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Utilization of Deoxyuridine and 5-Methyluridine for the Biosynthesis of Thymine by the Rat

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5-Methyluridine and deoxyuridine were prepared from thymine-2-^{14}C and uracil-2-^{14}C, respectively, by the action of a nucleoside phosphorylase from an extract of lyophilized *Escherichia coli*. The compounds were identified by light absorption curves, sugar reactions and titration with metaperiodate.

The two labeled nucleosides were injected into partially hepatectomized rats and their utilization for pyrimidine synthesis in the liver and intestine was studied. The incorporation data were compared with values from parallel experiments with ^{14}C labeled uracil, uridine, uridine-5'-phosphate, thymine, thymidine and orotic acid.

Uracil showed a very small incorporation into PNA pyrimidines and DNA thymine. Uridine was utilized for all pyrimidines, the amount of isotope incorporation increasing rapidly with higher dose levels. Thymine and 5-methyluridine were slightly utilized for synthesis of DNA thymine in regenerating liver.

Deoxyuridine was in the liver and intestine incorporated into DNA thymine and to a much smaller extent into PNA uracil from intestine. In agreement with earlier experiments thymidine was exclusively incorporated into DNA thymine.

It is concluded that the ribose of uridine (or uridine-phosphate) is first reduced to deoxyribose forming deoxyuridine (phosphate) which accepts a methyl group to form thymidine (phosphate).

The free pyrimidine bases uracil, cytosine and thymine are very poorly or not at all utilized for the biosynthesis of polynucleotide pyrimidines by the rat [1-5]. Cytidine and to a smaller extent uridine, however, are readily utilized for the synthesis of both PNA * and DNA * pyrimidines [6], as are deoxycytidine and thymidine for DNA pyrimidines [7]. From the incorporation of the ^{15}N labeled ribosides into DNA pyrimidines in contrast to the metabolic inertness of the corresponding free pyrimidines we came to the conclusion that the rat is able to convert pyrimidine-attached ribose to deoxyribose without rupture of the glycosidic linkage [6]. Using cytidine-^{14}C labeled in both the base

* The following abbrevations are used in this paper: Pentosenucleic acid, PNA; Deoxypentosenucleic acid, DNA; Uridine-5'-phosphate, UMP; Adenosine triphosphate, ATP.

and ribose Rose and Schweigert [8] demonstrated the intact incorporation of this nucleoside into PNA and DNA pyrimidine nucleosides and thus definitively established a reaction of this type.

The present work represents a first attempt to find possible intermediates in the conversion of a riboside to a deoxyriboside. With the aid of a pyrimidine nucleoside phosphorylase from an extract of *E. coli* [9, 10] it was possible to synthesize labeled deoxyuridine from uracil-2-^{14}C + deoxyribose-1-phosphate and labeled 5-methyluridine from thymine-2-^{14}C + ribose-1-phosphate. The incorporations of these compounds into the polynucleotides from regenerating liver and intestine were studied at different dose levels and compared with corresponding incorporation experiments using labeled thymine, thymidine, uracil, uridine, UMP and orotic acid.

MATERIALS AND METHODS

Uracil-2-^{14}C * was synthesized according to Johnson and Flint [11], *orotic acid-2-^{14}C* according to Nyc and Mitchell [12], *thymine-2-^{14}C* according to Scherp [13].

Ribose-1-phosphate was prepared as the di*cyclo*hexylammonium salt according to Klenow [14], *deoxyribose-1-phosphate* according to Friedkin and Roberts [15]. In the latter case the phosphorylase from *E. coli* was used instead of Friedkin's liver enzyme.

For the preparation of the nucleosides the *nucleoside phosphorylase* activity from *E. coli* [9, 10] was used. A cell free extract of the lyophilized bacteria was prepared as described earlier [16]. The extract was diluted with an equal volume of water, precipitated in the cold with one tenth volume of M $MnCl_2$ and allowed to stand for 30 minutes at 0° in order to remove nucleic acids. After centrifugation the clear supernatant was dialyzed for four hours against a total of four liters of 0.01 M phosphate buffer, pH 7.0 and then lyophilized. The dry powder was stored at −15° in a desiccator without appreciable loss of activity for several weeks.

Chromatographic separation of free pyrimidines from corresponding ribosides or deoxyribosides. For this purpose ion exchange on Dowex-2, formate (200−400 mesh) was used. A column of 2 cm diameter and 15 cm length was sufficient for the separation of 50−500 μmoles of pyrimidine compounds. A 4 × 15 cm column was used for larger amounts. Before starting the chromatogram the pH of the solution was adjusted to *ca.* 10 by addition of concentrated ammonia. The solution was introduced to the column by gravity and elution carried out with ammonium formate, pH 9.0 (formate concentration = 0.1 M). The pyrimidine nucleoside or deoxynucleoside emerged between 4 and 7 column volumes, immediately followed by the free base. This method satisfactorily separated nucleosides from the corresponding free bases, but did not resolve either the nucleosides or the free pyrimidines.

Fractions containing the same pyrimidine compound were combined and after evaporation to dryness *in vacuo* passed through a Dowex-50 column (H^+ form, 2 × 10 cm) to remove ammonia. The column was washed with water until the effluent was free from light absorption at 260 mμ. Formic acid was removed from the effluent by repeated evaporation *in vacuo*. The dry residue was dissolved in water and used directly for the experiments.

Further resolution was attained by a combination of this method and chromatography on starch columns with water saturated butanol [17]. In this way a mixture of thymine, thymidine, uracil and deoxyuridine was separated. First pyrimidines were separated from deoxyribosides by ion exchange chromatography. Subsequently the two compounds in each group were separated by starch chromatography. Under these conditions deoxyuridine had an R value of 0.81.

Starch chromatography with butanol-water also allowed direct separation of thymine from 5-methyluridine. The R-value for the latter compound was 0.60 (thymine = 1.48).

* We wish to thank Dr. H. L. Smith for the synthesis of the labeled uracil.

Preparation of bases from polynucleotides. PNA and DNA were prepared and separated according to Hammarsten [18]. DNA was hydrolyzed at 175° with formic acid [19] and the bases separated by chromatography on Dowex 50 [20, 21]. Since thymine was eluted in the front with water, contamination was possible. In several cases it was therefore rechromatographed on starch [17]. However, the specific activity of the thymine before and after starch chromatography never showed significant differences.

The PNA nucleotides were hydrolyzed with N HCl for one hour at 100°. After removal of HCl and adjustment of pH to 9—10 the mixture of purines and pyrimidine nucleotides was chromatographed on a Dowex-2-chloride column (diameter 0.9 cm, length 15 cm) according to the principles of Cohn [22]. Elution was started with 0.0025 N HCl. Two peaks showing absorption around 260 mμ were obtained. The first consisted of a mixture of adenine and guanine; the second was cytidylic acid. After elution of the cytidylic acid peak, the uridylic acid was eluted with 0.02 N HCl. In one experiment a separation of PNA purines was desired. This was achieved by chromatography on Dowex-50 (H^+ form, solvent 3 N HCl) after the Dowex-2 chromatogram.

Measurement of radioactivity. Fractions containing the same purine or pyrimidine compound were combined after chromatography and the acid removed by repeated evaporation *in vacuo*. The residue was dissolved in 5 ml of water. Aliquots were diluted with 0.05 M phosphate buffer, pH 7.2, for light absorption measurements. From the absorption at 260 mμ the concentration of each compound was calculated [23, 24]. Radioactivity measurements were carried out in a Tracerlab Sc-18 windowless flow counter on infinitely thin samples (*ca.* 1 μmole of compound) of non diluted aliquots.

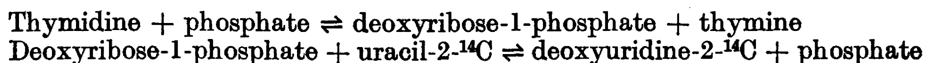
RESULTS

Enzymic synthesis of labeled nucleosides

5-Methyluridine. A typical preparation is given below. Thymine-2-^{14}C (30 mg), dissolved in 5 ml of 0.05 M phosphate buffer, pH 7.0, was added to 63 mg (125 μmoles) of di*cyclo*hexylammonium ribose-1-phosphate (85 % pure) and 40 mg of lyophilized enzyme. Incubation was carried out at 37°. In preliminary experiments it had been found that better yields of riboside were obtained in phosphate than in tris buffer, probably because phosphate inhibits breakdown of ribose-1-phosphate [10].

The reaction was stopped by immersion in boiling water for one minute when there was no further decrease in light absorption at 300 mμ. The light absorption was measured on aliquots of the reaction mixture diluted with 0.1 N NaOH (*cf.* Ref. [15]). Usually the reaction was complete after 4 hours. Separation of the nucleoside from the remaining thymine was carried out by ion exchange chromatography. 80 μmoles of riboside (yield *ca.* 60 % based on ribose) and 130 μmoles of thymine were recovered.

Deoxyuridine. This deoxynucleoside was prepared by two different methods. One way consisted of incubation of uracil-2-^{14}C with deoxyribose-1-phosphate and enzyme in the manner described above for 5-methyluridine. The yield based on deoxyribose was 40—50 %. In the second method deoxyribose-1-phosphate was not isolated, but was formed and reacted by the following reactions:



Thymidine + phosphate $\rightleftharpoons$ deoxyribose-1-phosphate + thymine
Deoxyribose-1-phosphate + uracil-2-^{14}C $\rightleftharpoons$ deoxyuridine-2-^{14}C + phosphate

Uracil-2-^{14}C (400 μmoles) was dissolved in 15 ml of 0.05 M phosphate buffer, pH 7.0. Thymidine (400 μmoles) and 150 mg of enzyme were added and incubation was carried out at 37° for 6 hours. By a combination of ion

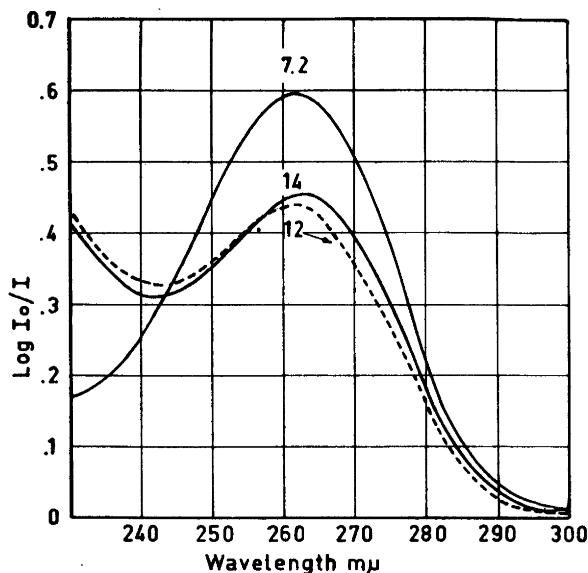


Fig. 1. Ultra violet absorption of deoxyuridine in aqueous solution at pH values indicated.

exchange and starch chromatography deoxyuridine (120 μmoles), uracil, thymidine and thymine were separated.

The second method was less time consuming than the first one and even though the yield of deoxyuridine was lower (*ca.* 30 %) it was used when larger amounts were prepared. The recovery of nonreacted isotopic uracil was almost quantitative.

Thymidine. This deoxyriboside was prepared by incubation of thymine-2-^{14}C (200 μmoles, 140 000 ct/min/μmole) with nonlabeled thymidine (100 μmoles) and 50 mg of enzyme in 5 ml of 0.1 M phosphate buffer, pH 7.0, for 5 hours. The thymidine-^{14}C formed during the exchange reaction was isolated by ion exchange chromatography. It amounted to 86 μmoles and had a specific radioactivity of 80 000 ct/min./μmole.

Uridine. Our enzyme preparation from *E. coli* had a surprisingly low phosphorylytic activity towards uridine. Labeled uridine was therefore prepared from orotic acid-2-^{14}C *via* UMP. The enzymic synthesis of UMP was carried out in a crude extract of rat liver from ribose-5-phosphate, ATP, and orotic acid as described earlier [25]. The protein free perchloric acid supernatant was hydrolyzed for 1 hour at 100° to break pyrophosphate linkages. UMP was purified by chromatography with formic acid on Dowex-2 [26]. After removal of the formic acid UMP was dephosphorylated with snake venom phosphatase [26] and the resulting uridine was then chromatographed as described in the experimental part.

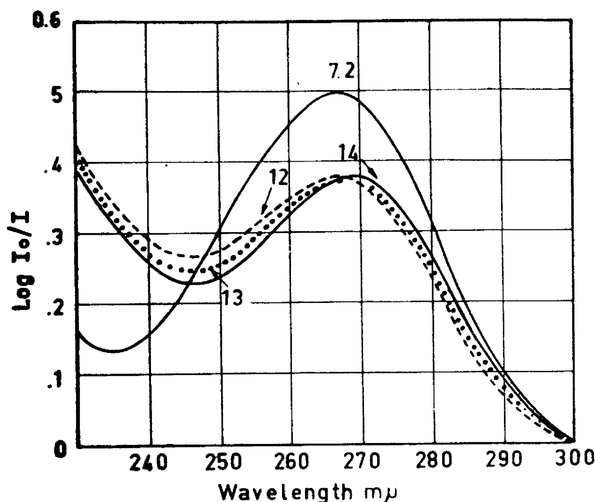


Fig. 2. Ultra violet absorption of 5-methyluridine in aqueous solution at pH values indicated.

Characterization of 5-methyluridine and deoxyuridine

Ultra violet curves. Light absorption curves of the compounds were measured at different pH in a Beckman Spectrophotometer, model DU (Figs. 1 and 2). Since the compounds were not crystalline values for log I_0/I are given rather than molecular extinction coefficients *. The curves for deoxyuridine (Fig. 1) show the same maxima and minima and the same isosbestic points as given by Fox and Shugar [27] for deoxyuridine obtained by chemical deamination of deoxycytidine.

No similar light absorption curves for 5-methyluridine have been found in the literature. The extensive studies of Fox and Shugar [24,27] have demonstrated that the nature of the sugar attached to a pyrimidine in nucleosidic linkage influences in certain systematic ways the light absorption of the pyrimidine, especially in the alkaline range. Thus according to the predictions by the cited authors 5-methyluridine and uridine would be expected to show similar changes at pH 12—14 (reflecting dissociation of sugar hydroxyls). The presence of the methyl group on the pyrimidine ring would largely result in a bathochromic shift of the curves.

The curves of Fig. 2 fulfil these conditions. The first isosbestic point at 2 475 Å is identical with that of thymidine and represents the dissociation of the 6-OH-group. This point indicates that the compound, in analogy with thymidine, has a furanose structure, since thymine pyranosides show an isos-

* The molar extinction coefficients could be calculated from nitrogen analyses of the solutions which were used for light absorption measurements. Under those circumstances values of 9 800 (at 267 mμ and pH 7.2) and 10 200 (at 262 mμ and pH 7.2) were obtained for 5-methyluridine and deoxyuridine respectively.

bestic point at shorter wave lengths [27]. In comparison to thymidine [27] the sugar dissociation of the compound starts at lower pH, as indicated by the difference between the pH 12 and pH 13 curves. Furthermore, the shift of the isosbestic point from 2 475 Å to 2 685 Å is larger than the corresponding shift for thymidine (2 475 Å to 2 640 Å). Both of these results show that the compound is a riboside and not a deoxyriboside.

The light absorption data are thus in full accordance with a formulation of the compound as 5-methyluracil-ribofuranoside.

Sugar reactions. 5-Methyluridine gave a positive orcinol test for ribose [28]. Color development was not complete after two hours and was considerably lower than that of adenosine. Parallel experiments with uridine showed similar results.

When deoxyuridine was compared with thymidine in the cysteine-sulfuric acid test for deoxyribose [29] it was found that deoxyuridine after one hour showed 83 % of the amount of color obtained from an equimolar amount of thymidine.

Paper chromatography. Each ^{14}C compound used in the animal experiments showed a single radioactive and light absorbing (Mineralite lamp) spot on paper in all the tested solvents. A summary of the different R_F-values is given in Table 1.

Table 1. R_F values of ^{14}C-pyrimidines and nucleosides. The spots were localized by radioactivity and by ultra violet absorption (except in the pyridine containing solvent).

	Butanol : water 87 : 13	Butanol : conc. NH_3 75 : 25	*t*-butanol : pyridine : water 65 : 25 : 15
Uracil	0.34	0.20	0.60
Thymine	0.49	0.41	0.67
Uridine	0.17	0.07	
Thymidine	0.48	0.41	0.74
Deoxyuridine	0.34	0.15	0.71
5-Methyluridine	0.31	0.20	0.66

Metaperiodate titration. The furanose structure of the nucleosides was established by titration with metaperiodate as described by Manson and Lampen [30]. Deoxyuridine did not consume any periodate during 24 hours. 5-Methyluridine (21 μmoles) consumed 20.2 μmoles of periodate under the same conditions.

Incorporation experiments

Thymine derivatives. White albino rats weighing 150—155 g each were subjected to partial hepatectomy by the technique used in this laboratory [31]. Each isotopic compound was dissolved in a total of 4 ml of physiological sodium chloride and injected subcutaneously in the back of a single rat 24 and 26 hours after the operation. Five hours after the last injection the animal was killed. The liver and the thoroughly washed small intestine were immediately removed and put into alcohol.

The pyrimidines of the polynucleotides from the regenerating liver and the small intestine were prepared and analyzed for ^{14}C with the results shown in Table 2. The purines are not included in the table since all analyses here and in the experiments with uracil derivatives showed insignificant amounts of isotope. Similar experiments with orotic acid are also included in the table for comparison.

Table 2. Incorporation of thymine-^{14}C compounds and orotic acid-^{14}C into regenerating liver and intestine. Specific activities of precursors (count/min/μmole): thymine: 140 000; thymidine: 14 000; 5-methyluridine: 140 000; orotic acid: 310 000. The values in the table are given for a specific activity of 100 000 count/min/μmole in the precursors.

Isolated	Precursor	thymine		thymidine		5-methyl-uridine		orotic acid	
	μmoles injected	6	40	6	40	6	40	6	40
Liver	*PNA:* uridylic acid	5	15	0	0	15	15	1 600	6 500
	cytidylic acid	5	7	25	0	0	10	600	5 200
	DNA: thymine	30	340	290	7 500	70	380	600	1 500
	cytosine	10	25	25	0	5	0	420	1 000
Intestine	*PNA:* uridylic acid	0	20	30	—	15	20	45	380
	cytidylic acid	0	15	10	5	0	10	35	270
	DNA: thymine	5	15	2 200	4 400	20	0	45	230
	cytosine	0	7	0	25	0	0	25	100

Thymidine was a very efficient precursor for DNA thymine in both liver and intestine. Thymine and 5-methyluridine showed a similar behaviour in that both were utilized to a significant extent for the synthesis of DNA thymine by liver but not by the intestine. Orotic acid was well utilized for the synthesis of all liver pyrimidines. The same general pattern was obtained in the intestine, although the amount of ^{14}C incorporation there was much smaller.

Uracil derivatives. Table 3 shows similar experiments with different uracil containing compounds. Free uracil showed a small but significant incorporation into PNA uracil in some experiments. There was a still smaller utilization of uracil for PNA cytosine and DNA thymine. Uridine was significantly incorporated into all pyrimidines at doses of 40 and 100 μmoles, with maximum incorporation into PNA uracil. Deoxyuridine was found to be well utilized for DNA thymine synthesis by both liver and intestine. In the intestine it was also slightly incorporated into PNA uracil. Uridine-5-phosphate was not significantly utilized for pyrimidine synthesis in the liver, but was somewhat more efficient than uridine for PNA uracil synthesis in the intestine.

Table 3. Incorporation of uracil-^{14}C compounds into regenerating liver and intestine. Specific activities of precursors (count/min/µmole): Uracil: 98 000 ct.; uridine: 62 000; deoxyuridine; 98 000; UMP: 38 000. The values in the table are given for a specific activity of 100 000 count/min/µmole in the precursor.

Isolated	Precursor	uracil		uridine			deoxy-uridine		UMP
	µmoles injected	40	100	6	40	100	6	40	40
Liver	*PNA:* uridylic acid	10,0	100	10	150	250	3	25	30
	cytidylic acid	20,0	60	1	50	170	0	15	0
	DNA: thymine	10,30	60	15	70	330	180	530	20
	cytosine	3,10	0	3	50	130	10	0	0
Intestine	*PNA:* uridylic acid	—,25	80	15	200	440	60	90	400
	cytidylic acid	30,3	50	10	130	180	10	10	30
	DNA: thymine	40,15	40	0	20	160	185	680	30
	cytosine	40,3	0	0	30	130	5	10	30

One rat received a total of 100 µmoles of deoxyuridine-^{14}C divided into 6 doses during 3 days. Incorporation in the intestine was again limited to DNA thymine (and PNA uracil) as shown in Table 4.

DISCUSSION

The first consideration which must be discussed is that of the chemical nature and purity of the enzymically synthesized nucleosides. Crystallization of the substances was not carried out and elementary analyses and melting points are therefore not available. Identification of the compounds as 5-methyluridine and deoxyuridine rests on the following points: (a) sugar reactions, which show the presence of ribose and deoxyribose respectively, (b) metaperiodate titration, which showed that the compounds had a furanose structure, (c) light absorption data at different pH and, (d) the mode of synthesis.

Table 4. Incorporation of deoxyuridine-^{14}C (98 000 ct/min./µmole) into intestinal nucleic acids of a non-hepatectomized rat.

		specific activity (ct/min./µmole)
PNA:	adenine	0
	guanine	0
	uridylic acid	70
	cytidylic acid	30
DNA:	adenine	0
	guanine	0
	thymine	2 300
	cytosine	20

In addition to the rate of polynucleotide synthesis the relative incorporation of the various isotopic compounds might be determined by other uncontrolled effects such as the permeability of the cells for the precursor, the size of the "precursor pool" in the body and the presence of competing processes which keep the precursor from reaching the site within the cell where it is utilized for polynucleotide synthesis. In the case of the nucleosides this last factor may have played a very important role because of the high concentration of nucleoside phosphorylases [32] and possibly hydrolases in most organs. The demonstrated specificity of some of these enzymes for uridine might possibly explain the lower incorporation of this nucleoside in comparison to cytidine [6]. The possibility of the metabolic inhomogeneity of the polynucleotides [33] introduces one more reason why great caution must be exercised when interpreting the "effectiveness" of different precursors.

A general idea of the extent to which some of these factors influence the observed amounts of incorporation might be obtained by comparing the utilization of a precursor at different dose levels. This was done in the present investigation not with the purpose to gain information about rates of polynucleotide biosynthesis but in order to find out if the administered precursor could be considered as an intermediate in the conversion of ribose to deoxyribose derivatives. One reason why these incorporation experiments do not measure rates of pyrimidine synthesis is that one cannot decide, whether a net synthesis or an exchange reaction is observed.

The results with the *thymine-^{14}C compounds* (Table 2) confirmed the earlier demonstrated specific incorporation of thymidine-^{15}N into DNA thymine. Thymidine-^{14}C incorporation was of the same order of magnitude as that of orotic acid in the liver, while in the intestine orotic acid incorporation was much smaller. This was largely the result of the low utilization of orotic acid in extra hepatic organs in experiments of relatively short durance.[34]

Thymine and 5-methyluridine showed the same general incorporation pattern. In confirmation of the finding of Holmes *et al.* [5] some utilization of thymine-^{14}C was found for DNA thymine biosynthesis in regenerating liver (but not in the intestine), especially at the higher dose level. The similarity of the results between the two substances makes it seem likely that 5-methyluridine was incorporated after breakdown to free thymine.

All *uracil-^{14}C compounds* were incorporated to some extent. In some experiments free uracil was significantly incorporated into PNA pyrimidines and into DNA thymine. The incorporation was very small and the possibility exists that it occurred after breakdown to CO_2. In all these experiments, however, the purines showed no significant incorporation of isotope, which is evidence for a specific incorporation of the labeled uracil.

Uridine was incorporated at the two higher dose levels into all polynucleotide pyrimidines five to ten times more effectively than free uracil. The mechanism of uridine incorporation did not therefore involve conversion to free uracil. The results were closely dependent on the amount of uridine injected, and at the lowest dose level no incorporation could be observed. This and the corresponding result with thymidine incorporation in liver might be explained by the action of nucleoside phosphorylase on the injected precursors. At low levels the nucleosides might have been almost completely split

by the enzyme and therefore were not available for polynucleotide synthesis. At higher doses increasing amounts of the injected nucleoside would be available for synthetic processes. These considerations make impossible direct comparison of nucleoside incorporation as was done earlier with uridine and cytidine [6].

UMP was slightly more effectively incorporated than uridine in the intestine, but incorporation in the liver was very small. In this case the well known low permeability of nucleotides might explain the low incorporation. It is not possible therefore to conclude from *in vivo* experiments whether the incorporation of uridine takes place via uridine-5-phosphate.

A type of incorporation was found with deoxyuridine which was similar to that of thymidine rather than uridine. It was almost exclusively incorporated into DNA thymine both in regenerating liver and intestine. A small but significant conversion to PNA uracil was observed in the intestine but not in liver.

The incorporation data are in accordance with the view that uridine (or a derivative of uridine) is transformed by the rat to DNA thymine and that deoxyuridine (or derivative) and thymidine (or derivative) are intermediates in this process. The first step involves a reduction at C_2 of ribose while the second step consists of the attachment of a methyl group to the uracil ring. Deoxyuridine might also arise through deamination of deoxycytidine. The possibility of phosphorylated derivatives as intermediates for these reactions rather than nucleosides must be strongly considered. In liver preparations uridine is quite rapidly phosphorylated to UMP in the presence of ATP *. Phosphorylated intermediates rather than nucleosides are furthermore indicated in pyrimidine biosynthesis from orotic acid [35, 25] and in purine biosynthesis [36, 37]. This question can be answered only from experiments at the enzyme level.

An attempt to summarize the available incorporation data is made in Fig. 3. It is probable that the diagram will have to be modified by future results,

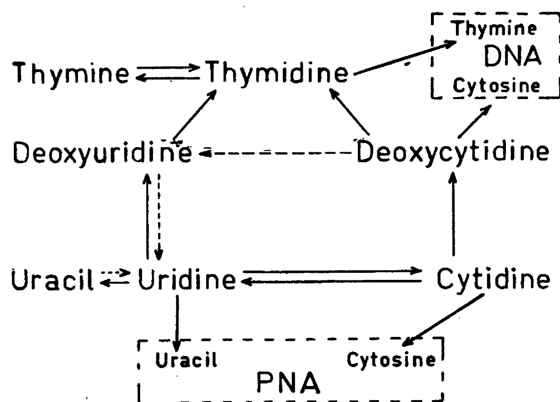


Fig. 3. Possible interrelations between nucleosides in the rat.

* Unpublished experiments.

especially with respect to the level of phosphorylation of the intermediates involved.

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Note added in proof. Friedkin and Roberts (*Federation Proc.* **14** (1955) 215) have recently briefly described the incorporation of deoxyuridine into DNA thymine from suspensions of chick embryo and bone marrow. These observations are in accordance with the intermediate position of deoxyuridine in DNA synthesis as pictured in Fig. 3.

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