sherman strain rats, each weighing *ca.* 140 g., were given single intraperitoneal injections of 9-β-D-ribofuranosylpurine-8-C^{14} (I-8-C^{14}) (prepared by diluting I-8-C^{14} [4] with non-radioactive I to give material with a specific activity of 2280 c.p.m./μmole) at the level of 100 mg./kg. After 4 hr. the livers were excised under ether anesthesia and immediately frozen on Dry Ice to give 70 g. of material. All of the subsequent manipulations were done at 4°. The livers were extracted with 3% and then with 2% perchloric acid. The combined extracts were neutralized with 5 *N* potassium hydroxide and clarified by filtration through a Celite pad. The clear filtrate (520 ml.) contained 8840 optical density units[12] and radioactivity totaling 156,000 c.p.m. The material was fractionated by gradient elution chromatography which made use of a 1-liter mixing chamber and a 21.5 × 2 cm. column of Dowex-1 ion exchange resin (formate). After the entire sample of liver perchloric acid-soluble material had been absorbed on the resin, the column was washed with water until the optical density at 260 mμ of the effluent had fallen to 0.06. The water wash contained 1300 optical density units and a total of 9000 c.p.m. The column was then successively eluted with 2.5 *N* formic acid (fractions 1–100, 2 ml. each; fractions 101–520, 4 ml. each), 4.0 *N* formic acid (fractions 521–640, 5 ml. each), 8.0 *N* formic acid (fractions 641–820, 10 ml. each), 8.0 *N* formic acid + 0.4 *M* ammonium formate (fractions 821–900, 10 ml. each) and finally 8.0 *N* formic acid + 1 *M* ammonium formate (fractions 901–960, 10 ml. each). Major peaks of radioactive material were located in tubes 471–500, 760–800 and 881–900. Radioactive fractions were combined and lyophilized in groups consisting of tubes 471–480, 481–489, 490–499, 761–800 and 881–900. Spectral studies indicated that the pooled fractions 471–480 contained the largest amount of a derivative of I uncontaminated by other materials.

The Identification of 9-β-D-Ribofuranosylpurine-5′-phosphate (II).—The R_f values (ascending) of synthetic II, of the major component of fractions 471–480, of 9-β-D-ribofuranosylpurine and of adenosine-5′-phosphate, are, respectively, as follows: (A) 0.50, 0.50, 0.74, 0.35 (1% aqueous ammonium sulfate 33⅓%, isopropyl alcohol 66⅔%)[13]; (B) 0.83, 0.84, 0.72, 0.67 (5% aqueous monobasic sodium phosphate layered with isoamyl alcohol[14]; (C) 0.44, 0.45, 0.58, 0.48 (isopropyl alcohol 75 ml., water 25 ml., trichloroacetic acid 5 g., 28% ammonia 0.3 ml.)[15]; (D) 0.39, 0.39, 0.65, 0.29 (glacial acetic acid 20%, *n*-butyl alcohol 50%, water 30%). The unknown material corresponding to II in each of the above solvent systems gave positive tests for *cis*-glycol groups[9] and for organically bound phosphate[10] (except in solvent B). The spectrum of the material eluted from papers developed using solvent A was identical with that of II, in 0.1 *N* hydrochloric acid, water at *p*H *ca.* 5 and 0.1 *N* sodium hydroxide. The specific activity, calculated with the ε for synthetic II, of the material eluted from papers developed using solvent D was 2100 c.p.m./μmole. Radioautographs prepared from papers developed in solvents B and D showed that the only detectable radioactivity was associated with the material with R_f values corresponding to II, although faint AMP spots were detectable by inspection with ultraviolet light.

Electrophoresis was carried out at *p*H 8.25 as described.[6] The principal component of fractions 471–480, 9-β-D-ribofuranosylpurine-5′-phosphate, and adenosine-5′-phosphate migrated 9 to 10 cm. toward the anode, while 9-β-D-ribofuranosylpurine migrated 1.4 cm. toward the cathode.

The only other component detected in fractions 471–480 during these studies was a small amount of adenosine-5′-phosphate.

The Partial Identification of 9-β-D-Ribofuranosylpurine-5′-diphosphate.—The *p*H of the material in pooled fractions 761–800 was adjusted to 5 with ammonium hydroxide and submitted to electrophoresis. The conditions were the same as those above except that the *p*H of the buffer was 5.25 and the duration of the run was 75 minutes. Seven compounds clearly separated. Guanosine-5′-diphosphate was used as a marker because previous experience[4] indicated that it would be the major contaminant of the metabolite. The guanosine-5′-diphosphate and the major rapidly moving component of the mixture migrated 15 to 16 cm. toward the anode. The latter material gave a positive test for *cis*-glycol groups; its spectrum in 0.1 *N* hydrochloric acid, water and 0.1 *N* sodium hydroxide was very similar to that of 9-β-D-ribofuranosylpurine. The specific activity of the material (assuming an ε at 263 mμ equal to that of the monophosphate) was 1900 ± 200 c.p.m.[16]

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(12) Optical density units equal the optical density at 260 mμ times the volume in ml.

(13) N. Anand, V. M. Clark, R. H. Hall and A. R. Todd, *J. Chem. Soc.*, 3665 (1952).

(14) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(15) J. P. Ebel, *Bull. soc. chim. France*, 1089 (1953).

(16) A correction factor for the salt present was determined by plating small amounts of 9-β-D-ribofuranosylpyrine-8-C^{14} in the presence of buffer. In view of this correction factor and the use of an uncertain ϵ_{max}, there is no significant difference between this specific activity and those of the II and the administered I.

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[CONTRIBUTION FROM THE CHEMICAL PHYSICS SECTION, DIVISION OF INDUSTRIAL CHEMISTRY, COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION]

Tosyl-α-amino Acids. I. Degradation of the Acid Chlorides and Azides by Aqueous Alkali

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The decompositions resulting from the treatment of certain tosyl (*p*-toluenesulfonyl)-α-amino acid chlorides and azides with aqueous alkali are described and the mechanisms discussed.

In 1952, Wiley, *et al.*,[1a] observed that α-(benzenesulfonamido)-phenylacetyl chloride undergoes decomposition when treated with aqueous sodium hydroxide forming benzenesulfonamide, benzaldehyde and carbon monoxide. Wiley and Davis[1b] later

(1) (a) R. H. Wiley, H. L. Davis, D. E. Gensheimer, N. R. Smith, THIS JOURNAL, **74**, 936 (1952); (b) R. H. Wiley and R. P. Davis, *ibid.*, **76**, 3496 (1954).