209. *A Synthesis of Adenine labelled with* ^{14}C.

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Adenine hydrochloride, labelled at position 8 with ^{14}C, has been synthesised in high isotopic yield.

In view of the importance of adenine, its derivatives, and related compounds in biological systems, it seemed desirable to synthesise this purine with incorporation of a radioactive tracer, *viz.*, ^{14}C. Such a compound could then be used in experiments concerned with the mode of synthesis of nucleic acid and in studies of the mechanism of bacterial growth and reproduction.

The possibility of introducing isotopic carbon into the $C_{(8)}$ position of the adenine molecule merits particular consideration inasmuch as this step represents the final procedure in the adenine synthesis and therefore should give a high final yield of isotopic adenine. Cavalieri and Brown (*J. Amer. Chem. Soc.*, 1949, **71**, 2246) have recently explored this type of synthesis and called attention to the difficulty that during cyclisation of 4 : 6-diamino-5-formamidopyrimidine sulphate to adenine sulphate, using formamide as solvent, an extensive exchange between the formamido-group and formamide takes place. They attribute the exchange to a reversible ammonolytic cleavage, the ammonia arising from the thermal decomposition of the formamide (Freer and Sherman, *Amer. Chem. J.*, 1898, **20**, 223) : $H{\cdot}CO{\cdot}NH_2 = CO + NH_3$.

On using dimethylformamide as the solvent for cyclisation it has been found that extensive decomposition again takes place. To overcome this difficulty it was decided to use a substituted formamide of enhanced stability whose boiling point was well above the temperature required for cyclisation (165—170°). The cyclisation could then be carried out in an open tube, any products of solvent decomposition volatilising before aminolysis and subsequent exchange could take place. In consultation with Professor A. R. Todd, our choice fell on 4-*formylmorpholine*.

By using this as the solvent, adenine hydrochloride, labelled in the 8 position with ^{14}C, has been prepared by cyclisation of the correspondingly labelled 4 : 6-diamino-5-formamidopyrimidine hydrochloride, obtained by formylation of 4 : 5 : 6-triaminopyrimidine hydrochloride with ^{14}C-labelled formic acid. It is noteworthy that this formylation can be carried out by using aqueous solutions of formic acid having concentrations as low as 5%. The adenine hydrochloride which crystallised out after cyclisation showed a molar radioactivity which was 79—80% of that of the 4 : 6-diamino-5-formamidopyrimidine. Obviously only a minor fraction of the latter product had exchanged its isotopic carbon with the solvent during cyclisation. In the present preparation, the isotopic formic acid used for $C_{(8)}$ contained 1·65% excess of ^{14}C, corresponding to 1 mc. per millimole, and the adenine hydrochloride prepared therefore possessed activity corresponding to 0·79 mc. per millimole. Inasmuch as the material supplied from the Isotope Division at Oak Ridge contained 4 mc. per millimole, and that we diluted it fourfold, the present procedure should allow the preparation of ^{14}C-adenine which carries at least 3 mc. per millimole.

The identity of the adenine hydrochloride obtained on cyclisation was established by absolute ultra-violet spectrophotometry and by the differential ultra-violet spectrophotometry based on the spectral changes brought about by addition of adenase and xanthine oxidase (cf. Kalckar, Harvey Lectures, 1949—1950, Harvey Society, New York). Under these conditions adenine is converted into uric acid. The synthetic product was also shown to function as a growth factor for *L. casei* in the absence of folic acid (Snell and Mitchell, *Proc. Nat. Acad. Sci.*, 1941, **27**, 1). Paper chromatography showed that the radioactive adenine had an R_F value identical with that of a standard adenine preparation (Hoffmann–La Roche). With an adenine preparation having a ^{14}C content of 0·79 mc. per millimole elutions from paper-chromatographic

spots have shown that 0·5 μg. of the material can readily be determined quantitatively with a Geiger–Müller tube having a window thickness of 3·5 mg. per sq. cm. Since tubes with a window thickness of 1—2 mg. per sq. cm. are quite commonly used, samples containing less than 0·05 μg. of the above-mentioned preparation of ^{14}C-adenine could still be analysed for radioactivity.

Experimental.

4-Formylmorpholine.—Morpholine was heated under reflux for 1 hour with a 10% excess of 70% formic acid, and the reaction mixture was then fractionated to yield *4-formylmorpholine*, b. p. 242° (119°/20 mm.) (Found: C, 52·6; H, 7·9; N, 12·2. $C_5H_9O_2N$ requires C, 52·2; H, 7·9; N, 12·2%).

Formylation of 4 : 5 : 6-Triaminopyrimidine Hydrochloride by using ^{14}C-Labelled Formic Acid.—In the initial experiments the formic acid solution used in the formylation was prepared by using potassium formate obtained by catalytic reduction of potassium hydrogen carbonate with hydrogen and palladium-black at 100 atm. and 70° (Melville, Rachele, and Keller, *J. Biol. Chem.*, 1947, **169**, 419). Subsequently, ^{14}C-labelled sodium formate purchased from the U.S. Atomic Energy Commission (Oak Ridge) was used.

To a solution of sodium formate (66·4 mg., 0·98 m.-mol.; containing 0·97 mc. of ^{14}C) in water (0·75 c.c.) was added an equivalent of hydrochloric acid (0·2 c.c. of 5N.). This solution of formic acid was well stirred, and then anhydrous 4 : 5 : 6-triaminopyrimidine hydrochloride (161·5 mg., 1 m.-mol.) was cautiously added, stirring being continued until the solid passed completely into solution. After the solution had been left at room temperature for 15 minutes, 4 : 6-diamino-5-formamidopyrimidine hydrochloride began to crystallise in long colourless needles. The solution was kept at room temperature for 2 hours and then at 0° for 2 hours, and the product was filtered on a pre-cooled sintered filter. The contents of the reaction tube were washed out with ethanol (5 × 0·3 c.c.), and the product dried at 70° for 1 hour (122·4 mg., 67%). The filtrate and the ethanol washings were combined and kept at 0° to produce a second crop of the desired product (12 mg.; total yield, 73%).

The product was characterised by comparison of its ultra-violet absorption spectrum with that of an authentic specimen. The difference between the spectra of the formylated and the unformylated product is particularly pronounced at neutral reaction. The maximum for 4 : 5 : 6-triaminopyrimidine hydrochloride at pH 7 was at 277 mμ. ($\varepsilon_{mol.}$ = 8000); that for the 5-formylated product at pH 7 was found to be at 258 mμ. ($\varepsilon_{mol.}$, estimated for two isotopic samples = 4600 and 4560, respectively; authentic specimen, 4600).

The formylated product undergoes a spectral shift of the maximum to 265 mμ. on addition of 1 drop of concentrated sulphuric acid. This phenomenon is due to de-formylation, the maximum of 4 : 5 : 6-triaminopyrimidine hydrochloride in 3N-sulphuric acid being at 265 mμ.

Measurement of the radioactivity of the 4 : 6-diamino-5-formamidopyrimidine hydrochloride, after correction for the background, gave a value of 1·02 × 10^7 counts per minute per m.-mol. As the initial sodium formate had an activity of 1·08 × 10^7 counts per minute per m.-mol., the radioactive yield on the formylation stage was 95%.

Adenine Hydrochloride labelled with ^{14}C.—^{14}C-Labelled 4 : 6-diamino-5-formamidopyrimidine hydrochloride (117·4 mg.) was placed in an unsealed tube (length 200 mm., diam. 8 mm.) together with freshly distilled 4-formylmorpholine (0·75 c.c.). The mixture was heated to 200° by an infra-red lamp and kept at this temperature for 80 minutes, although the evolution of water vapour appeared to cease after 55 minutes. The mixture was cooled to room temperature and kept at 0° overnight. The adenine hydrochloride which had then separated was filtered off, washed with ethanol (2 c.c.), and dried at 70° for 1 hour (57 mg., 53%). Wash-out dilution with non-isotopic adenine hydrochloride gave a further 8 mg. (total yield, 65 mg., 60%).

The first crop of material was recrystallised from hydrochloric acid (0·75 c.c. of 2N.), Norite-A being used in small amounts as a decolorising agent. The product, adenine hydrochloride hemihydrate, crystallised as long colourless needles (50·5 mg.) (Found, for synthetic non-isotopic material: C, 32·8; H, 3·8; N, 39·0. Calc. for $C_5H_5N_5,HCl,\frac{1}{2}H_2O$: C, 33·2; H, 3·9; N, 38·8%).

The identity of the product was confirmed by comparison of its ultra-violet absorption spectrum in N/20-hydrochloric acid ($\lambda_{max.}$ = 263 mμ., $\varepsilon_{max.}$ = 13,100) with that for an authentic sample ($\lambda_{max.}$ = 263 mμ., $\varepsilon_{max.}$ = 13,200), both values being calculated in terms of the free base (cf. Loofbourow and Stimson, *J.*, 1940, 844).

Paper Chromatography of the Radioactive Adenine.—Radioactive adenine hydrochloride (6·6 μg.) was placed on a paper chromatogram and developed with butanol–water (Hotchkiss, *J. Biol. Chem.*, 1948, **175**, 315) containing two drops of concentrated ammonia solution. The R_F value was 0·39. For a commercial sample of non-isotopic material (Hoffmann–La Roche) a value of 0·40 was obtained. The R_F value of 4 : 6-diamino-5-formamidopyrimidine hydrochloride in the same solvent mixture was 0·14.

The extent of the adenine spots on the paper was determined by use of the Mineralight lamp (Ultra-violet Products, Inc., Los Angeles, Calif.).

The radioactive spot was excised and eluted by boiling for 1 minute with formic acid (1 c.c. of 0·1N.); 0·1 c.c. of this eluate was evaporated on an aluminium tray under the infra-red lamp, and the radioactivity of the residue determined. Activity of sample = 188 counts per minute. Theoretical for 0·49 μg. of adenine = 206 counts per minute. Hence, recovery from paper = 91%.

This work was supported by grants to Dr. H. M. Kalckar from the Rockefeller Foundation, the Carlsberg Foundation, and Lederle Laboratories Division, American Cyanamid Company. One of us (V. M. C.) took part in the work under the auspices of Rask-Ørsted Fondet. We thank Dr. E. Hoff-Jørgensen, Department of Biochemistry, University of Copenhagen, for performing the microbiological assay.

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[*Received, December* 14*th*, 1949.]