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SOME BIOLOGICAL AND BIOCHEMICAL PROPERTIES OF 5-IODOCYTOSINE*

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Abstract—5-Iodocytosine exerted a lethal effect in mice in dosages at which the corresponding ribonucleoside, deoxyribonucleoside, or the analogous derivatives of uracil were innocuous. This toxicity was best prevented by the co-administration of 5-methylcytosine, whereas cytosine, cytidine, and deoxycytidine exerted a lesser antidotal activity; in addition, certain halogenated derivatives of uracil exerted some protective activity, whereas uracil and thymine were relatively inert. A marked difference has been observed between the metabolic fates of 5-iodouracil and 5-iodocytosine; catabolism of 5-iodocytosine resulted in higher levels of iodinated acyclic derivatives. Possible schemes for the catabolism of 5-iodocytosine, compatible with the experimental observations, have been discussed.

CERTAIN halogenated pyrimidine derivatives inhibit the reproduction of various neoplasms in mice and man, of mammalian cells in culture, of bacteria, and of viruses *in vitro* and *in vivo*.[1–4] Whereas the deoxyribonucleosides, 5-iodo-2′-deoxyuridine[5] and 5-iodo-2′-deoxycytidine,[6] inhibit the reproduction of certain transplanted neoplasms in mice, the analogous iodinated pyrimidines, 5-iodouracil (IU) and 5-iodocytosine (IC), exert no significant antitumor activity.[7] These results were not unexpected in view of the failure of mammalian cells to utilize significantly either thymine or cytosine for the biosynthesis of DNA pyrimidines under conditions in which the corresponding deoxyribonucleosides are utilized efficiently. An unexpected finding, however, was the marked toxicity exerted by IC when this compound was administered to mice under conditions in which equimolar amounts of either IU or of the ribo- or deoxyribo-nucleosides of IU or IC were nontoxic. This report is concerned with an attempt to elucidate the biochemical basis for the pronounced toxicity of IC.

MATERIALS AND METHODS

Preparation of ^{125}*I-labeled 5-iodocytosine and 5-iodouracil*

These compounds were prepared by the method of Johnson and Johns[8] with minor modifications.[9] The radioisotope was obtained as ^{125}I-sodium iodide from the Oak Ridge National Laboratory, Oak Ridge, Tenn. The original specific activities of the radioactive compounds were: ^{125}I-IC, 4×10^5 counts per minute/μmole; ^{125}I-IU,

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2×10^5 cpm/μmole. Radioactivity was determined in a well-type scintillation γ-ray counter having a sodium iodide (Tl) crystal and a pulse-height analyzer; all radioactive materials were counted in a constant volume in tubes of uniform dimensions. Corrections were applied for background and decay as well as for the quenching observed in concentrated aqueous solutions of potassium iodide.[10]

Toxicity studies

Male BDF_1 mice weighing approximately 20 g were injected intraperitoneally with a single dose of the appropriate compound suspended by homogenization in an aqueous solution of sodium chloride (0·9%) containing gelatin (5% w/v). Toxicity was determined by death, which generally occurred within 48 hr after administration of the drug.

Studies in vivo

BDF_1 mice bearing 4-day old implants of the ascites cell form of the Ehrlich carcinoma or hepatoma 129 received a single intraperitoneal injection of ^{125}I-IC; groups of three mice were sacrificed either 45 min or 4 hr after injection, and the peritoneal fluid was collected. Tumor cells were separated from ascitic fluid by centrifugation. The combined nucleic acids from the tumor cells were fractionated by the Schmidt–Thannhauser procedure,[11] and the radioactivity in the cold acid-soluble [perchloric acid (PCA)], phospholipid, RNA, and DNA fractions was determined.

The cold acid-soluble extracts from the cells and those from the ascitic fluid were neutralized with KOH, centrifuged, and the supernatant fraction was analyzed by ion-exchange chromatography. Aliquots of these fractions, mixed with nonradioactive markers, were made alkaline (pH 10 to 11) with ammonium hydroxide, and the total radioactivity was determined; the solutions were applied to columns (10 × 1 cm) of Dowex-1-formate ion-exchange resin. The columns were washed with water until the pH of the eluate was approximately pH 7 and then were eluted in the manner described in Table 2.[12, 13] The radioactivity of the water washes and the eluted fractions (5 ml) was determined.

Studies in vitro

Tumor cells from mice bearing 4-day old Ehrlich ascites tumors were collected, washed once with an aqueous solution of sodium chloride (0·9%), and suspended in 9 volumes of Krebs III buffer (pH 7·2) containing 12 μmoles of ^{125}I-IC (approximately 2×10^6 cpm). The cell suspension was incubated in a Dubnoff metabolic shaker at 37° in air for 4 hr. The reaction was terminated by the addition of PCA to yield a final concentration of 0·5 M, and the resulting precipitate was collected by centrifugation. The cold PCA extracts were subjected to ion-exchange chromatography and, with the methods described above, the combined nucleic acids were isolated from the residue.

Excretion and metabolism

A single intraperitoneal dose of either ^{125}I-IC (LD_{50}) or an equimolar dose of ^{125}I-IU was administered to mice; these animals were placed individually in 600-ml beakers, and the urine was collected on Whatman 3MM filter paper protected by a wire mesh screen. During this time the animals received food and water *ad libitum*. At designated times the mice were killed by cervical dislocation, and the contents of the

bladder were added to the collected urine. Various tissues were excised, weighed, dissolved in hot sodium hydroxide (6 N), adjusted to a volume of 5 ml, and counted for total radioactivity. The carcass (residue after removal of spleen, kidneys, liver, intestine, stomach, heart, brain, and lungs) of each mouse was weighed, homogenized in a Waring blender with alkali, and heated until the tissues were dissolved; a 5-ml aliquot was then counted for radioactivity.

The radioactive urine was extracted from the filter paper by homogenization in the blender in a dilute ammonium hydroxide solution (pH 9). The homogenate was centrifuged, the residue washed, and an aliquot of the combined extracts subjected to analysis by ion-exchange chromatography.

Chromatography

For the analysis of the '6 M fraction' by paper chromatography, three solvent systems were used:

1, *n*-butanol–formic acid (90%)–water (77 : 10 : 13, v/v)[14]; 2, ethanol–methanol–conc. HCl–water (50 : 25 : 9 : 16, v/v)[15]; and 3, ammonium acetate (1 M)–ethanol (60 : 140, v/v).

A sample of the material isolated as the '6 M fraction', together with marker compounds, was applied to Whatman No. 1 paper and subjected to descending chromatography. The developed chromatograms were cut transversely into strips 1 cm wide and were counted for ^{125}I. Marker compounds were located by examining the paper with an ultraviolet 'Mineralight' lamp.

RESULTS

The relative toxicities of 5-iodouracil and 5-iodocytosine, as well as those of the corresponding ribonucleosides, were compared in normal mice; the results are presented in Table 1. A single intraperitoneal dose of 100 mg IC/kg led to the death of 60% of a group of 10–15 animals. In contrast, IU at a level corresponding to five times the LD_{60} of IC (500 mg IU/kg) was not lethal to the mice. Furthermore, the ribonucleosides of both IU and IC did not cause the toxicity observed with IC, and previous work from this laboratory has shown that mice also can tolerate relatively high dosages of the deoxyribonucleoside of either IC[6] or IU.[13]

The reported utilization of 5-methylcytosine for the biosynthesis of soluble RNA in HeLa cells,[16] suggested that possibly a biochemical antagonism may exist between 5-methylcytosine and IC, analogous to that observed between derivatives of thymine (5-methyluracil) and IU. Since such effects conceivably could account for the marked toxicity observed with IC, it was important to determine whether this compound or its metabolic derivatives were incorporated into RNA. The metabolism of ^{125}I-IC was studied in rapidly proliferating mammalian cells both *in vivo* and *in vitro*. These experiments, carried out with murine neoplastic cells, demonstrated that no radioactivity was associated with either the combined nucleic acid fraction or the protein when neoplastic cells were exposed to labeled drug both *in vivo* and *in vitro*. The chromatographic analysis of the cold acid-soluble extracts from the cells and from the ascites fluid (Table 2) showed that, *in vivo*, no stable phosphorylated derivatives of IC were present and that the majority of the metabolites reflected catabolism rather than anabolic modification of IC; this metabolic pattern was observed in both types of tumor. When ^{125}I-IC was incubated with Ehrlich ascites cells *in vitro*, an environment

TABLE 1. THE TOXICITY OF 5-IODOCYTOSINE, 5-IODOURACIL, AND THEIR NUCLEOSIDES IN BDF_1 MICE*

Compound	Dose (mg/kg)	Percentage killed
Iodocytosine	400	100
	300	100
	200	95
	100	60
	50	20
Iodouracil	500	0
	400	0
	300	10
	200	0
	100	0
	50	0
Iodocytidine	155	0
	78	0
Iodouridine	370	0
Iododeoxycytidine†	3,500	0
Iododeoxyuridine†	2,500	80

* Mice were given a single i.p. injection of the indicated compound suspended in 5% gelatin–physiological saline. Each value represents the results obtained with 10 to 15 mice.

† Data from Ref. 6.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY DERIVED FROM ^{125}I-IODOCYTOSINE AMONG VARIOUS METABOLITES IN MOUSE EHRLICH AND HEPATOMA 129 ASCITES CELLS

Eluant	Isolated metabolite*	Percentage of total radioactivity: Ascites fluid				Cells			
		Ehrlich carcinoma		Hepatoma 129		Ehrlich carcinoma		Hepatoma 129	
		0·75 hr	4 hr	0·75 hr	4 hr	0·75 hr	4 hr	0·75 hr	4 hr
Formic acid, 0·007 M	ICR	0	0	0	0	0	0	0	0
	IC	86	45	84	47	97	78	97	95
	IUR	1	4	1	3	1	0	<1	<1
	IU	1	1	<1	2	0	0	0	<1
Formic acid, 1·0 M	ICMP	0	0	0	0	0	0	0	0
Ammonium formate, 1·0 M	IUMP	0	0	0	0	0	0	0	0
Formic acid, 6·0 M	6 M fraction	5	21	3	18	<1	<1	<1	<1
Potassium iodide, 6·0 M	Iodide	7	29	12	30	2	22	3	5

Mice bearing 4-day-old Ehrlich or hepatoma 129 ascites cells were given a single i.p. injection of 5 mg ^{125}I-IC (approx. $1{\cdot}4 \times 10^6$ cpm). At the times indicated after injection, the mice were sacrificed and the peritoneal fluid was collected and fractionated as described in the text. The acid-soluble extracts from the cells and from the ascites fluid were analyzed by ion-exchange chromatography.

* ICR = iodocytidine; IUR = iodouridine; ICMP, IUMP = 5′-monophosphates of iodocytidine and iodouridine respectively.

in which the cells were unable to reproduce, no metabolism of the compound occurred, the radioactivity in the cells being recovered almost quantitatively (99·7%) as IC. Under similar conditions 5-iodo-2′-deoxyuridine,[17] 5-iodo-2′-deoxycytidine,[6] and 5-fluorouracil[14] undergo considerable metabolic alteration.

A comparison was made of the metabolism of radioactive IC and IU, labeled with ^{125}I, and of the distribution of the label between the excreta and host tissues of normal mice. Groups of mice were sacrificed at 7, 12, and 24 hr after administration of the labeled compounds. Several parameters were investigated for each group: (1) the total radioactivity in the urine and feces collected in the indicated time interval; (2) the distribution of radioactivity of the urine among the various fractions obtained by ion-exchange chromatography; (3) the total radioactivity present in several organs (liver, spleen, heart, kidney, stomach, brain, lung, and small intestine); and (4) the radioactivity in the carcass. The distributions of radioactivity from IC and IU are shown in Table 3. An analysis of the radioactivity in the excised organs showed the

TABLE 3. THE DISTRIBUTION OF RADIOACTIVITY IN TISSUES AND EXCRETA OF MICE GIVEN EITHER ^{125}I-IODOCYTOSINE OR ^{125}I-IODOURACIL

Hours after injection	Percentage of administered dose					
	Tissues		Urine		Feces	
	IC	IU	IC	IU	IC	IU
7	28	15	72	81	<1	4
12	28	11	69	85	3	4
24	21	8	76	85	4	7

The mice were given either ^{125}I-labeled IC (approx. $1{\cdot}5 \times 10^8$ cpm) or IU (approx. 5×10^6 cpm) as a single i.p. injection. Groups of three mice were sacrificed at the indicated times and analyzed for radioactivity by the methods described in the text. Each value represents the average percentage of radioactivity recovered from 3 mice; recovery of radioactivity was between 85% and 105%.

same relative distribution of label in the organs from IC- and IU-treated mice and gave no indication of a tissue localization that could explain the toxicity of IC; accordingly, the amounts of radioactivity in the excised tissues and the carcasses have been combined to give the total retention of drug by the host. The results indicated that the urinary excretion of the label derived from either compound reached 70% to 80% of the administered dose within 7 hr and did not increase significantly thereafter. This is in agreement with earlier work on the metabolism of other iodinated pyrimidine derivatives.[13] Excretion of label in the urine from the IC-treated mice was less than that from the IU-treated mice; consequently, the amount of radioactivity retained by the host was higher in the IC-treated mice.

Chromatographic analysis of urine, 24 hr after injection of isotopic IC or IU, disclosed a significant difference in the metabolism of the two compounds (Table 4). Although the distribution of metabolites was similar for both compounds, the quantitative relationships were markedly different; thus, virtually all the label in the urine of IU-treated mice was present as iodide, whereas unchanged IC accounted for almost as much radioactivity as did free iodide in the urine of mice treated with IC. Furthermore,

considerably more label appeared in the '6 M fraction' from IC-treated animals than from those receiving IU. The distribution of radioactivity in the urine of mice at either 7 or 12 hr after the administration of the drugs was similar to that shown in Table 4.

TABLE 4. RADIOACTIVE METABOLITES IN THE URINE OF MICE GIVEN EITHER ^{125}I-IODOCYTOSINE OR ^{125}I-IODOURACIL

Isolated metabolite	Percentage of administered dose	
	IC	IU
ICR	0·1	
IC	33·4	
IUR	0·4	0·1
IU	0·2	6·8
6 M fraction	4·2	0·2
Iodide	36·5	78·2

The urine of mice given a single i.p. injection either of ^{125}I-IC or ^{125}I-IU was analyzed by ion-exchange chromatography 24 hr after drug administration. The results, which represent the average of 3 mice, are expressed as the percentage of the administered radioactivity in each chromatographically isolated metabolite.

TABLE 5. THE EFFECT OF VARIOUS PYRIMIDINE DERIVATIVES ON THE TOXICITY OF 5-IODOCYTOSINE FOR MICE

Pyrimidine derivative	Dose ratio, μmole IC/μmole pyrimidine derivatives	Lethality (%)
None*		92
Cytosine	1	0
	2	60
5-Methylcytosine	1	0
	2	0
	10	100
5-Fluorocytosine	1	30
Uracil	1	90
	2	100
Thymine	1	80
5-Iodouracil	1	5
	2	90
5-Bromouracil	1	10
Cytidine	1	50
2′-Deoxycytidine	1	40
5-Methyl-2′-deoxycytidine	2	80

BDF_1 mice were given a single i.p. injection of IC (200 mg/kg) either alone or simultaneously with a pyrimidine derivative in physiological saline–gelatin. Each figure represents the average of results from 10 to 30 mice.

* Mice received treatment with IC alone.

To gain information concerning the mechanism of IC-induced toxicity, an attempt was made to prevent the lethality caused by IC in mice by the simultaneous administration of various pyrimidines and nucleosides; the results are presented in Table 5. Cytosine and 5-methylcytosine were the best antagonists studied, the methylated base being completely effective at a half-molar ratio; 5-fluorocytosine was a less potent antidotal agent. Uracil and thymine were not significantly effective in reversing the toxicity of IC, whereas both 5-iodouracil and 5-bromouracil exhibited an unexpected ability to antagonize the toxicity caused by IC. In addition, the ribonucleosides and deoxyribonucleosides of the cytosine series were slightly less effective than the parent pyrimidines.

The effect on the excretion of label by mice receiving ^{125}I-IC of two of these pyrimidines, 5-methylcytosine, a compound capable of preventing the IC-induced toxicity, and thymine, a compound incapable of reversing toxicity of IC, was determined. The urine from mice given ^{125}I-IC, with or without simultaneous administration of

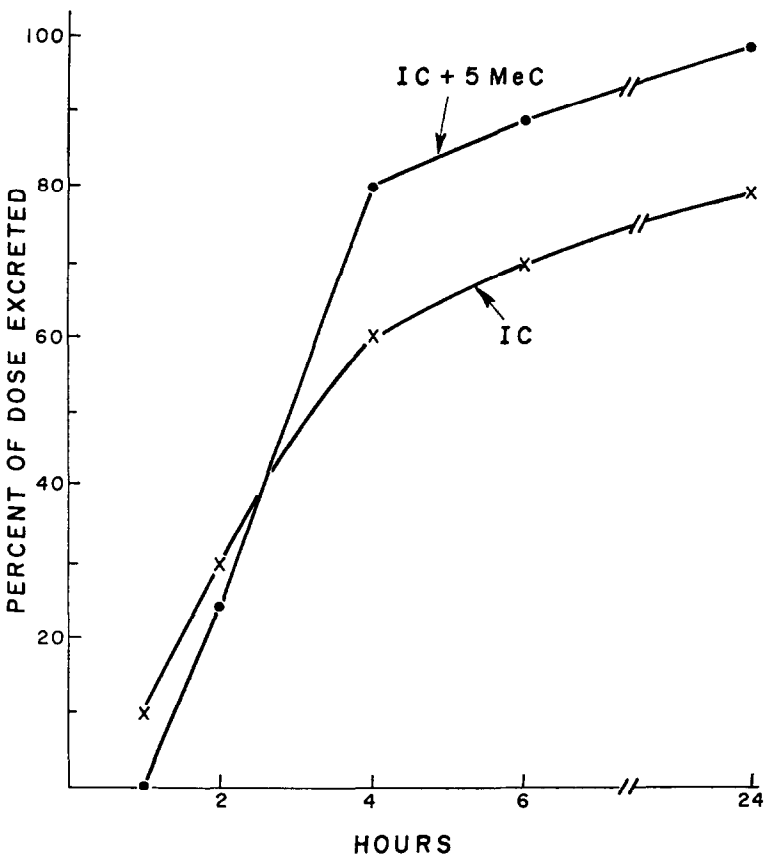

FIG. 1. The rate of urinary excretion of radioactivity from mice given ^{125}I-IC with and without 5-methylcytosine. Two groups of 3 mice each were given a single intraperitoneal injection of either ^{125}I-IC (2×10^6 cpm) with an equimolar amount of 5-methylcytosine, or ^{125}I-IC alone. Each mouse was placed in a 600-ml beaker and the urine collected on filter paper protected by wire mesh. At the indicated times, the filter paper was assayed for total radioactivity. Each value represents the average of 3 mice.

5-methylcytosine, was collected at designated intervals for 24 hr, and radioactivity in the urine was measured. Figure 1 shows that the excretion of label in the absence of 5-methylcytosine was rapid for about 4 hr after injection of the drug and then decreased to approximately 1/30th of the initial rate for the subsequent 20 hr. The simultaneous administration of 5-methylcytosine increased significantly the amount of label excreted. Although the initial high rate of excretion showed no meaningful change, it was maintained for a longer period. The increased urinary excretion of label in the presence of 5-methylcytosine is reflected in a lower radioactivity in the tissues after 24 hr; the proportion retained dropped from about 20% of administered dose (IC alone) to about 0·2% of administered dose (IC plus 5-methylcytosine). In contrast, thymine, when given simultaneously with ^{125}I-IC, had no significant effect on the amount of label excreted in the urine or on that remaining in the tissues.

5-Methylcytosine could antagonize the toxic effects of IC by causing an increased excretion of this compound. Since this would yield a decreased concentration of tissue metabolites of IC, the possibility that one (or possibly several) of the metabolites of IC is the toxic agent was considered. Of the various compounds isolated from the urine of IC-treated mice, IU, 5-iodouridine (IUR), 5-iodocytidine (ICR), and iodide are nontoxic in the amounts involved.

The chemical nature of the '6 M fraction' has not been established; but analysis by both anion-exchange and paper chromatography indicates that the fraction contains at least two iodinated components. In one system (solvent 1) three radioactive components were obtained (Table 6). None of these radioactive materials

TABLE 6. CHROMATOGRAPHY OF '6 M FRACTION' DERIVED FROM MICE GIVEN ^{125}I-IC

Material	Solvent 1		Solvent 2		Solvent 3	
	R_f	Radioactivity (%)	R_f	Radioactivity (%)	R_f	Radioactivity (%)
'6 M fraction'	0·027	6·6	0·55	92·4	0·46	8·4
	0·23	90·6	0·80	7·6	0·74	91·6
	0·46	2·8				
Iodocytosine	0·08		0·58		0·73	
Fluorouracil	0·34					
Iodouracil	0·48		0·63		0·74	
Iodoacetamide	0·67		0·77		0·87	
Iodoacetic acid	0·83				0·81	

Urine was collected for a 24-hr period after i.p. injection of 2 mg ^{125}I-IC to each mouse. The '6 M fraction' was isolated by ion-exchange chromatography of the urine (see Table 2) and further analyzed by chromatography on Whatman No. 1 paper in the solvents described (see Methods).

migrated consistently with iodoacetic acid, iodoacetamide, or any other marker compound. Although the '6 M fraction' has been toxic to mice when injected intraperitoneally, this result was not obtained consistently; therefore, toxicity could not be ascribed definitely to this fraction.

DISCUSSION

The present investigation has attempted to correlate some of the biochemical properties of IC with its pharmacological effects and, in particular, to account for the differences in the toxicities of IC and IU on the basis of differences in their metabolic

fates. The results obtained with neoplastic cells showed that IC resembles cytosine in its relative inability to be incorporated into the nucleic acids, the major metabolic alterations of IC being of a catabolic nature.

An analysis of the ^{125}I-labeled products in the urine from mice given either ^{125}I-IC or ^{125}I-IU demonstrated that IC gives rise to a larger quantity of iodinated acyclic compounds (the '6 M fraction'). It is possible that the higher toxicity of IC as compared with IU may be a result of the production of an increased amount of certain catabolites occurring in the '6 M fraction'. A correlation between toxicity of some halogenated pyrimidines and their catabolites has been made by Philips *et al.*[18] Since uracil may be degraded to β-alanine and finally to acetate in rats,[19] Philips and his co-workers have suggested that fluoroacetate, similarly derived, may be responsible for some of the toxicity observed in animals receiving fluorouracil, dihydrofluorouracil, or α-fluoro-β-alanine. Mukherjee and Heidelberger,[14] however, have been unable to demonstrate fluoroacetate in the urine of animals given fluorouracil.

Regardless of the nature of the toxic agent, the higher level of iodinated acyclic derivatives derived from IC as compared with IU is anomalous. Since cytosine deaminase activity in mammalian systems is negligible,[20] and cytosine is not a substrate for dihydropyrimidine dehydrogenase,[21] catabolism of cytosine cannot occur by conversion to either uracil or 5 : 6-dihydrocytosine. The catabolic behavior of IC is in contrast to that of both cytosine and 5-iodo- or 5-bromo-uracil; thus, at least 4% of IC is degraded to iodinated acyclic compounds, and unlike the halogenated uracil derivatives, IC is relatively resistant to dehalogenation, only 36% of iodide being produced.

Iodocytosine, in contrast to cytosine, may indeed be a substrate for a cellular deaminase, since iodouracil is formed to some extent (Table 4). If iodouracil were the major initial catabolic product, however, an explanation is required to account for the subsequent quantitative alteration in the formation of acyclic compounds (Table 4). The known and hypothetical pathways for the catabolism of IC, IU, and uracil are depicted in Fig. 2. The hypothetical metabolic sequences in Fig. 2 are a logical extension of the observations and suggestions of Mukerhjee and Heidelberger[14] and Philips *et al.*[18]

One explanation for these observations would be to assume that, in contrast to cytosine, IC may be a substrate for dihydropyrimidine dehydrogenase, and thus the initial catabolic product would not be IU but 5 : 6-dihydro-5-iodocytosine. This compound could conceivably be resistant both to hydrolytic deamination,[22] by virtue of the electronic properties of the 5-iodo substituent, and to dehalogenation due to the 4-amino group. Since 5 : 6-dihydro-5-halogenopyrimidines are susceptible to both ring cleavage and dehalogenation,[23] a decrease in dehalogenation could possibly lead to an increase in ring cleavage to iodinated acyclic compounds.

Another possibility is that the degradation of iodocytosine does indeed take place via iodouracil and that the subsequent deiodination is inhibited by either iodocytosine itself or one of its derivatives. Hence, a greater proportion of 5 : 6-dihydro-iodouracil is cleaved to iodinated acyclic products.

The ability of various derivatives of cytosine to prevent the toxicity caused by IC in mice may reflect either competition for entry into the cell or for an enzymatic site; however, preliminary experiments suggest that IC competes with 5-methylcytosine for transport into the cell. The simple 2 : 4-diketo-pyrimidines, uracil and thymine, would

not be expected either to share or to inhibit the transport mechanism for cytosine derivatives and thus would not reverse toxicity. The prevention of IC-induced lethality by 5-halogenated uracils is more difficult to explain; if it is assumed that they share with uracil and thymine a common mechanism for entry into the cell, then the substituted uracils also would not be expected to compete with IC. Accordingly, this antagonism would appear to arise later in the metabolic sequence.

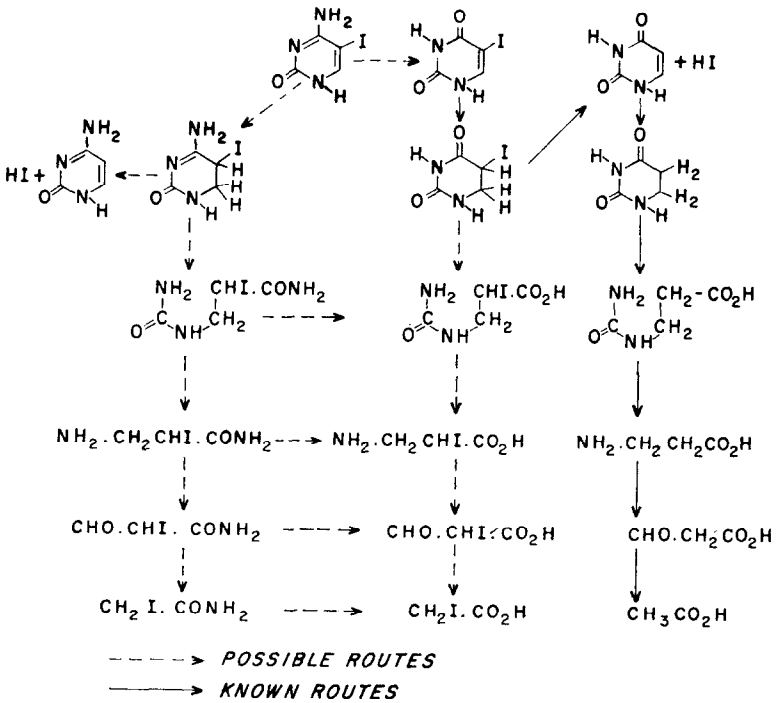

FIG. 2. Proposed scheme for the degradation of iodinated pyrimidines.

This investigation has revealed a toxicity of IC that could not have been predicted from the known characteristics of derivatives of IC or their deaminated analogs. This toxicity appears to be associated with a catabolic pathway for IC that is different from that observed with other pyrimidines and may be of fundamental biochemical interest. To define more precisely the enzymatic reactions involved, studies in a cell-free system are in progress.

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