[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF KANSAS SCHOOL OF MEDICINE]

Dehalogenation of Substituted Pyrimidines *in vivo*[1]

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This work was undertaken to investigate the reported deiodination of 5-iodothiouracil to produce thiouracil in dogs and humans. A number of 5-iodo- and 5-bromopyrimidines have been found to undergo the same type of dehalogenation in the rat, and reasons are given for assuming that the nuclear dehalogenations proceed *via* an elimination reaction following metabolic reduction of the 5:6 double bond.

Introduction

It has been found that 5-iodothiouracil undergoes partial deiodination in dogs[2] and humans[3] in such a way as to produce thiouracil in the urine. On chemical grounds, this kind of deiodination would not be expected, since 5-halogeno substituents in rings of this type are markedly inert so long as the 5:6 double bond remains intact, and can be displaced only by vigorous chemical treatment.[4,5] Tissue preparations, as described by Bray, *et al.*,[6] for nonenzymatic sulfhydryl displacement of chlorine in organic compounds, have no effect on 5-iodothiouracil. It is necessary to assume, therefore, that the compound is altered metabolically in some way prior to deiodination, despite the fact that it represents an abnormal substrate. Hence, the object of this work was to determine whether *in vivo* dehalogenation of the kind described is specific either for iodo derivatives, or for a particular pyrimidine ring, and to make some study of the dehalogenation mechanism.

By testing a number of 5-halogenopyrimidines in intact rats, it was found that a number of iodo and bromo (but not chloro) derivatives undergo some degree of dehalogenation *in vivo*, and in every instance the over-all result could be represented as replacement of halogen by hydrogen. In considering the possible mechanisms for such a replacement, the formation of dihydropyrimidines seemed an attractive hypothesis, and was justified by recent findings regarding the metabolic products of uracil and thymine,[7,8] the demonstration of an orotic acid reductase,[9] and the occurrence of dihydrouracil in beef spleen.[10] The dihydropyrimidines undergo ring hydrolysis in dilute sodium hydroxide solution to give ureido acids,[11] but some time ago Fischer and Roeder found that 5-bromo-6-methylhydrouracil spontaneously eliminates hydrogen bromide in such media to yield 6-methyluracil instead of the expected 5-hydroxy derivative.[12] Ring opening apparently was not observed.

Whether either reaction could be obtained under physiological conditions with the saturated compounds was determined by preparing solutions of 5-bromo- and 5-chlorohydrouracil and of 5-bromo-6-methylhydrouracil in isotonic phosphate buffer at *p*H 7.4, and maintaining the solutions at 37–38°. The bromo derivatives slowly lost hydrogen bromide to yield the unsaturated pyrimidines, while the chloro derivative showed evidence of ring opening, but no loss of hydrogen chloride.[13] Thus, results obtained in the animal work with 5-halogenopyrimidines, paralleled those obtained with the 5-halogenohydropyrimidines *in vitro*.

While direct evidence for reduction of 5-halogenopyrimidines *in vivo* was not obtained, the hypothesis of reduction prior to dehalogenation is in accord with present knowledge of pyrimidine metabolism, and provides the simplest explanation for the observed results. It may be noted that any nucleophilic displacement of the halogen *in vivo* would produce a different 5-substituted pyrimidine which would then have to undergo a further reaction to account for the urinary products actually found.

Experimental

Preparation of Compounds.—All halogenopyrimidines used were prepared in this Laboratory by methods described in the literature by ourselves and by others. A new derivative, 5-chlorohydrouracil, was prepared in 31% yield by direct chlorination of 5,6-dihydrouracil in glacial acetic acid at 100°. After recrystallization from 95% alcohol, the compound melted at 215°. *Anal.* Calcd. for $C_4H_5O_2N_2Cl$: Cl, 23.87; N, 18.86. Found: Cl, 23.74; N, 18.91.

Animal Testing.—Compounds were injected subcutaneously into Sprague Dawley rats, 8–16 weeks of age, and the urine subsequently examined for excreted pyrimidines. Depending on solubility, the compounds were either dissolved in isotonic phosphate buffer at *p*H 7.4, or prepared as olive oil mulls by grinding in a glass homogenizer. It was found necessary to inject the experimental animals with approximately 20 mg. (0.17 mmole) of uracil per day in order for this pyrimidine to appear consistently in the urine, hence, in the absence of other criteria, this molar quantity was adopted as the standard dosage for the various halogeno derivatives. Higher dosages were tested when the

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(13) The loss of hydrogen bromide from these compounds is presumed to take place *via* an E_2 elimination reaction initiated by loss of a proton at C_6 to a hydroxyl group of the medium. The binding pair of electrons simultaneously turns in to establish the 5:6 double bond and displace the negative bromide ion. This mechanism would be favored by tendency of the ring to regain conjugation. Chlorine, being more firmly bound, resists displacement, hence, through inductive effect, should favor ring opening by facilitating hydroxyl attack at the adjacent carbonyl carbon. A previous analysis of the ring indicates that the C_6 position is most apt to suffer electron deficiency, hence to lose a proton. (*Cf.* R. A. West and H. W. Barrett, THIS JOURNAL, **76**, 3146 (1954).)

original pyrimidine failed to appear in the urine after giving the standard dosage, or when no dehalogenated product could be found. Five to nine animals were used for each compound, and urine collected for 24 hours from animals caged in large Buchner funnels. The urine was treated as described below, then subjected to paper chromatography. Pyrimidines were located on the paper by their absorption of ultraviolet light at 2500 Å.

Treatment of Urine Samples.—Pooled urine was concentrated to a small volume in a stream of dry air with gentle heating, and chromatographed on paper. When the fluorescent substances normally present in the concentrates interfered with pyrimidine detection, a partial separation was performed. By adding known pyrimidines to normal urine, it was found that methylated derivatives of thiouracil, and all dimethyl derivatives, would be removed by acidifying the urine to *p*H 4 with HCl, concentrating carefully to a paste, and extracting the paste several times with hot ethanol and decanting. The chloromethyl and 4-chloro (as well as any dimethyl derivatives) were removed by decomposing urea with urease, bringing to convenient volume, then extracting 6–7 hr. with ether at *p*H 4–5 in a small liquid–liquid extractor. All extracts were evaporated to dryness, and the residue taken up in a minimum of warm water for chromatography. When necessary, all other pyrimidines were separated by column chromatography on alumina prepared by shaking Fisher Chromatographic grade with 5% acetic acid (10 ml./g.), then washing with distilled water till neutral. A 1 × 15 cm. column was prepared with distilled water, which was also used as eluent. The urine was treated with urease, then desalted with Amberlite resins IRC 50H and IR4B, to give a solution of *p*H 5–6 which was concentrated to about 15 ml. and transferred to the column. The colored materials in rat urine are separated into 4 bands by such a column, and most pyrimidines are removed either ahead of, or with, the most rapidly moving yellow band. In this work, all eluate was collected as a single fraction till the second yellow band approached the bottom of the column, at which point all pyrimidines were removed. As before, the eluate was concentrated to a small volume and chromatographed on paper. When methylated pyrimidines were administered, both extraction and alumina chromatography were used, either successively on an entire urine sample, or on aliquots.

Paper Chromatography.—One-dimensional, ascending chromatography was carried out at 22–25° with 0.01–0.03 ml. of solution on acid-washed Whatman #40 filter paper. The solvent systems used consisted of *n*-butanol saturated with (1) 0.2 *N* HCl; (2) 2 *N* NH_4OH; (3) 10% aqueous urea. All pyrimidines used were chromatographed in triplicate, both as pure compounds and as mixtures containing the pyrimidine and an expected metabolite, and with two or three solvent systems to obtain standard R_f values. With a solvent front of 28 cm., the maximum variation of R_f's amounted to 0.02 unit.

When a urine sample was chromatographed, one spot containing the administered pyrimidine, and another containing an expected metabolite, were also placed on the same paper. Identification of a urinary constituent was accepted only if the substance was properly resolved, and gave an R_f identical to that of a known compound in two different solvent systems. Since it was not known how much of a given pyrimidine would be retained in the tissues or degraded to unrecognizable products over a 24-hour period, only rough quantitations were obtained by comparing the size and density of spots with known standards. One μg. of uracil or of 6-methyluracil in a spot no larger than 2 cm. in diameter was readily identified, while at least 15 μg. of orotic acid was required in a spot of the same size. Amounts of other pyrimidines required for detection varied between these limits. One mg. of uracil added to 50 ml. of urine was readily detected after concentrating the eluate from alumina to 10 ml. and using 0.01–0.02 ml. for paper chromatography. Results with orotic acid were not entirely satisfactory, as this compound and its derivatives chromatographed poorly on paper with the solvents used, and they absorb weakly at 2500 Å.

No pyrimidines were found in normal urine from untreated rats. Two substances were found which absorbed ultraviolet light, but they were easily separated and distinguished from the pyrimidines on the paper chromatograms. The separation procedures described removed one or both of these substances completely.

Results

Standard R_f values obtained with known pyrimidines are shown in Table I. All these compounds were tested in rats, and those which underwent detectable dehalogenation are shown in Table II. A (+) sign indicates the excreted substance present in greatest amount. No pyrimidine could be found by the ultraviolet method when 5,6-dihydropyrimidines were injected in quantities up to 40 mg., and no change in the absorption spectrum was found after 48 hours when they were maintained *in vitro* at 37–38° in isotonic phosphate buffer at *p*H 7.4. Administration of 5-chlorohydrouracil also failed to produce unsaturated pyrimidine in the urine, but after 48 hours of the *in vitro* treatment, the extinction coefficient showed a marked decrease in the region below 260 mμ. Eventually, the absorption spectrum approached that of β-ureidopropionic acid (ε 180 at 230 mμ, almost zero at 250 mμ). Hence, the saturated chloro derivative is assumed to undergo ring hydrolysis under the conditions described. None of the 5-chloro derivatives underwent detectable dehalogenation in the animals.

TABLE I

R_f VALUES OF PYRIMIDINES

Compounds	Solvent, *n*-butanol saturated with: 0.2 *N* HCl	2 *N* NH_4OH	10% aqueous urea
Cytosine	0.098	0.25	0.26
Thymine	.54	.47	.60
Uracil	.35	.24	.43
5-Aminouracil	.05	.20	.19
5-Hydroxyuracil	.84	0	.095
Barbituric acid	.60	.17	.58
5-Chlorouracil	.56	.25	..
5-Bromouracil	.57	.26	.56
5-Iodouracil	.58	.27	.58
6-Methyluracil	.45	.43	.47
6-Methyl-5-chlorouracil	.61	.31	..
6-Methyl-5-bromouracil	.63	.33	.62
6-Methyl-5-iodouracil	.68	.35	.67
5-Chloromethyluracil	.74	.58	..
2-Thiouracil	.57	.15	.29
5-Iodo-2-thiouracil	.75	.25	.71
2-Methylthiouracil	.75	.31	.67
5-Chloro-2-methylthiouracil	.82	.44	..
5-Bromo-2-methylthiouracil	.85	.45	.39
5-Iodo-2-methylthiouracil	.83	.49	.73
4-Chloro-2-methylthiouracil	.83	.39	..
6-Methyl-2-thiouracil	.60	.25	.52
6-Methyl-2-methylthiouracil	.84	.46	.47
6-Methyl-5-chloro-2-methylthiouracil	.86	.54	..
6-Methyl-5-bromo-2-methylthiouracil	.86	.46	.61
6-Methyl-5-iodo-2-methylthiouracil	.88	.53	.69
6-Chloromethyl-2-methylthiouracil	.88	.55	.82
Orotic acid	.43	0	.11
5-Bromoörotic acid	.46	0	.21

The effect of the *in vitro* treatment on 5-bromohydrouracil and on 5-bromo-6-methylhydrouracil is shown in Figs. 1 and 2. Paper chromatography of the two solutions after 48 hours revealed the presence of uracil and 6-methyluracil, respectively. Because of local reaction which prevented absorp-

TABLE II

PYRIMIDINES DEHALOGENATED IN THE RAT

Pyridine injected	Dosage, mg.	Pyrimidine found in urine[a]
None (control)	..	None
5-Bromouracil	45	Uracil (+)
5-Iodouracil	60	Uracil (+)
6-Methyl-5-bromouracil	40	6-Methyluracil
6-Methyl-5-iodouracil	45	6-Methyluracil
5-Bromo-2-methylthiouracil	30	2-Methylthiouracil
5-Iodo-2-methylthiouracil	40	2-Methylthiouracil, uracil
5-Iodothiouracil	40	Thiouracil, 5-iodouracil[b]
6-Methyl-5-bromo-2-methylthiouracil	30	6-Methyl-2-methylthiouracil
6-Methyl-5-iodo-2-methylthiouracil	40	6-Methyl-2-methylthiouracil
4-Chloro-2-methylthiouracil[c]	60	2-Methylthiouracil (+) uracil, unknown
5-Chloromethyluracil[c]	35	Uracil (+)
5-Chloromethyl-2-methylthiouracil	40	6-Methyluracil (+) 2-methylthiouracil
5-Bromohydrouracil[c,d]	30	Uracil (+)
5-Bromo-6-methylhydrouracil[c,d]	40	6-Methyluracil (+)
5-Bromoörotic acid	35	Unknown (+) orotic acid ?

[a] Unless otherwise noted, the injected pyrimidine was also found in the urine. A (+) sign indicates the urinary pyrimidine present in largest amount. [b] Found in urine after injection of 5-iodo-2-thiouracil synthesized with I^{131}. [c] Toxic by subcutaneous injection, injected pyrimidine not found in urine. [d] Not detectable by ultraviolet method.

tion, these derivatives could not be tested by subcutaneous injection of aqueous solutions. Intraperitoneal injection of the compounds as olive oil mulls caused strong temporary abdominal swelling, but the dehydrobrominated products were excreted. Elimination of HBr *in vivo* appeared to be fairly rapid for these compounds. The 4-chloro- and 5-chloromethyl derivatives also seemed to lose halogen rapidly, and were toxic. The former compound was lethal within 10 hours for all rats in the dosage given, while the latter caused severe diarrhea which persisted for 36 hours. The halogen in these compounds is somewhat labile, and was replaced by hydroxyl when heated in neutral aqueous media.

Iodine and bromine at the C_5-position appeared to be removed to about the same extent from pyrimidines of the same structure, *e.g.*, 5-iodo- or 5-bromouracil. Nuclear dehalogenation was generally more extensive with less substituted derivatives, and was greatest with 5-bromohydrouracil and 5-bromo-6-methylhydrouracil. In addition to the dehalogenations, several other substituents on the pyrimidine ring were removed to some extent *in vivo*. Thus, 5-aminouracil was extensively converted to uracil plus an unknown substance, and none of the original pyrimidine appeared in the urine when dosages up to 60 mg. were given. As shown in Table II, a 6-methyl, a 2-thiomethyl or 2-thio group and a methyl group attached to sulfur at C_2 were removed from some structures, but apparently not from others. However, when the injected pyrimidine contained one of these groups in addition to nuclear iodine or bromine, it was expected that some urinary pyrimidine would be found with the halogen still present, but with the other substituent removed. For example, after injection of 5-iodo-2-thiouracil, both 2-thiouracil and 5-iodouracil were expected in the urine, but the latter compound was not found by the ultraviolet method. Similar results were obtained with

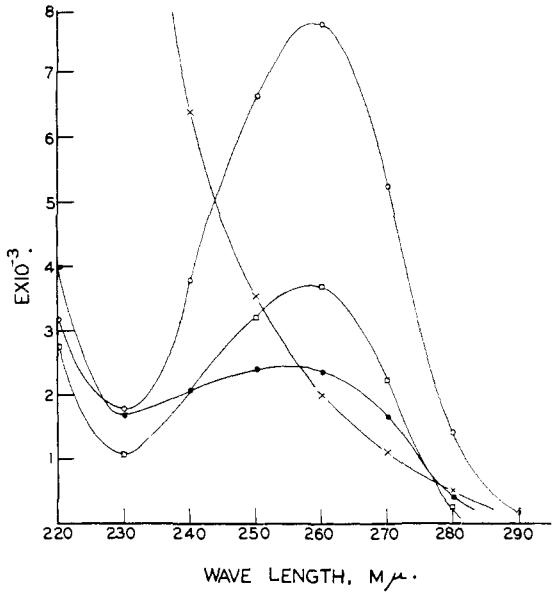

Fig. 1.—Ultraviolet absorption spectra. All solutions 10^{-4} *M* and at *p*H 7.4. Uracil —O—; freshly prepared solution of 5-bromohydrouracil —x—; 5-bromohydrouracil after 24 hours —●—, and after 48 hours —□—, at 37–38°.

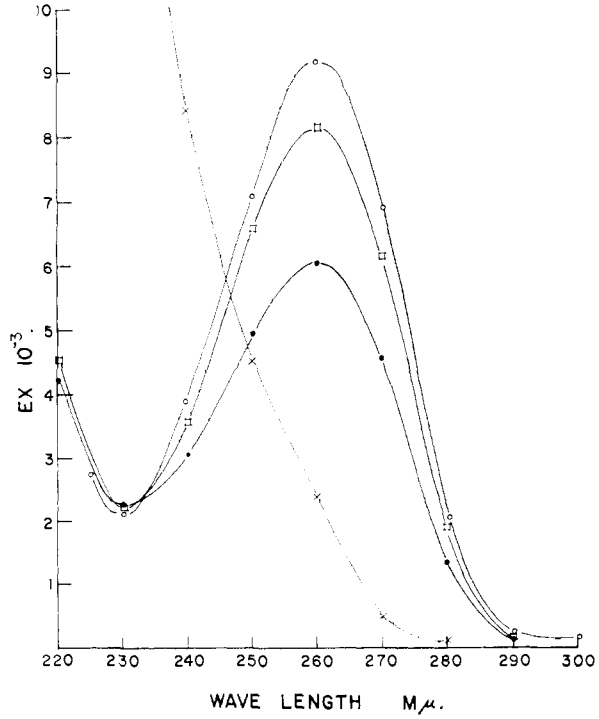

Fig. 2.—Ultraviolet absorption spectra. All solutions 10^{-4} *M* and at *p*H 7.4. 6-Methyluracil —O—; freshly prepared solution of 5-bromo-6-methylhydrouracil—x—; 5-bromo-6-methylhydrouracil after 24 hours —●—, and after 28 hours —□—, at 37–38°.

all pyrimidine derivatives which contained some removable group in addition to the nuclear halogen, hence it appeared that no other substituent could be removed *in vivo* from such derivatives prior to dehalogenation. However, after injection of 5-iodo-2-thiouracil synthesized with I^{131}, radioactive 5-iodouracil was found in the urine, but in amounts too small to be detected by other than tracer methods. This compound was not excreted following injection of KI^{131} or of KI^{131} plus uracil.

Quantitative results as obtained in the paper chromatography revealed that the concentration of 2-thiouracil in the urine was a fourth to a third that of 5-iodo- or 5-bromo-2-thiouracil after administration of either halogenopyrimidine. In view of the total quantity of pyrimidines excreted in 24 hours, uracil (but not thiouracil) derivatives appeared to undergo considerable ring hydrolysis *in vivo*. None of the pyrimidines tested in the animal work gave rise to detectable quantities of barbituric or isobarbituric acid in the urine. Administration of excess thymine or uracil (15–20 mg./day) resulted in excretion of the same pyrimidine only.

Discussion

Release of chloride *in vivo* from the 4-chloro and from the two chloromethyl derivatives was expected, since chlorine in the former compound is readily replaceable by hydroxyl in aqueous media, and is fairly labile in the other two. Also, uracil was expected from the 4-chloro derivative, but not from the 5-chloromethyl derivative. In metabolic degradation of the latter, it is possible that 5-hydroxymethyluracil was produced as an intermediate, since the hydroxymethyl group is labile, and the compound is degraded to formaldehyde and uracil by heating in aqueous solution.[14] The lability of this hydroxymethyl group may be of further significance since the rat has been shown to utilize the hydroxymethyl group of serine to form the methyl group of thymine.[15] Hence, an intermediate containing this group may be formed in the biosynthesis, but not in the degradation, of thymine in this species. It was expected that 6-chloromethyluracil would also produce a hydroxymethyl derivative *in vivo*, but none was found in the urine.

Since 2-thiouracil has been reported to antagonize the catabolism of uracil by surviving liver slices *in vitro*,[16] it was recognized that any of the abnormal pyrimidines used in this work might cause uracil excretion by acting as antagonists *in vivo*. However, there seems little doubt that the urinary uracil found after administration of several halogenopyrimidines arose from these compounds rather than from endogenous sources. Aside from uracil itself and the 5-amino derivative, comparatively large amounts of urinary uracil were found only when the administered compound was readily dehalogenated to give uracil directly without requiring further changes in the molecule. 2-Thiouracil, up to 60 mg., caused only slight excretion of uracil on two occasions, while none was excreted after injection of 5-chlorouracil or 5,6-dihydrouracil. The question of origin did not arise when abnormal pyrimidines were found in the urine of treated animals.

With regard to metabolic reduction of uracil and thymine, Fink, *et al.*,[17] first suggested a reductive pathway, and these workers later demonstrated that thymine, and, more rapidly, dihydrothymine, are converted to β-ureido- and to β-aminoisobutyric acid by the intact rat and by rat liver slices.[7,18] Di Carlo, *et al.*,[19] working with yeasts, eliminated the possibility of oxidative attack on the double bond by these organisms, and suggested reduction of uracil to 5,6-dihydrouracil, carboxylation to 5,6-dihydroörotic acid, then ring hydrolysis. Dihydrouracil, but not uracil, has been demonstrated to give rise to β-ureidopropionic acid and to β-alanine in rats.[8] Hence, reduction of pyrimidines at some stage in their metabolism is established, but there appears to be some uncertainty regarding the stage at which reduction occurs; *i.e.*, whether it is the intact ring, or a hydrolytic product that is reduced. Difficulties in establishing the reduction stage are due, aside from other factors, to the rapid breakdown of free pyrimidines *in vivo*[20,21] and to chemical difficulties involved in detecting dihydropyrimidines and their hydrolytic products.[7] On the basis of our results, we feel that reduction of the intact ring is most probably with the 5-halogeno compounds used here, since reduction of a β-amino- or of a β-ureido acid would result in a compound containing an active α-halogen. Removal of this halogen would then be expected to occur *via* nucleophilic displacement; recyclization of the resulting product would give a further 5-substituted derivative instead of the products found. In view of these considerations, and of the *in vitro* results, assumption of a prior reduction of the pyrimidine ring to permit spontaneous elimination of hydrogen halide under physiological conditions, provides the simplest and most logical mechanism to account for our results with the 5-halogenopyrimidines. It is interesting that a variety of substituents on the ring did not prevent metabolic attack, hence, whether or not the above assumption is substantiated, it is hoped that the compounds described here will be found of further use in studies of pyrimidine metabolism.

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