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CONTRIBUTION OF HYDROURACIL AND ITS DERIVATIVES TO PYRIMIDINE BIOSYNTHESIS

II. MECHANISM STUDIES

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SUMMARY

In an attempt to elucidate the mechanism of RNA formation from CβA and HU the relative incorporation of a number of derivatives was determined. From the data it is clear that the ribotides of CβA and HU are much superior to the parent compounds and to orotate or CO_2 as precursors of RNA. The ribosides of CβA and HU were incorporated somewhat better than the parent compounds.

To clarify the intermediate steps in the sequence, the appearance of [^{14}C]CβA and [^{14}C]HU into their ribotides was demonstrated. A reaction involving DPN and HURP to give URP was demonstrated and attempts to purify the system were made. Apparently, TPN and/or the flavins are not involved.

Evidence for the cyclization of CARP to HURP was obtained with the suggestion that a more exergonic condition than simple reversal of hydrolysis is required.

Abbreviations used in this paper are as follows: HU, 4,5-dihydrouracil; CβA, carbamoyl-β-alanine; C-Asp, carbamoyl aspartate; URP, uridylic acid; HURP, 4,5-dihydrouridylic acid; CAR, carbamoyl-β-alanine riboside, CARP, carbamoyl-β-alanine ribotide; HUR, 4,5-dihydrouridine; RNA, ribonucleic acid; DPN, TPN, di or triphosphopyridine nucleotide; DPNH, TPNH, reduced di or triphosphopyridine nucleotide.

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INTRODUCTION

Biochemical studies on the hydropyrimidines and related compounds have concentrated mainly on their degradations[1-8]. Until recently [9-13] studies on the properties of hydropyrimidine nucleosides and nucleotides have been neglected.

Hydropyrimidine nucleotides have been demonstrated from the action of rat liver slices on cytidine[12] but their role, apart from the presumed relation to pyrimidine nucleotide degradation, remained uncertain. A number of enzymic reactions of the pyrimidine nucleotides have been tested with the hydropyrimidine analogs[9-11] and some of these analogs have been tested for utilization in feeding experiments[13].

In the previous paper of this series[14] it was demonstrated that dihydrouracil and CβA are incorporated into RNA by a number of tissues and species. Although the mechanism of this incorporation was not discussed, it was apparent from the conditions of the test system and from the results that the incorporation was not mediated by orotate, nor to a major extent by CO_2. The purpose of the experiments described in this paper was to compare a number of other suspected precursors of RNA with orotate for efficiency as precursors, and to deduce relationships, leading to a better understanding of both the orotate and hydropyrimidine pathways of RNA biogenesis.

MATERIALS

Some of the reagents used in this study were prepared as described previously[14]. [6-^{14}C]5'-URP was prepared from [6-^{14}C]orotate[15]. All hydropyrimidine compounds were prepared by the method of COHN[16] and the corresponding carbamoyl components by alkaline scission of the hydropyrimidine ring[17]. Each compound was purified by paper sorbography or by elution from a Dowex 1 × 8 100–200 mesh column using a linear-gradient elution system and 0.1 *M* NaCl–0.1 *M* HCl as the concentrated eluant. [^{14}C]uridine and cytidine were obtained from Schwarz Laboratories, Inc. 5'-Nucleotidase was prepared by the method of HEPPEL AND HILMOE[18].

METHODS

The use of acetone fractions of liver preparations for the incorporation of compounds into RNA was described previously[14]. The standard conditions, previously defined, were used in the studies reported here, unless otherwise specified.

Experiments testing for interconversions of the precursors were performed in the same manner as for RNA labelling except that no exogenous RNA was added and the reactions were stopped after 60 min incubation by the addition of an equal volume of 2-propanol. After centrifugation of the insoluble matter, the separations of components in the supernatant were performed by methods indicated above.

RESULTS

The data of Table I illustrate the relative efficiencies of the precursors tested. Noteworthy are the values for CO_2 and uracil in this system. In the experiments described, orotate is superior to CβA or HU: this was invariably the case when chicken liver acetone fractions 3 and 4 were used in combination and again provides a more

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rigorous test of the hydropyrimidine system. Although in a series of ten experiments one preparation differed markedly from another in total incorporating capability, as is shown here by two examples, the relative magnitudes of incorporations for the compounds tested remained virtually constant.

It is tempting to suggest that CARP is prior to HURP in the sequence of RNA formation although the data do not stand as proof, nor has CARP been proved to be a physiological intermediate. Nonetheless both CARP and HURP are clearly more proximate to RNA than orotate, HU or CβA in the test system. It seems possible that CARP could be formed from HURP by a reversible cleavage of the HU-ring analogous to the HU ⟷ CβA interconversion.

A number of attempts to detect interconversion relations between the various HU derivatives and between CβA and orotate or C-Asp were made. Standard in-

TABLE I

SPECIFIC INCORPORATION* OF POSSIBLE RNA PRECURSORS

Chicken liver acetone fractions 3 and 4 were combined using standard conditions[11], except that a pool of $NaHCO_3$ was not included.

Expt. 1	HCO_3^-	3.0	5′CARP	83
	CβA	6.4	5′HURP	99
	HU	8.4	5′URP	340
	orotate	30.0		
Expt. 2	Uracil	0.0	CβAR	2.8
	HU	1.1	HUR	2.1
	orotate	4.5	Cytidine	3.9
	Uridine	3.0	Dihydrocytidine	2.9
			5′URP	30.0

* Counts/min/mg RNA/μC precursor.

corporation conditions were used and the compounds, one of which was labeled with ^{14}C, were added in equal amounts. By this means, no significant incorporation into orotate or C-Asp from CβA was detected. It has also been shown that the conversion of C-Asp to CβA proceeds at a very slow rate[8].

The conversion of [^{14}C]CβA to CARP and [^{14}C]HU to HURP was tested; when labeled substrates with specific activities of $7.5 \cdot 10^3$ counts/min/μmole were used with the test system, CARP and HURP with specific activities of 467 and 2620 counts/min/μmole, respectively, were isolated from the corresponding conversion mixtures. Isolations were performed using paper sorbography. The two solvents were 2-propanol–water–28 % NH_3 (60:40:1, by volume) and 2-propanol–5 *M* HCl (3:1, by volume).

The presence in the test system of enzymes able to degrade the nucleotides makes the quantitative study of the interconversions difficult and equivocal. Thus, for one example, when [^{14}C]URP was incubated in the presence of a pool of HURP, only 1 % of the [^{14}C] was found in the HURP whereas 7 % occurred in the HU.

Figs. 1 and 2 summarize a number of experiments in which the direct interaction of URP and DPN and their reduced derivatives were studied. In Fig. 1 is seen the relative specificity of the system for DPNH, TPNH oxidation being scarcely stimulated by URP. Compared to the effect of 5′-URP, the oxidation of DPNH is not

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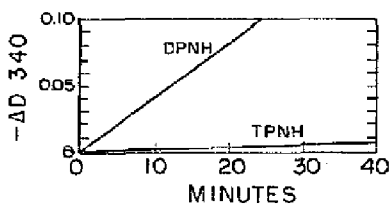


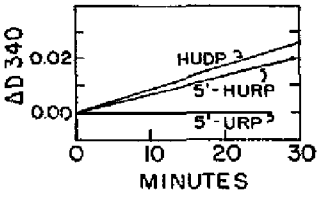
Fig. 1. Oxidation of reduced pyridine nucleotides in the presence of 5′-URP. The reaction mixture contained the following components at the indicated concentrations: 1 mg ml enzyme preparation (chicken liver acetone fraction 4), 0.1 m*M* DPNH or TPNH, 10 m*M* Tris maleate (pH 7.98), 4 m*M* cysteine and 0.5 m*M* 5′-URP. Light path, 1 cm. The control cuvets lacked 5′-URP. In the absence of TPNH or DPNH, there was no change in light absorption at 340 mμ due to the presence of URP. Temperature 38°.

significantly stimulated by uracil, uridine, 3′-URP or UTP while UDP is about as effective as 5′-URP.

Fig. 2 illustrates the reduction of DPN in the presence of 5′-HURP, HUDP and 5′-URP. It is apparent that only the reduced nucleotides are effective. In general the oxidation of DPNH was about 5 times as fast as the reverse reaction.

Although chicken preparations were originally used, rat liver proved to have more of the activity sought, which has also been detected in pigeon and dog liver, sucrose supernatants, beef liver acetone powder extracts and ascites cells. No significant activity was detected in rat intestinal mucosa, kidney or spleen, with HURP and either DPN or TPN.

Fig. 2. Reduction of DPN in the presence of hydrouracil nucleotides. The reaction mixture contained the following components at the indicated concentrations: 0.7 mg/ml enzyme preparation, 0.3 m*M* DPN, 10 m*M* Tris maleate (pH 7.98), 4 m*M* cysteine, 6 m*M* nicotinamide and 1 m*M* 5′-HURP, HUDP or 5′-URP. The control cuvet lacked the uracil nucleotides and there was no change in light absorption due to the uracil nucleotides in the absence of DPN. Temperature, 38°.



Attempts to purify the system were largely unsuccessful because of the instability of the system which failed to be preserved by Triton X-100, acetyl-tryptophane, 5′-HURP, 5′-URP, 3′-HURP, 3′-URP, stearate, dog brain cephalin or sucrose. The most successful method was the acetone fractionation described earlier[14], the bulk of the activity appearing in the last acetone fraction. Although the fractionation was devised with chicken liver preparations, it also worked satisfactorily with rat liver preparations. The active system was purified further by re-fractionation with acetone but did not survive more than a couple of hours at 0° and could not be frozen overnight with retention of activity.

The possible involvement of the flavins was considered since semi-purified preparations of the reductase have an absorption spectrum suggestive of the presence of flavins. Accordingly flavin mononucleotide and flavin adenine dinucleotide were tested with the liver preparation and HURP in the presence and absence of added DPN. No significant reduction in the flavin absorption peak was induced by HURP.

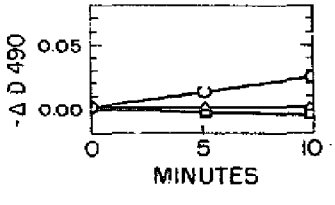


Fig. 3. Consumption of 5′-CARP by liver preparation. The complete reaction mixture contained the following components at the indicated concentrations: 20 mg/ml rat liver sucrose supernate, 5 m*M* 5′-CARP, 25 m*M* potassium citrate (pH 6.50), 4 m*M* ATP and 0.1 mg/ml 5′-nucleotidase. The curves represented are for systems which are complete (○), lacking ATP (□) and lacking ATP and nucleotidase (△). Temperature, 38°. The method of ARCHIBALD[23] was used to follow the disappearance of CARP.

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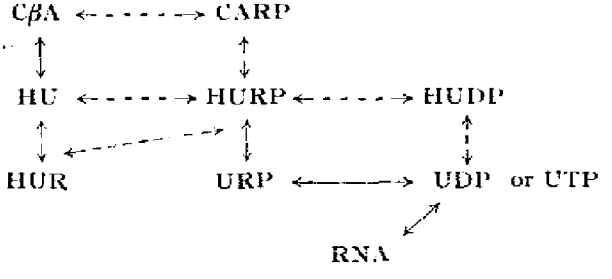
That 5'-HURP is converted to 5'-URP was shown in an experiment in which 2 μmoles 5'-HURP and 1 μmole DPN were incubated aerobically with 30 mg of a rat liver supernate preparation in a volume of 1 ml for 60 min at 38° and pH 7.6. From this mixture 0.55 μmole 5'-URP was isolated. No residual HURP could be found. If DPN or HURP were omitted no URP was found.

In Fig. 3 the results of an attempt to detect the presumed ring closure of CARP to form HURP are given. The principle involved is that 5'-nucleotidase does not attack CARP whereas HURP is the best of substrates we tested for this enzyme. The results suggest that a conversion of the CARP to the nucleotidase-susceptible HURP takes place. Parallel results were obtained when phosphate release was followed rather than carbamoyl group disappearance. Phosphate release, compared to the appropriate controls, was depressed in the absence of 5'-nucleotidase, CARP or ATP.

DISCUSSION

From a consideration of the data of Table I and the other data (see also ref.[14]), it is clear that neither uracil nor CO_2 mediate the transformation of HU into RNA nor is it probable that orotate or C-Asp mediate. The fact that HU is a precursor superior to CβA implies that it is a more proximate intermediate of RNA. The equilibrium of the CβA-HU transformation is in favor of ring cleavage at the pH used[6], and if CβA is a more remote precursor, it would be less well incorporated than HU. Ring closure of course, might be favored by further reactions decreasing the concentration of free HU such as ribotidation, or perhaps an exergonic ring closure reaction exists, as implied by the data of Fig. 3, in addition to the simple hydrolytic equilibrium.

From the direct and indirect relationships between the compounds related to HU and proved to be precursors of RNA, a plausible reaction sequence may be postulated:



where the broken arrows indicate relationships the absence of intermediates of which is unproved by direct isolation of all reactants. Admittedly some of the evidence is presumptive, the intermediates not having been isolated or proved to be the most proximal intermediates. Alternate pathways for RNA biosynthesis for *Neurospora* have been suggested[19] and similar mechanisms may apply in animal tissues.

In the interconversion of the uracil and HU-nucleotides, HUDP or UDP was not always a less effective substrate than the corresponding monophosphate. In addition to the enzyme sought, there was an abundance of phosphatases, kinases and nucleoside diphosphokinase in the liver preparations; hence the dinucleotides may have functioned through the monophosphates, or *vice versa*. Only a purification of

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the enzyme system would decide the issue, however, the preservation of the activity must precede the use of further devices for purifying the enzyme system.

The activation of HU may conceivably proceed in the same manner as that of uracil but not necessarily with the same distribution between the reported alternatives[21]. Similarly CβA may be ribotidated by analogous reactions. Except for the remotely related ribotidation of glycine[22] the authors are not aware of any reaction such as the ribotidation of β-alanine. On this basis, this reaction and subsequent carbamoylation of the β-alanine derivative are not regarded as highly probable.

The finding by VISSER *et al.*[13] that HUR appears in the urine of animals after feeding 3'-HURP is not sufficient to imply the formation of 5'-HURP from HUR. Nevertheless riboside formation may precede ribotide formation.

One may speculate at length about the significance of HU incorporation (and uracil degradation[20]) since, like all other physiological reactions, it must have some significance. It is perfectly possible that its efficient operation may depend upon a specific intracellular spacial relationship of the component enzymes, as may be the case in other synthetic reactions (*e.g.* oxidative phosphorylation, fatty acid synthesis, etc.) when competing reactions interfere with the demonstration of a given synthetic reaction due to disruption of cell structure or intracellular relationships. On the contrary it may be that it is as weak within the cell as it is without. In addition it is quite possible that there exist metabolic systems (other than microbiological mutants) in which hydropyrimidines are the preferred precursors of RNA, and which have not yet been examined in this respect.

ACKNOWLEDGEMENT

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