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Transformylation in the Synthesis of Adenine-8-C^{14}

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It has been possible to demonstrate by means of C^{14}-labeling that when 4,6-diamino-5-formamidopyrimidine is heated in N-formylmorpholine, half the carbon atoms in position 8 of the resultant adenine come from the formamidopyrimidine and half from the N-formylmorpholine. These results can best be accounted for by assuming the intermediary formation of 6-amino-4,5-diformamidopyrimidine which then randomly loses one of the formyl groups to yield adenine. To synthesize adenine-8-C^{14} and avoid the 50% loss of isotope inherent in the use of formylmorpholine, two methods have been found satisfactory: (1) 4,5,6-triaminopyrimidine sulfate heated with an equimolar amount of formyl-labeled N-formylmorpholine gives adenine in 92% yield; (2) 4,6-diamino-5-formamidopyrimidine sulfate labeled in the formyl carbon heated with diethanolamine forms adenine in 75% yield.

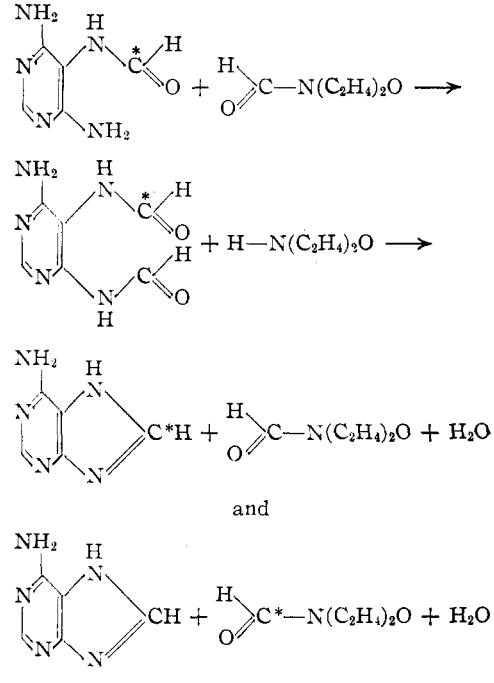
Adenine labeled with C^{13} or C^{14} is a useful tool for the study of nucleic acid metabolism, and accordingly several isotopic syntheses have been described.[1,2,3] Of these, the condensation of 4,5,6-triaminopyrimidine with labeled formic acid introduces the isotope in the last step of the synthesis and thus should result in the most efficient utilization of the tracer. However, Cavalieri and Brown[2] observed that when the condensation product, 4,6-diamino-5-formamidopyrimidine, was heated in formamide to effect ring closure, there was an extensive exchange of the formamido group with the formamide solvent so that the adenine formed contained only 25% of the expected isotope concentration. Clark and Kalckar[3] reported that this exchange could be eliminated by using a substituted formamide as solvent, *i.e.*, N-formylmorpholine.

We have studied the course of adenine synthesis in N-formylmorpholine in some detail and have found that exchange between the formamido group and the solvent does occur, but that it is of a rather special type which might be called reversible transformylation.

From the data of experiments 1 and 2 summarized in Table I, it is apparent that adenine obtained by heating formyl-labeled 4,6-diamino-5-formamidopyrimidine in N-formylmorpholine has 50% of the expected isotope concentration. Carrying out the ring closure in the absence of oxygen resulted in a higher yield of adenine accompanied by less darkening of the reaction mixture, but the isotope concentration was still half the theoretical value. This led us to the assumption that N-formylmorpholine transfers a second formyl group to the formamido compound resulting in the intermediate formation of 6-amino-4,5-diformamidopyrimidine. The latter then loses one or the other of the formyl groups at random to yield adenine which has half the isotope concentration of the original 5-formamido compound. That N-formylmorpholine can act as a source of formyl groups is not unexpected since Galat and Elion[4] have shown that formamide reacts readily with amines to produce substituted formamides and ammonia. And, in fact, in experiment 5 where the triaminopyrimidine was heated with N-formylmorpholine as the sole source of formyl groups, the chemical yield of adenine was 92%.

The postulated participation of the solvent was confirmed by using labeled N-formylmorpholine. When adenine was formed, in experiments 3 and 4, from unlabeled 4,6-diamino-5-formamidopyrimidine by heating in formyl-labeled N-formylmorpholine, it had, as expected, 50% of the isotope concentration of the labeled precursor. This was essentially independent of the molar excess of N-formylmorpholine.

Since no liberated formic acid could be detected by the method of Benedict and Harrop,[5] the most probable sequence of reactions in adenine synthesis by this method is



The 50% isotope dilution observed by us is contrary to the 20% dilution reported by Clark and Kalckar.[3] The reason for this conflict is not immediately apparent, although two possibilities suggest themselves. (1) The use of the formamido compound as the sulfate rather than the hydrochloride may have influenced the observed result, although this seems relatively improbable. (2) The method of C^{14} assay may have been the cause of the discrepancy. We have been able to get C^{14} counts accurate to within 5% only by completely combusting the sample and counting it as an infinitely thick layer of $BaCO_3$. In attempting

(1) L. F. Cavalieri, J. F. Tinker and A. Bendich, THIS JOURNAL, **71**, 533 (1949).

(2) L. F. Cavalieri and G. B. Brown, *ibid.*, **71**, 2246 (1949).

(3) V. M. Clark and H. M. Kalckar, *J. Chem. Soc.*, 1029 (1950).

(4) A. Galat and G. Elion, THIS JOURNAL, **65**, 1566 (1943).

(5) E. M. Benedict and G. A. Harrop, *J. Biol. Chem.*, **54**, 443 (1922).

TABLE I

ISOTOPE YIELD IN INTRODUCTION OF 8-CARBON INTO ADENINE

Expt.	Solute,[a] pyrimidine sulfate	Solvent[a]	Mole fraction of solute in solvent	Atmosphere	Yield of adenine,[b] %	Relative specific activity of adenine[c]
1	4,6-Diamino-5-formamido-C^{14}-	N-Formylmorpholine	0.90	N_2	93	51
2	4,6-Diamino-5-formamido-C^{14}-	N-Formylmorpholine	.09	Air	56	50
3	4,6-Diamino-5-formamido-	N-Formyl-C^{14}-morpholine	.5	N_2	88	43
4	4,6-Diamino-5-formamido-	N-Formyl-C^{14}-morpholine	.09	N_2	..	48
5	4,5,6-Triamino-	N-Formyl-C^{14}-morpholine	.5	N_2	92	106
6	4,6-Diamino-5-formamido-C^{14}-	Morpholine:nitrobenzene (1:10)	.09	N_2	41	102
7	4,6-Diamino-5-formamido-C^{14}-	Diethanolamine	.04	N_2	75	98

[a] Designation -C^{14}- indicates formyl group is labeled. [b] Yield is based upon labeled precursor indicated in the solute, solvent columns. [c] Relative specific activity is the specific activity of the 8-carbon atom compared to the labeled carbon atom of the precursor taken as 100. Specific activity in these experiments is defined as the counts per min. of an infinitely thick (20 mg./sq. cm.) layer of $BaCO_3$ as measured in a 1.4 sq. cm. dish in a windowless flow counter.

to count thin samples of uncombusted compounds we have observed inaccuracies as high as 30%, and if this method was used by Clark and Kalckar it might account for their high value.

When only one source of formyl groups was present during ring closure, no dilution of the isotope occurred. Thus 4,5,6-triaminopyrimidine heated with labeled N-formylmorpholine resulted in a 92% yield of adenine having the theoretical isotope content. A similar result was obtained from labeled 4,6-diamino-5-formamidopyrimidine when the solvent was a morpholine–nitrobenzene mixture (1:10) or simply diethanolamine. With diethanolamine as a solvent we have been able to obtain 75% yields of adenine from 4,6-diamino-5-formamidopyrimidine with no dilution of the isotope.

Experimental

4,5,6-Triaminopyrimidine.—The method of Bendich, Tinker and Brown[6] was followed to prepare 2-thiol-5-nitroso-4,6-diaminopyrimidine from malononitrile and thiourea. This was converted to 4,5,6-triaminopyrimidine by reduction with hydrogen and Raney nickel as follows: A suspension of 1 g. of the thiolpyrimidine and 1 g. of Raney nickel in 80 ml. absolute ethanol was shaken in a hydrogenator for 15 hours at 100° and 60 atmospheres pressure. After filtering, the solution was taken to dryness under nitrogen at reduced pressure. The residue was dissolved in 0.1 *M* H_2SO_4, decolorized with charcoal, and concentrated in vacuum to initiate crystallization. Yield of white needles of $C_4H_7N_5 \cdot H_2SO_4 \cdot H_2O$ was 49%.

4,6-Diamino-5-formamidopyrimidine.—The method of Clark and Kalckar[3] was modified somewhat in order to use the less soluble triaminopyrimidine sulfate. To a solution of 1.05 mM. of sodium formate in 1.7 ml. of 0.07 *M* HCl was added 1.05 mM. of the triaminopyrimidine sulfate. The mixture was heated to 50–70° and stirred until completely dissolved. After 2 hours, the solution was cooled and kept in the ice-box overnight. The crystals were centrifuged, washed with 0.2-ml. portions of ethanol, and dried in vacuum to yield 77% of 4,6-diamino-5-formamidopyrimidine sulfate.

N-Formylmorpholine.—Médard[7] has prepared formylmorpholine by refluxing an aqueous solution of formic acid with morpholine. In carrying this out on a small scale with tracer formate, 3.7 mM. of sodium formate was dissolved in 2.5 ml. of water, followed by the addition of 0.37 ml. of 12 *M* HCl and 4 mM. morpholine. The mixture, which contained a heavy precipitate of NaCl, was heated under reflux for 2 hours over an oil-bath at 130–150°. The liquid phase, together with dioxane washings of the crystalline residue, was transferred to a microdistillation apparatus, and after removing the low-boiling components under nitrogen at 100°, the residue was vacuum distilled to yield 2.1 mM. (59%) of colorless N-formylmorpholine b.p. 235° uncor. The relative specific activity as compared to the initial formate was 0.19 (theoretical, 0.20).

Adenine.—All syntheses were carried out on a 0.1 to 1 mM. scale. In experiments 1–4 adenine was prepared from 4,6-diamino-5-formamidopyrimidine sulfate by heating with freshly vacuum distilled N-formylmorpholine at 200° for 80 min. as described by Clark and Kalckar.[3] It was observed that a higher yield (over 90%) of a cleaner product was obtained by excluding air and maintaining a slow stream of nitrogen through the test-tube. After heating, the reaction mixture was cooled, diluted with water, and the adenine precipitated as the silver salt. This was decomposed with 1 *M* HCl and the adenine obtained in pure form by passage through a cation exchange column of Nalcite HCR as previously described.[8] The use of ion exchange purification eliminated the need for decolorizing with charcoal, a procedure accompanied by significant losses in small scale preparations.

The procedure was the same in experiments 5–7 with the following modifications. In experiment 5, the triaminopyrimidine sulfate was used instead of the 5-formamido derivative. In experiment 6, the solvent was a 1:10 mixture of morpholine and nitrobenzene. After heating under reflux for 15 min. in a 200° oil-bath, the condenser was removed and a stream of nitrogen introduced until the solvent was almost completely evaporated away. The residue was then taken up in water and purified as described above.

The most satisfactory solvent found for ring closure was diethanolamine, as indicated in experiment 7; 1.09 mM. of 4,6-diamino-5-formamidopyrimidine sulfate was suspended in 2.5 ml. of diethanolamine and heated for 90 min. at 210° under nitrogen. Adenine was isolated as described above; yield was 0.82 mM. (75%).

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(6) A. Bendich, J. F. Tinker and G. B. Brown, THIS JOURNAL, **70**, 3109 (1948).

(7) L. Médard, *Bull. soc. chim. France*, [5] **3**, 1343 (1936).

(8) R. Abrams, *Arch. Biochem.*, **30**, 44 (1951).