Biochemical Pharmacology, 1962, Vol. 11, pp. 323–336. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE METABOLISM OF THYMINE AND 6-AZATHYMINE*

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(*Received* 22 *November* 1961; *accepted* 18 *December* 1961)

Abstract—The administration of 6-azathymine to the mouse led to the urinary elimination not only of free azathymine, but also of various metabolites of it. Of these metabolites, two have been identified: (a) the ribonucleoside, (b) a side-chain oxidation product of azathymine, tentatively identified as 5-hydroxymethyl-6-azauracil (6-hydroxymethyl-*as*-triazine-3:5-(2H:4H)-dione). Other metabolic derivatives of azathymine were produced, but their nature has not been established conclusively. The administration of azathymine also resulted in the excretion of relatively large amounts of free uracil. Following the administration of 6-azathymine-5-^{14}C to mice, radioactivity was found in all tissues investigated, not only in the form of free azathymine, but also as metabolic derivatives. A study of the influence of mouse liver slices or homogenates on radioactive azathymine and thymine indicated that each of these compounds is converted to the corresponding ribonucleoside and, to a lesser degree, to the analogous 2′-deoxyribonucleoside.

INTRODUCTION

THESE studies are an extension of earlier studies which were concerned with the investigation of the mechanism of action of a thymine analog, 6-azathymine (6-methyl-*asym*-triazine-3 : 5-(2H : 4H)-dione), a substance in which a nitrogen atom replaces the carbon and hydrogen in position-6 of thymine.[1–4] This pyrimidine analog is a competitive antagonist of the utilization of thymine and of thymidine for the growth of several micro-organisms. Thus, *Streptococcus faecalis* (ATCC 8043) not only converts azathymine to the deoxyribonucleoside, azathymidine, but, under certain circumstances, incorporates this analog into the deoxyribonucleic acid (DNA) of the cells. However, this latter process does not appear to be the mechanism whereby inhibition of microbial reproduction occurs, since incorporation can occur in the absence of inhibition of growth.[4]

Thymidine[5] but not free thymine[6–8] is utilized extensively by mammalian systems; accordingly, the chemotherapeutic potential of the deoxyribonucleoside of the analog was investigated. Ellison *et al.* have reported that azathymine *per se* produced no improvement in patients with either acute leukemia or a variety of solid tumors.[9] Since the renal excretion of azathymidine is very rapid in mice, an attempt was made to decrease the excretory rate by administering relatively large amounts of azathymine

* These studies were supported by grants from the National Cancer Institute (C-3076 and C-5262). One of us (R.A.G.) was the recipient of a Public Health Service Training Grant (CRTY-5012). Preliminary reports have been presented.[15, 17]

simultaneously, in order to maintain a high level of azathymidine in blood and other tissues. This approach was based on the hypothesis that the renal excretion of azathymidine might involve active transport. Although the desired objective was not attained, unidentified ultraviolet-absorbing areas were observed upon subjecting the urine to paper chromatography.

This report describes a study of the metabolism of azathymine by the mouse under various conditions, *in vivo* and *in vitro*, and the isolation, identification and biological assay of two metabolically formed derivatives of azathymine. Investigations have also been conducted on the metabolism of thymine by tissues of the mouse, *in vitro*.

EXPERIMENTAL

Materials

Three compounds were obtained from Dr. Ross Hall, formerly of the American Cyanamid Company, Pearl River, New York: (a) 6-azathymine, (b) a synthetically prepared ribonucleoside of azathymine[10], and (c) a sample of azathymidine formed enzymically from azathymine.[2] 6-Azathymine-5-^{14}C (6-methyl-*as*-triazine-3 : 5-(2H : 4H)-dione-6-^{14}C) was prepared in this department by a modification of published procedures.[11, 12] Thymine, thymidine and the other compounds were commercial preparations; their purity and identity were checked by standard spectrophotometric and microbial assay methods.

A heat-inactivated extract of liver was prepared by mincing 2 g of fresh mouse liver, transferring it to a beaker containing 6 ml of boiling water and heating for 2 min. After cooling in an ice-water bath, the material was ground in a Potter–Elvehjem homogenizer, centrifuged at 5000 **g** for 30 min; the supernatant fluid was decanted and frozen immediately.

Animals

DBA/2 mice (purchased from the Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine) and mice of the Swiss strain (S.W.R.) (purchased from the Millerton Research Farm, Millerton, New York) weighing 20–26 g were used.

Methods of analysis

Azathymine was determined with the Beckman spectrophotometer, model DU; 2′-deoxyribose by the method of Stumpf[13]; and ribose by the orcinol reaction, using the method of Mejbaum[14].

Methods of urine collection and chromatography

Urine was collected either by absorption on filter paper or in a bottle, using metabolism cages constructed to keep the mice separated from each other and from the collecting device by wire-screening. Following the administration of the compounds, the mice were denied access to food until sacrificed, at which time the urinary bladder was excised and residual urine was obtained. Either the water-eluate of the filter paper or the collected urine was concentrated and subjected to descending paper chromatography, using Whatman-3 paper and a solvent system composed of ethyl acetate-phosphate buffer (0·05 M, pH 6).[2]

Measurements of radioactivity

1. *Quantitative.* Suitable aliquots were evaporated to dryness in stainless steel planchets and the radioactivity was measured in either a manual Packard or a Nuclear automatic windowless flow counter.

2. *Qualitative.* Areas of 1 cm length were cut serially from paper chromatograms, inserted into stainless steel planchets and the radioactivity was measured in a windowless flow counter.

Preparation of cold acid-soluble and combined-nucleic acid fractions

The appropriate tissue was homogenized with 10 vols. of 7% trichloroacetic acid (TCA) in a Waring blendor or in a Potter–Elvehjem tube. After transfer to a 20 ml glass centrifuge tube, the homogenate was centrifuged at 500 **g** for 20 min; the supernatant fraction was decanted and stored at 0°, while the centrifugate was resuspended with 10 vols. of 7% TCA at 0° and centrifuged. The combined supernatant fluids are referred to as the cold acid-soluble fraction. After washing the centrifugate twice more with 10 vols. of 7% ice-cold TCA, it was resuspended in 10 vols. of 5% TCA and heated at 90° for 30 min in a water bath. After centrifugation, a supernatant fraction was obtained which was termed the combined-nucleic acid fraction. The TCA was extracted with ethyl ether from the two fractions and the residual ether was removed by heating in a bath of hot water.

TABLE 1. IDENTIFICATION OF URACIL AS A COMPONENT OF THE URINE OF MICE INJECTED WITH AZATHYMINE

Determination	Uracil	Urinary component
Spectra:		
Acid:		
Max	259	259
Min	227	227
Alkali		
Max	284	284
Min	241	241
Mobility ($R_f \times 100$):		
*Solvent 1	14	15
2	34	34
3	46	46
4	67	69
Growth response of *Lactobacillis bulgaricus*	++++	++++

* Composition of solvent systems by volume: solvent 1, upper phase from a mixture of ethyl acetate saturated with 0·05 M phosphate buffer, pH 6; solvent 2, *n*-butanol-concentrated ammonium hydroxide (100:1); solvent 3, *n*-butanol–glacial acetic acid–water (50:10:25); solvent 4, *iso*butyric acid–water–25% solution of ammonium hydroxide (400:208:0·4).

Method of microbiological assay

Two levels (1 and 10 μmoles) of the isolated azathymine derivatives were added to tubes which contained basal medium and constant amounts of thymine, thymidine or pteroylglutamic acid (PGA). The extent of growth was determined at 16 and 24 hr

after inoculation with *Streptococcus faecalis* (ATCC 8043) by measurement of turbidity in a Klett–Summerson photoelectric colorimeter (filter 62).

RESULTS

Metabolism of radioactive azathymine by the mouse in vivo

When 6-azathymine-5-^{14}C was administered to DBA/2 mice and the urine subjected to paper chromatography in the ethyl acetate-phosphate buffer system,[2] three radioactive areas (R_f : 0·00, 0·15, 0·63) were observed; that with an R_f of 0·63 was identified, on the basis of spectra and mobility, as free azathymine. The intermediate zone contained three components: (a) a non-radioactive substance which, on the basis of u.v.-spectra, mobility studies and microbial response, was identified as free uracil (Table 1); (b) a radioactive metabolite the u.v.-absorption of which resembled that of azathymidine[15] although it contained no deoxyribose; and (c) a radioactive compound, to be described in detail, the elementary analysis of which suggested that the methyl substituent of azathymine had been oxidized to an hydroxymethyl group (Tables 2 and

TABLE 2. ABSORPTION MAXIMA OF VARIOUS AZATHYMINE DERIVATIVES

Compound	1 N HCl	pH 11	1 N KOH
Synthetic azathymine ribonucleoside	264		254
Urinary azathymine ribonucleoside	262		254
Azathymine deoxyribonucleoside	265		252
Azathymine	260	245	288
3-Methylazathymine*	260		298
1:3-Dimethylazathymine*	274		273
Urinary 5-hydroxymethyl-6-azauracil	258	253	288

* Obtained from the literature.[10]

TABLE 3. MOBILITIES ON PAPER CHROMATOGRAMS IN VARIOUS SOLVENT SYSTEMS OF A SYNTHETIC PREPARATION OF AZATHYMINE RIBONUCLEOSIDE AND OF TWO METABOLIC DERIVATIVES OF AZATHYMINE: AZATHYMINE RIBONUCLEOSIDE AND 5-HYDROXYMETHYL-6-AZAURACIL, DERIVED FROM THE URINE OF MICE INJECTED WITH AZATHYMINE

Solvent system*	R_f values × 100†		
	Synthetic azathymine ribonucleoside	Urinary azathymine ribonucleoside	Urinary 5-hydroxy-methyl-6-azauracil
1	7	7	13
2	7	7	5
3	54	54	49
4	69	69	63
5	70	69	60

* Composition of solvent systems by volume: solvent 1, upper phase from a mixture of ethyl acetate saturated with 0·05 M phosphate buffer, pH 6; solvent 2, *n*-butanol–concentrated NH_4OH (100:1); solvent 3, *n*-butanol–glacial acetic acid–water (50:10:25); solvent 4, *n*-propanol–glacial acetic acid (50:50); solvent 5, *n*-propanol–concentrated hydrochloric acid–water (66:33:1).

† Whatman no. 1 chromatographic paper.

3). The u.v.-spectrum indicated that the N-atoms were unsubstituted. The first radioactive compound (b), R_f 0·15, gave a positive orcinol reaction (Fig. 1) and the u.v.-absorption spectrum (Table 2) and chromatographic mobility (Table 3) were similar to those of the synthetic ribofuranoside of azathymine (2-D-ribofuranosyl)-6-methyl-*asym*-triazine-3 : 5-(2H : 4H)-dione)[10]; an elementary analysis yielded a carbon-content of 41 per cent, a finding which agreed with the calculated value for azathymine ribonucleoside (41·5%). The radioactive material which remained at the point of origin had no mobility upon re-chromatography in butanol–water and no additional attempts were made to characterize this substance (or these substances).

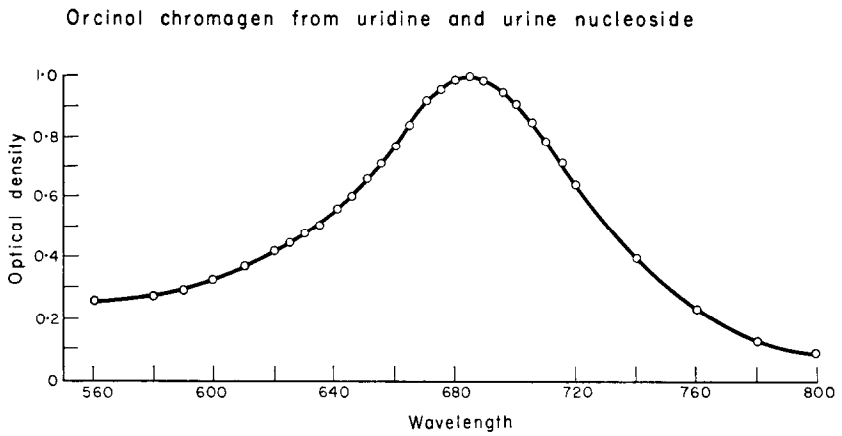

FIG. 1. Absorption spectra of chromagens derived from uridine and urinary 6-azathymineribonucleoside in the orcinol reaction.

Rate and pattern of urinary excretion of metabolic derivatives of radioactive azathymine

Each of 5 DBA/2 mice was injected with 2 μmoles of 6-azathymine-5-^{14}C; the urine was collected at 4, 8 and 24 hr intervals and chromatographed in the ethyl acetate-phosphate buffer system and the distribution of the radioactivity in the three areas was determined (Table 4). Within 4 hr essentially all of the free azathymine (R_f 0·63) was excreted, since there was no significant increase in this fraction over a 24 hr interval. The area with an R_f of 0·15, composed primarily of a mixture of the side-chain oxidation product and the ribonucleoside, gradually increased in content from an amount equivalent to that of the excreted free azathymine to about twice that amount within 24 hr; however, no attempt was made to determine the precise amounts of these two major metabolites. The metabolites of azathymine which possessed no mobility in this solvent system represented about 5 per cent of the radioactivity excreted within from 4 to 8 hr; however, within 24 hr, this fraction increased four- to five-fold.

Tissue distribution of radioactive azathymine

6-Azathymine-5-^{14}C (2 μmoles) was injected intraperitoneally into each of eight mice and radioactivity was found in all tissues analysed. These tissues (spleen, lung, kidney,

liver, heart, gastro-intestinal tract) were fractionated into a cold acid-soluble and a combined-nucleic acid fraction; the radioactivity was associated almost exclusively with the former (Table 5). The cold acid-soluble fraction was chromatographed in the ethyl acetate-phosphate buffer system and the distribution of the radioactivity on the paper chromatogram followed the pattern observed in urine. However, the liver had an additional radioactive peak which was subsequently identified as the 2′-deoxy-ribonucleoside of azathymine (azathymidine).

TABLE 4. URINARY RATE AND PATTERN OF EXCRETION OF AZATHYMINE AND ITS RADIOACTIVE METABOLIC DERIVATIVES IN MICE INJECTED WITH 6-AZATHYMINE-5-^{14}C*

Excretion time (hr)	Urinary excretion products†		
	R_f 0·63 (μmoles)	R_f 0·15 (μmoles)	R_f 0·00 (μmoles)
4	0·53	0·56	0·05
4	0·31	0·56	0·04
8	0·49	0·52	0·05
24	0·64	1·08	0·18

* Each mouse was injected intraperitoneally with 2 μmoles of 6-azathymine-5-^{14}C (8×10^5 counts/min per μmole).

† Separated into three areas by paper chromatography using the ethyl acetate-phosphate buffer system: R_f 0·00, unknown derivatives; R_f 0·15, 5-hydroxymethyl-6- azauracil and trace amounts of azathymine ribonucleoside; and R_f 0·63, free azathymine.

TABLE 5. TISSUE DISTRIBUTION OF RADIOACTIVITY IN MICE INJECTED WITH 6-AZATHYMINE-5-^{14}C

Tissue	Cold acid-soluble fraction		Combined-nucleic acid fraction	
	Total counts (counts/min)	Counts/100 mg* (counts/min)	Total counts (counts/min)	Counts/100 mg* (counts/min)
Liver	46,900	711	875	13
Lung	22,000	2550	80	9
Spleen	3,570	1320	120	5
G.I.†	247,000	1720	550	7
Kidney	45,400	3200	90	6
Heart	8300	1100	40	5

* Wet weight.
† Gastrointenstinal tract.

Metabolism of radioactive azathymine by mouse liver in vitro

Incubation mixtures composed of 1 g of homogenized or sliced mouse liver in tris buffer (3·0 ml, 0·01 M, pH 7·4) and 6-azathymine-5-^{14}C (3·65 μmoles, 7·3 μc) in 20 ml beakers were placed in a Dubnoff metabolic shaker at 37° for 2 hr under an atmosphere of air. The contents of each beaker were then separated into cold acid-soluble and combined-nucleic acid fractions. Accordingly, aliquots were taken from these fractions

and subjected to chromatography in the ethyl acetate-phosphate buffer system and the distribution of radioactivity was determined. Fig. 2 shows that the radioactivity of cold acid-soluble fractions separated into four areas tentatively identified: AzT, unreacted free azathymine; X, unknown metabolites; R, azathymine ribonucleoside; dR, azathymine 2′-deoxyribonucleoside. The area corresponding to the 2′-deoxyribonucleoside of azathymine (dR) formed with slices was greater than that produced by the homogenate. The radioactivity found in the combined-nucleic acid fraction was negligible, in agreement with the above *in vivo*-experiment. Unlabeled markers (azathymine ribonucleoside, azathymidine, and azathymine) were added to an aliquot of the acid-soluble fraction derived from the incubated liver slices and then subjected to paper chromatography in the ethyl acetate-phosphate buffer system. The superimpostion of the radioactive areas with the known markers gave presumptive evidence of their identity.

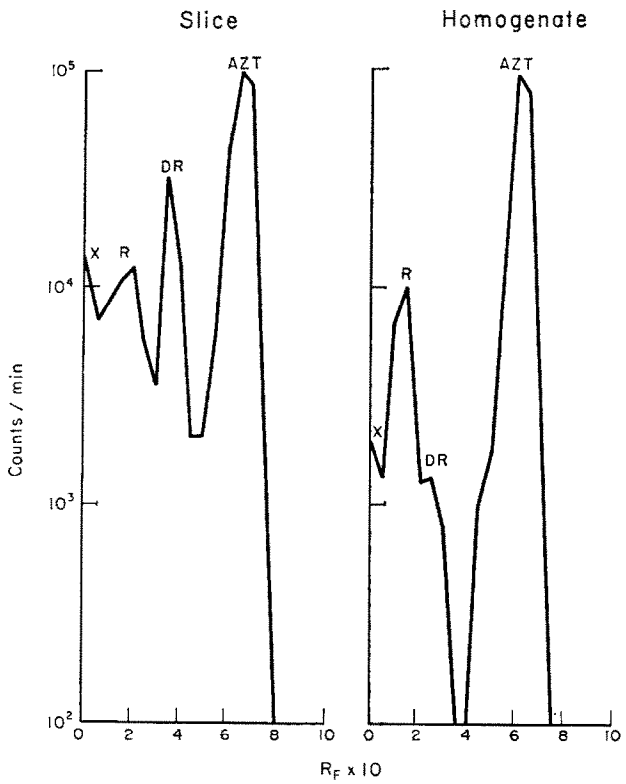

FIG. 2. Chromatographic separation of the cold acid soluble fraction of mouse liver slices or homogenate following incubation with radioactive 6-azathymine-5-^{14}C. The solvent system was ethyl acetate saturated with phosphate buffer (0·05 M, pH 6).

Preparation of the ribonucleoside and the side-chain oxidation product of azathymine

Each of 126 mice (females) was injected intraperitoneally with 100 μmoles of azathymine and the urine collected for 24 hr, during which period food but not water was restricted. Acetone (200 ml) was added to a filtered concentrate of the urine and the mixture was kept at 0° for 1 hr. The suspension was filtered and the acetone

removed by gentle heating in a bath of hot water. The filtrate was subjected to descending paper chromatography in the ethyl acetate-phosphate buffer system using Whatman-3 paper.

The area at R_f 0·00 was removed and new filter paper stapled on in its place. Chromatography was continued in the same direction using the butanol–water system. These chromatograms demonstrated three large u.v.-absorbing areas: azathymine, uracil, and the desired metabolites of azathymine. The area which contained the last-named was removed, eluted with water in a Waring blendor, and the eluate was concentrated to a small volume. The concentrate was subjected to re-chromatography in the butanol–water system in order to insure the separation of any contaminating uracil. Once again the area which contained the desired azathymine metabolites was cut from each paper and eluted with water in a Waring blendor. The eluate was made alkaline with 15 N NH_4OH (35 ml per l.), adsorbed on a Dowex-1 formate ion-exchange column and eluted with gradually increasing concentrations of formic acid. U.v.-absorbing material appeared in the effluent when the concentration of formic acid reached 0·01 N. This effluent was concentrated, lyophilized and subjected to chromatography in the butanol–water and the ethyl acetate-phosphate buffer systems. The latter showed separation of the material into two u.v.-absorbing areas. The minor component corresponded to that of azathymine ribonucleoside and the major component to that of the side-chain oxidation derivative of azathymine (5-hydroxymethyl-6-azauracil). These substances were eluted independently and subjected to ion-exchange chromatography on Dowex-1 formate columns; each was eluted by formic acid when the concentration reached 0·01 N. The yield of the side-chain oxidative product was approximately 107 mg. The elementary analysis was: C, 32·52; H, 3·36; O, 34·72; N, 27·60; calculated: C, 33·6; H, 3·5; O, 33·6; N, 29·4. The slight discrepancy between the analysis and that calculated for 5-hydroxymethyl-6-azauracil can be explained by the finding of a residue of 4·03 per cent in the preparation. The compound had a melting point range of 198°–204 °C and spectral data are shown in Table 2. The R_f values in five solvent systems are shown in Table 3.

The yield of azathymine ribonucleoside was about 2·0 mg. An orcinol reaction was performed, using uridine as the control and, as can be seen from Fig. 1, uridine and the ribonucleoside of azathymine gave identical chromagens on the basis of their spectra. Although the chromagens formed were identical, the amount produced per μmole of nucleoside was different: 1 μmole of ribose was equivalent to 2·5 μmole of uridine, 9·2 μmole of the synthetic, and 9·4 μmole of the urinary, azathymine ribonucleoside. The biologically formed azathymine ribonucleoside and that prepared synthetically had identical R_f values in five solvent systems (Table 3). The spectral data for the biologically prepared azathymine ribonucleoside agreed well with those of the synthetic preparation (Table 2).

Biological activity of the isolated metabolites of azathymine

Both azathymine ribonucleoside and the side-chain oxidation metabolite were biologically inactive in the systems investigated. Each, in contrast to azathymidine[16], failed to inhibit the utilization of formate-^{14}C in the biosynthesis of the methyl groups of DNA-thymine by Ehrlich ascites tumor cells *in vitro*. Neither compound inhibited the growth of *Streptococcus faecalis* in media supplemented with thymine, thymidine or folic acid, even at molar levels 400 times those of the pyrimidine metabolites.

Metabolism of thymine by mouse liver in vitro

Reaction mixtures composed of 1 g homogenized or sliced mouse liver in tris buffer (3·0 ml, 0·01 M, pH 7·4), and thymine-2-^{14}C (3 μmoles, 3μc) were incubated aerobically in 20 ml beakers in a Dubnoff metabolic shaker at 37° for 2 hr. The contents of each beaker were then separated into cold acid-soluble and combined-nucleic acid fractions. Suitable aliquots were subjected to paper chromatography in the ethyl acetate-phosphate buffer system and the distribution of radioactivity on the chromatogram was determined (Fig. 3). The combined-nucleic acid fraction contained no significant amount of radioactivity.

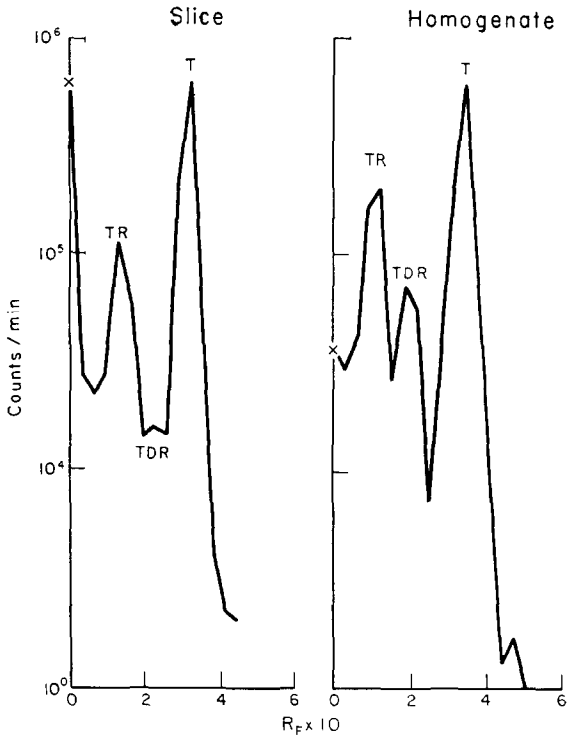

FIG. 3. Chromatographic separation of the cold acid soluble fraction of mouse liver slices or homogenate following incubation with radioactive thymine-2-^{14}C. The solvent system was ethyl acetate saturated with phosphate buffer (0·05 M, pH 6).

In addition to the radioactivity at the origin (X), there were three other radioactive areas on each chromatogram, arbitrarily designated thymine ribonucleoside (TR), thymidine (TdR), and thymine (T). The TR and TdR areas were eluted and digested in 12 N perchloric acid at 100° for 1 hr in order to prove that these radioactive areas were derivatives of thymine. Non-radioactive thymine was added to the digestants prior to chromatography in the ethyl acetate-phosphate buffer system. The radioactive area coincided with that which, with thymine, absorbs in the ultraviolet. Identification of TR and TdR as metabolic products of the incubation reaction, as

well as of free thymine, was made by comparison of the mobilities of these radioactive areas with those of known added markers: TR, TdR and T, in six solvent systems (Table 6). Since each marker contained all the radioactivity, sufficient evidence of identity probably has been obtained.

The formation of thymine ribonucleoside or of thymidine from radioactive thymine by homogenates or slices of liver from mice could be markedly influenced[17]; thus, the

TABLE 6. IDENTIFICATION OF THYMINE DERIVATIVES FORMED FROM THYMINE BY MOUSE LIVER *in vitro**

Compound	Solvent system†	R_f value 100	
		Isolated‡	Standard§
Thymidine	1	73	73
	3	65	65
	4	67	67
	6	34	34
Thymine ribonucleoside	1	66	66
	2	70	70
	4	53	53
	6	24	24
Thymine	1	69	69
	2	86	86
	5	46	46
	6	51	51

* Details of incubations are described in the text. The appropriate non-radioactive derivative was added to the biosynthesized components prior to chromatography.

† Composition of solvent systems[32] by volume: solvent 1, *tert.*-butanol–methyl ethyl ketone–water–ammonium hydroxide (40:30:20:10); solvent 2, upper phase from a mixture of water–*sec.*-butanol–*tert.*-butanol (47·5: 42·5:8·5); solvent 3, *tert.*-butanol–methyl ethyl ketone–formic acid–water (40:30:15:15); solvent 4, *tert.*-butanol–methyl ethyl ketone–water–formic acid (44:44:11:0·264); solvent 5, upper phase from a mixture of *n*-butanol–water–ammonium hydroxide (60:30:10); and solvent 6, upper phase from a mixture of ethyl acetate–water–formic acid (60:35:5). When a two-phase system was used, a 20-ml beaker containing 15 ml of the lower phase was placed in the bottom of the chromatographic jar.

‡ The R_f of the isolated component was determined from the mobility of its radioactivity.

§ The R_f of the added non-radioactive standard was determined by u.v.-absorption.

addition of nonradioactive thymidine or thymidine-5′-phosphate to the reaction mixture decreased the formation of radioactive thymine ribonucleoside, whereas the formation of radioactive thymidine increased. Under the same conditions, the inclusion of non-radioactive thymine ribonucleoside increased the formation of radioactive thymine ribonucleoside. Neither azathymine ribonucleoside nor azathymidine inhibited the formation of thymine ribonucleoside.[17]

A sucrose (0·25 M) homogenate of liver from mice was separated by differential centrifugation into nuclei, mitochondria, microsomes and a particle-free fraction, and the major enzymic activity was observed in the particle-free fraction (obtained at 100,000 g, 1 hr).

In an attempt to elucidate the mechanism of deoxyribonucleoside formation, studies were performed with uracil derivatives since radioactive thymine ribonucleoside had not been available in 1957. Uniformly labeled ^{14}C-uridine was incubated with the particle-free fraction (100,000 g) of a homogenate of mouse liver in Tris buffer (0·05 M, pH 7·4). Following incubation for 1 hr, the cold acid-soluble extract was

TABLE 7. SPECIFIC ACTIVITIES OF THE 2′-DEOXYURIDINE AND ITS URACIL MOIETY DERIVED FROM THE INCUBATION OF TOTALLY LABELED URIDINE WITH A PARTICLE-FREE HOMOGENATE OF MOUSE LIVER

Isolated compound	Specific activity		Ratio nucleoside:uracil
	Nucleoside (counts/min per μmole)	Uracil (counts/min per μmole)	
Uridine	250,000	99,300	2·5
2′-Deoxyuridine	3260	3020	1·07

chromatographed in the manner described above; the compounds found included unhydrolysed uridine and radioactive uracil, but 2′-deoxyuridine was not present. When non-radioactive 2′-deoxyuridine was included in the reaction mixture, however, radioactive 2′-deoxyuridine was isolated as well. The specific activity of the uracil and 2′-deoxyuridine formed were determined, as well as that of the uracil obtained after hydrolysis with perchloric acid (12 N, 100°, 1 hr). The results, shown in Table 7, indicate that the radioactivity present in the 2′-deoxyuridine is derived from the uracil moiety and, hence, that the formation of the 2′-deoxynucleoside involved the preliminary cleavage to free uracil rather than direct conversion of the ribonucleoside to the deoxyribonucleoside.

DISCUSSION

A study of the metabolism of azathymine both *in vitro* and *in vivo* revealed that mammalian enzymes, as do their counterparts in microbial systems, accept this compound as a substrate. For many bacteria, azathymine and azathymidine are potent inhibitors of growth. Mantagazza *et al.* reported[18] that azathymine (5-methyl-6-azauracil) exerts a marked effect on the central nervous system in various mammalian systems. This was confirmed and extended to other 5-alkyl derivatives of this triazine series by Welch and co-workers[19, 20], who observed a direct relationship between the hypnotic activity in mice and the length of the side-chain on carbon-5 (numbered as a pyrimidine). Since in the present investigation the administration of radioactive azathymine to the mouse resulted in the formation of azathymine ribonucleoside, azathymidine and 5-hydroxymethyl-6-azauracil, as well as other non-characterized derivatives, it would appear to be difficult to relate the hypnotic activity[19, 20] to any single structural species. However, the fact that lengthening the side-chain, as in 5-ethyl-6-azauracil, leads to a disappearance of the capacity of these triazines to alter the catabolism of uracil[21] and that substituents other than hydrogen in position-5

reduce the inhibitory effects of 6-azauracil on the growth of tumors[19] strongly suggest that the hypnotic activity of these triazines is an intrinsic property of the unaltered compounds.

6-Azauracil is subjected to similar metabolic alterations observed with the 5-methyl derivative (6-azathymine). In a review[22] of the metabolism of 6-azauracil (*asym*-triazine-3 : 5-(2H : 4H)-dione) in mammalian systems, the formation is discussed of the ribonucleoside and the urinary excretion of the catabolites, glyoxylic acid semicarbazone, oxalic acid, an unidentified acid, and carbon dioxide. Also, the presence in the tissues and urine of various deoxyribonucleosides must be noted: 2′-deoxycytidine,[23, 24] 5-methyl-2′-deoxycytidine[24] and smaller amounts of 2′-deoxyuridine.[24]

Azathymine-^{14}C was not found in DNA even though its 2′-deoxyribonucleoside was formed in the liver of the mouse. This may be explained by the very unfavorable inhibition index which azathymidine possesses.[17, 25] It requires an excess of 1000-fold of azathymidine to inhibit the utilization of thymidine for the bio-synthesis of DNA-thymine. Thus, the incorporation of azathymidine into DNA can be readily prevented by a small pool of thymidylic acid.

It has been demonstrated that relatively large amounts of free uracil are excreted in the urine by the mouse upon administration of azathymine. The co-administration of azathymine and radioactive orotic acid resulted in a very marked increase in the excretion of radioactive uracil[15, 23] and studies *in vitro* with a particle-free preparation of rat liver have revealed that the enzyme(s) known to be involved in the degradation of uracil are inhibited by azathymine.[15, 23] Since it has been previously demonstrated[4] that the incorporation of azathymine into the DNA of *S. faecalis* was not necessarily related to the degree of observed inhibition, it would be of interest to investigate whether the mechanism of action of azathymine in microbial systems might be via the inhibition of a uracil-containing coenzyme. The recent isolation of thymidine diphospho-sugar derivatives[26–30] point to this area also as a possible site of inhibition by azathymine or its derivatives in microbial systems.

Thymine ribonucleoside has been reported previously to be formed enzymically by nucleoside phosphorylase,[31] as well as to be present in the urine of rats[32] and in the RNA of several microbial species.[33] De Verdier and Potter have reported that the formation of the ribonucleoside of thymine, as well as of thymidine, by homogenates of rat liver involve a transfer of ribose and 2-deoxyribose, respectively (through the mediation of their 1-phosphates), since labeled nucleosides were formed only from the radioactive base (thymine or uracil) when the appropriate unlabeled nucleoside was included in the reaction mixtures.[34] In our studies, previously reported in preliminary form[17] and in more detail in this report, the formation of the ribonucleoside and to a lesser extent of the 2′-deoxyribonucleoside of thymine and of azathymine by a particle-free preparation of mouse liver, has been observed in the absence of added nucleoside. However, the yield of the radioactive nucleoside can be markedly increased by inclusion of the corresponding nucleoside. This is probably a result of supplying for condensation with a base acceptor, a large source of pentose-1-phosphate which is used by the nucleoside phosphorylase known to be present.[31] Against the view that the additions might supply a nucleoside for transpentosidations is the very marked decrease in the specific activity of the uracil moiety of the formed 2′-deoxyribonucleoside (see Table 7). This can be explained only by cleavage of uridine

to free uracil, with resultant admixture with the endogenous pool of uracil and subsequent formation of the 2′-deoxyribonucleoside.

Thus, it is clear that pyrimidine-ribonucleosides and -2′-deoxyribonucleosides may be formed in mammalian systems via several routes; these include nucleoside phosphorolysis and transpentosidation.[31, 34–36] In addition, 2′-deoxyribonucleosides may be formed by reduction of the intact riboside,[37] apparently at the diphosphate level.[38] Although pyrimidine ribonucleosides may be formed by condensation of a pyrimidine with 1-pyrophosphoryl-ribose-5-phosphate, there is no evidence for a pathway involving a condensation with a similar phosphorylated derivative of 2-deoxyribose. In contrast to the initial formation *de novo* of the ribofuranosides of purines, the *de novo*-formation of pyrimidine nucleosides has not been observed.

Acknowledgement—We wish to express our sincere appreciation for the technical assistance of Mrs. Angela S. Sleddon.

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