INHIBITORY MECHANISM OF 5-FLUOROOROTATE ACTION IN RAT LIVER*

A.Čihák and H.C.Pitot**

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague 6

5-Fluoroorotic acid is subjected *in vivo* in rat liver to rapid metabolic transformations and is incorporated into a fraction of ribonucleic acids which differs from ribosomal and transfer RNA. It has been found that this heterodisperse RNA is bound to polyribosomes in the cytoplasm and behaves in a manner similar to the rapidly labelled RNA present in informosome.

The administration of 5-fluoroorotic acid results in a considerable inhibition of dietary as well as of hormonal induction of amino acid metabolizing enzymes in rat liver. Tyrosine α-ketoglutarate transaminase is an exception: Following the analogue its activity is increased, this enhancement is not associated with an increase in enzyme synthesis *de novo*. The possible mechanism of 5-fluoroorotate action is discussed.

5-Fluoroorotic acid undergoes in liver the same metabolic changes as orotic acid. Initial studies concerning inhibitory mechanism of this compound demonstrated the inhibition of orotidylic acid decarboxylase by the newly formed 5-fluoroorotidine 5′-phosphate and the incorporation of the fluoro analogue into liver ribonucleic acids[1–4]. In view of the effect of 5-fluoroorotic acid on the amino acid metabolizing enzymes induced in rat liver under different conditions[5,6], further studies were undertaken on the mechanism of action of this compound.

The results obtained and described herein suggest that the molecular mechanism of the inhibitory effect of 5-fluoroorotic acid is entirelly different from that caused by other known purine and pyrimidine antimetabolites. At the same time we have observed that following the administration of these analogues the changes in the level of induced liver enzymes are frequently quite similar[7]. In this paper are presented

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** Permanent address: McArdle Laboratory for Cancer Research, University of Wisconsin, Madison.

new data concerning the inhibitory mechanism of 5-fluoroorotic acid and the changes of various amino acid metabolizing enzymes in rat liver following its administration.

EXPERIMENTAL

Materials

5-Fluoroorotic acid was obtained through the courtesy of Hoffman La Roche-Inc., 5-fluoroorotic-[2-^{14}C] acid was a product of Tracerlab. Cortisone acetate was obtained from the Upjohn Company and casein hydrolysate from General Biochemicals. Orotic-[6-^{14}C] acid and dithiothreitol were from Calbiochem and Sephadex G 200 was a product of Pharmacia-Sweden. Sodium deoxycholate was a product of Matheson-Coleman and Bell, while disodium ethylenediaminetetraacetic acid was a product of Fischer Scientific Co.

Holtzman male rats weighing from 160—180 g were used. Adrenalectomized male rats of the same weight were obtained from the Endocrine Laboratory, Madison. Animals were maintained on laboratory chow diet (minimum 20% of crude protein) *ad libitum* or on a 12·5 and 0% protein diet, respectively as indicated. In all experiments animals were fasted overnight and the experiments started between 7 and 8 a.m.

Dietary Induction

Intubation of casein hydrolysate was carried out in ether narcosis. Intact animals were intubated with 3 ml of 33% solution of casein hydrolysate while in case of adrenalectomized rats 5 ml of a 10% solution was given. 5-Fluoroorotic acid freshly dissolved in 0·05M-NaOH and neutralized with 1M-HCl was injected *i.p.* in a maximal volume of 0·4—0·5 ml usually in a dose level of 0·1 g per kg. Control animals received the same volume of saline.

At different time intervals after administration of the inducer or 5-fluoroorotic acid groups of 5—6 animals were sacrified by cervical dislocation and the livers removed. Homogenization of cooled livers was carried out by a Polytron homogenizer in 3 volumes of 0·25M-sucrose in 0·05M-Tris-HCl buffer (pH 7·2) with 0·01M-$MgCl_2$, 0·025M-KCl, 10^{-4}M pyridoxal phosphate and 10^{-3}M dithiothreitol. Homogenates were centrifuged (1 h, 105·000 *g*, 4°C) and S_3-supernatant fractions were stored for 1—3 days at —70°C. The enzyme activity assay was carried out in freshly thawed high-speed supernatants using an automated combination unit described earlier[8].

Isolation and Sucrose Gradient Analysis of Rat Liver Cytoplasmic Ribonucleic Acids

Livers (5—6 g) which had been quickly removed were homogenized in 30 ml of ice-cold 0·25M-sucrose in TKM buffer, pH 7·6 (0·025M-Tris-HCl, 0·05M-KCl and 0·005M-$MgCl_2$) using a Potter-Elvehjem loose fitted glass homogenizer with a Teflon pestle. The homogenate was centrifuged (12 000 r.p.m., 10 min, 4°C) and the supernatant diluted to 70 ml with 0·25M sucrose in TKM buffer and centrifuged (30 000 r.p.m., 2 h, 4°C). The pellet was homogenized by hand in 20 ml of ice-cold 0·005M phosphate buffer (pH 7·4) with $5 \cdot 10^{-4}$M-$MgCl_2$, 2·3 ml of 15% lauryltrimethylammonium chloride were added, mixed well and let sit for 5 min before adding 1 ml of 1M-$MgSO_4$. The suspension was mixed and let stand for 20 min at 0°C before centrifugation (1 500 r.p.m., 10 min, 4°C). The sediment was washed with 5 ml of 0·01M-Tris-HCl (pH 7·4) with 10^{-3}M-$MgCl_2$ and 0·05% of lauryltrimethylammonium chloride and centrifuged (1 500 r.p.m., 10 min, 4°C). The sediment was mixed with 1·70 ml of 0·1M-Tris-HCl (pH 9·0), 0·1 ml of 0·15M-EDTA and 0·2 ml of 5% sodium dodecyl sulfate and let stand for 5 min at 0°C before

adding 3 ml of 0·1M Tris-HCl (pH 9·0) containing 10% sodium 4-aminosalicylate. All operations were carried out at 4°C.

Phenol extraction of the above suspension[9,10] was done with 5 ml of a phenol–cresol mixture (30 min, 20°C) during mixing. After centrifugation (15000 r.p.m., 30 min, 4°C) the phenol layer and interphase were reextracted for 10 min while mixing with an additional 5 ml of 0·1M-Tris-HCl (pH 9·0) containing 6% sodium 4-aminosalicylate and centrifuged. The supernatants were combined, a half volume of phenol–cresol solution was added, mixed well for 10 min and spun in the same way. The supernatant after phenol extraction was further passed through a Sephadex G 200 column (1·5 × 10 cm) to remove low molecular weight components and transfer RNA. For elution 0·01M-Tris-HCl (pH 7·4) with 0·1M-KCl and 0·001M-$MgCl_2$ was used. Absorbance of fractions was read automatically at 260 nm using a Gilford model 2400 spectrophotometer. After precipitation with 2—3 volumes of cold ethanol (48 h) the precipitate was centrifuged at 2,000 r.p.m. for 10 min.

The sediment of partially purified ribonucleic acids was dissolved in the above buffer (pH 7·4) and about 0·2—0·3 ml of the solution (200 O. D. units at 260 nm per 1 ml) were layered over precooled 5—15% (w/v) linear sucrose gradients. Centrifugation (24,000 r.p.m., 17 h, 3°C) was carried out in an SW 25·3 rotor using a Beckman model L 2 ultracentrifuge. 0·5 ml fractions were collected by hand in an ice-bath. Absorbance of the fractions was read at 260 nm on a Gildford spectrophotometer and radioactivity was measured in a Packard Liquid Scintillation Counter using Scintisol.

Chromatography of Ribonucleic Acids on Methylated Albumin Kieselguhr Column

The column was prepared in a manner similar to Sueoka and Yamane[11] and separation of RNA was carried out according to the method of Mandel and Hershey[12]. A chromatographic column (3 × 10 cm) with a water jacket was used. The first layer of the column was formed from 6 g of Kieselguhr, mixed and boiled for a short time with 30 ml of 0·05M-Na_2HPO_4 (pH 6·7) with 0·2M-NaCl. After cooling to the suspension was added slowly with mixing 1·5 ml of a 1% solution of methylated albumin. The upper layer was formed by adding 1 g of Kieselguhr in 20 ml of the same buffer. The washing of the column as well as the collection of samples was done at 37°C. Ribonucleic acids dissolved in 0·01M-Tris-HCl (pH 7·4) with 0·1M-KCl and 10^{-3}M-$MgCl_2$ were layered onto the column and it was washed with 150 ml of 0·2M buffered sodium chloride. A continuous gradient formed by addition of 1·2M buffered saline was used and 4·8 ml fractions were collected at a flow rate of 48 ml per h.

Sucrose Gradient Analysis of EDTA-Dissociated Ribosomal Subunits

Liver polyribosomes were isolated[13] from rat livers which had been homogenized ten times in 2 volumes (w/v) of 0·44M sucrose in TKM-buffer, pH 7·6 (0·05M-Tris-HCl with 0·07M-KCl and 0·002M-$MgCl_2$). Homogenates were centrifuged at 13000 r.p.m. for 10 min (4°C) and the pellets with the lower layer of the supernatant were discarded. The remaining supernatants were adjusted to 1·25% in sodium deoxycholate and incubated 30 min at 0°C. The detergent-treated supernatants were layered over a two-step gradient of 3 ml of 2M sucrose in TKM overlayed by 4 ml of 1·3M sucrose in the same buffer. Centrifugation was carried out in a Spinco type 50 Ti rotor at 50 000 r.p.m. for 135 min at 4°C using a Beckman model L 2 65-B ultracentrifuge. Supernatants were discarded and the tubes walls were wiped, the sediments rinsed with ice-cold TKM buffer and wiped free of any remaining solution.

Total liver polyribosomes were suspended in 0·05M-Tris-HCl buffer, pH 7·6 with 0·07M-KCl and 0·01M-EDTA. The suspension was homogenized by hand and the debri removed by centri-

fugation (15000 r.p.m., 10 min, 4°C). The EDTA-dissociated ribosomal subunits were separated on isokinetic 5—20% (w/v) sucrose density gradients[14] in the above buffer. Centrifugation (17 ml, 12 O.D. units at 260 nm) was carried out for 12 h at 24000 r.p.m. (4°C) in an SW 25·3 rotor using a Beckman model L 2 ultracentrifuge. The absorbance of 0·5 ml fractions was measured at 260 nm with a Gildford model 2400 spectrophotometer. The radioactivity of samples was measured as described above.

RESULTS

Incorporation of 5-Fluoroorotic-[2-^{14}C] Acid into Rat Liver Ribonucleic Acids

5-Fluoroorotic acid administered *in vivo* is phosphoribosylated in the liver and 5-fluorouridine 5'-phosphate appearing in the acid-soluble pool is taken up into ribonucleic acids. The degree of incorporation is about 30—40% lower than that

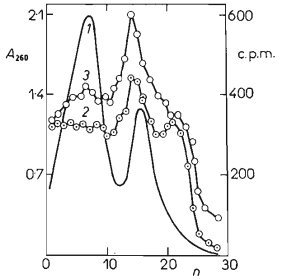

FIG. 1

Sucrose Gradient Analysis of Cytoplasmic RNA from Rat Liver Labelled with 5-Fluoro-orotic-[2-^{14}C] Acid

5-Fluoroorotic-[2-^{14}C] acid (8 μCi/2 μmol per animal) was applied *i.p.* 3 (*2*) and 5 h (*3*) before killing, resp. Isolated ribonucleic acids were analyzed on 5—15% linear sucrose gradient using Beckman model L 2 ultra-centrifuge (16 h, 24000 r.p.m., 4°C). Fractions of 0·5 ml were collected. *1* Optical density at 260 nm.

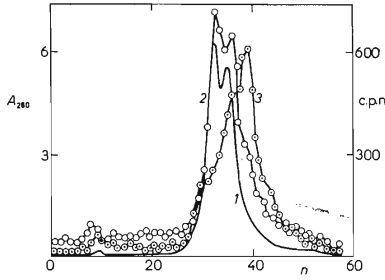

FIG. 2

Separation of Cytoplasmic RNA from Rat Liver on Methylated Albumin Kieselguhr Column

Orotic-[6-^{14}C] (*2*) and 5-fluoroorotic-[2-^{14}C] acid (*3*) (8 μCi/2 μmol per animal), resp. were administered 3 h before killing. From livers isolated ribonucleic acids were purified on a Sephadex G-200 column, precipitated with cold ethanol and dissolved in 0·01M Tris-HCl buffer (pH 7·4) with 0·1M-KCl and 0·001M-Mg^{2+} ions. Separation on the column was carried out at 37°C using 0·4—1·2M-NaCl in phosphate buffer (pH 6·4). Fractions of 4·7 ml (10 fractions per 1 h) were collected. *1* See Fig. 1.

of orotic acid[15]. In contrast to the natural precursor the incorporation is not substantially affected even by a high dosage of actinomycin D. Fig. 1 presents data on the sucrose gradient analysis of cytoplasmic ribonucleic acids isolated from rat liver 3 and 5 h after the administration of radioactive 5-fluoroorotic acid.

It is evident that even after 5 h no incorporation of the analogue into ribosomal ribonucleic acids occurs; the radioactivity appears in the region which corresponds[16,17] to the fraction of rapidly labelled RNA. Ribosomal RNAs and the fraction of rapidly labelled ribonucleic acids were separated further on methylated albumin Kieselguhr columns. The elution profile of the total cytoplasmic RNA following the incorporation of orotic-[6-^{14}C] and 5-fluoroorotic-[2-^{14}C] acid under identical conditions is given in Fig. 2. Whereas the radioactivity after labelling with orotic-[6-^{14}C] acid follows the UV-absorbancy of ribosomal RNAs, the radioactivity after 5-fluoroorotic-[2-^{14}C] acid is found to follow an entirely different pattern.

Actinomycin D even at high dosage does not influence the incorporation of the radioactivity from 5-fluoroorotic-[2-^{14}C] acid into this RNA. On the contrary, in the presence of actinomycin D the labelling of ribosomal ribonucleic acids was completely blocked. It was proposed[15] that 5-fluoroorotic acid is used for the synthesis of rapidly labelled RNA. This is in agreement with the effect of the antibiotic on the low- and high-ionic strength activated DNA-dependent RNA-polymerase[18] and with the inhibition of the synthesis of ribosomal RNAs by actinomycin D without involvement of messenger RNA synthesis[19,20].

While examining the release of the fraction of RNA labelled with 5-fluoroorotic-[2-^{14}C] acid from nucleus to cytoplasm, it was found that the radioactivity bound to 45 S nuclear particles is not transferred to free 45 S cytoplasmic particles[21]. In comparison with the labelling after orotic-[6-^{14}C] acid the radioactivity of free cytoplasmic ribosomal subunits after 5-fluoroorotic-[2-^{14}C] acid administration under identical conditions is considerably lower. The amount of radioactivity bound to polyribosomes in rat liver cytoplasm following orotic-[6-^{14}C] and 5-fluoroorotic-[2-^{14}C] acid is rather similar although the distribution of the label among individual polyribosomes is completely different[15].

Following a 3 h labelling period with orotic-[6-^{14}C] acid the radioactivity of individual polyribosomes shows a pattern such that the maximum amount of the label is located in monosomes and the minimum in the heaviest polyribosomes. In the case of 5-fluoroorotic-[2-^{14}C] acid administration the maximum labelling under identical conditions is restricted to tetra – hexa polyribosomes which are also capable of the maximal binding of transfer ribonucleic acids following pulse-labelling with leucine-[^{3}H]. Similar uneven distribution of polyribosome bound radioactivity was observed after a short-term labelling with orotic-[6-^{14}C] acid[22]. In the case of a long-term incorporation the uptake into ribosomal RNAs prevails, and the polyribosome radioactivity has a direct relation to its weight. The administration of 5-fluoroorotic acid which does not lead to the incorporation into ribosomal ribonucleic acids even

after long time intervals (Fig. 1) thus causes an uneven labelling of polyribosomes when given for longer time intervals.

Non-ribosomal, rapidly labelled ribonucleic acids are bound to polyribosomes[23,24] in the cytoplasm, probably as a particle[22,25,30]. EDTA treatment of rat liver polyribosomes result in their dissociation[26] and a fraction of non-ribosomal RNAs is released. The distribution on sucrose gradients of dissociated ribosomal subunits labelled with orotic-[6-^{14}C] and 5-fluoroorotic-[2-^{14}C] acid, respectively, is shown in Fig. 3. Whereas orotic-[6-^{14}C] acid is incorporated mainly into ribosomal RNA the radioactivity pattern following 5-fluoroorotic-[2-^{14}C] acid has a completely different character. It seems that the fraction of non-ribosomal RNAs labelled with 5-fluoroorotic-[2-^{14}C] acid is heterogeneous in character as evidenc by its overall distribution along the sucrose gradient.

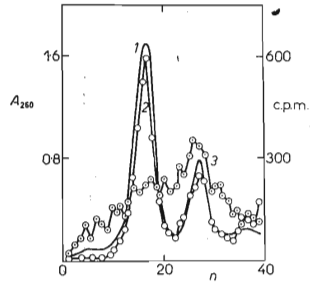

FIG. 3

Sucrose Gradient Sedimentation Analysis of EDTA-Dissociated Ribosomal Subunits

Rat liver polyribosomes isolated after 3 h of labelling with orotic-[6-^{14}C] (*2*) and 5-fluoroorotic-[2-^{14}C] acid (*3*) (8 μCi/2 μmol per animal), resp. were resuspended in 0·05M-Tris-HCl buffer (pH 7·6) with 0·07M-KCl and 0·01M-EDTA. Separation of dissociated subunits was carried out on isokinetic 5—20% sucrose density gradient in the same buffer in SW 25·3 rotor using Beckman model L 2 ultracentrifuge (12 h, 24000 r.p.m., 4°C). 0·5 ml fractions were collected. *1*, See Fig. 1.

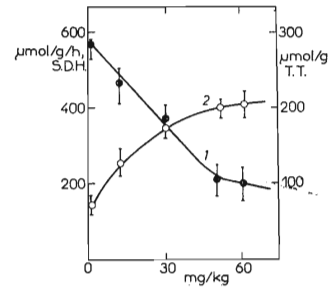

FIG. 4

Changes of Serine Dehydratase *1* and of Tyrosine α-Ketoglutarate Transaminase 2 Activities in Starved Rat Livers following 5-Fluoroorotic Acid Administration *in vivo*

Groups of 4—5 Holtzman male rats (180—200 g) kept 3 days on 12·5% protein diet were starved 14 h before the experiment. 5-Fluoroorotic acid was given i.p. 5 h before killing.

Effect of 5-Fluoroorotic Acid on Enzyme Induction in Rat Liver

After the finding of a selective incorporation of 5-fluoroorotic acid into a fraction of rapidly labelled RNAs it was interesting to investigate the effect of the analogue on enzyme synthesis *in vivo*. For this purpose we have chosen to study the dietary and hormonal induction of amino acid metabolizing enzymes in rat liver. The effect of 5-fluoroorotic acid on the dietary induction of serine dehydratase, ornithine δ-transaminase, histidase and tryptophan pyrrolase in rats kept on 0% protein diet is evident from Table I.

TABLE I

Dietary Induction of Some Amino Acid Metabolizing Enzymes in Rat Liver following 5-Fluoroorotic Acid

Groups of 5—12 Holtzman male rats (160 g) kept 5 days on 0% protein diet and starved 12 h before experiment (= control) were used. Dietary induction was carried out by intubation of 3 ml of 33% casein hydrolysate 5 h before sacrifice. 5-Fluoroorotic acid was given *i.p.* simultaneously with the inducer in a dose level of 0·1 g per kg.

Enzymes	Enzyme activity, μmol/g per h		
	Control	Induction	Following analogue
Serine dehydratase	64·4 ± 11·2 (12)	514·3 ± 68·2 (8)	178·5 ± 26·3 (6)
Ornithine δ-transaminase	15·2 ± 2·7 (6)	23·4 ± 6·2 (5)	20·1 ± 4·8 (5)
Histidase	10·6 ± 3·0 (6)	14·7 ± 2·5 (5)	5·7 ± 1·6 (5)
Tryptophan pyrrolase	1·0 ± 0·1 (8)	5·6 ± 0·9 (8)	2·7 ± 0·4 (6)

The activity of serine dehydratase is impaired not only under the conditions of induction but also in non-induced starved livers (Fig. 4). However, under identical conditions tyrosine α-oxoglutarate transaminase behaves in the opposite manner since its activity, followin g5-fluoroorotic acid, is enhanced. Through immunochemical techniques it was shown that the higher activity of this enzyme is not associated with an enhanced rate of synthesis *de novo*[7].

A similar difference in the response of serine dehydratase and tyrosine α-oxoglutarate transaminase activities after 5-fluoroorotic acid was observed during the hormonal induction of both enzymes. In case of the simultaneous or delayed administration of the analogue and cortisone the activity of serine dehydratase is lowered (Fig. 5). The activity of tyrosine α-oxoglutarate transaminase under similar conditions is considerably enhanced (Fig. 6), while the *de novo* enzyme synthesis remains un-

changed[7]. Similar differences in the activity of tyrosine α-oxoglutarate transaminase and of other amino acid metabolizing enzymes in rat liver has been found also after 5-azacytidine[7,27], 8-azaguanosine[28] and 5-fluorouracil[29], these analogues each differing in their inhibitory mechanisms.

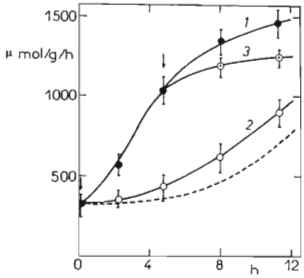

Fig. 5

Inhibition of Cortisone Induction of Serine Dehydratase in Rat Liver by 5-Fluoroorotic Acid

Groups of 4—7 Holtzman male adrenalectomized rats (160 g) kept 3 days on 12·5% protein diet were starved 10 h before cortisone (5 mg per animal) *1*. 5-Fluoroorotic acid (0·1 g per kg) was administered *i.p.* either simultaneously with cortisone *2* or 5 h later *3*. The dotted line represents the increase in enzyme activity of control animals during the experiment.

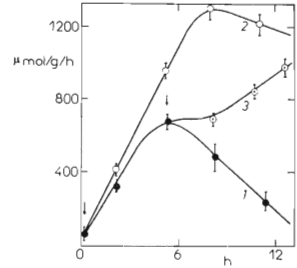

FIG. 6

Increase of Tyrosine α-oxoglutarate Transaminase in Rat Liver following 5-Fluoroorotic Acid Administration

Groups of 5—8 Holtzman male adrenalectomized rats (160—170 g) kept 3 days on 12·5% protein diet were starved 10 h before cortisone as in Fig. 5. *1* cortisone; *2* 5-fluoroorotic acid administered simultaneously with cortisone; and *3* 5-fluoroorotic acid administered 5 h after cortisone.

DISCUSSION

The findings reported in this paper demonstrate that the pyrimidine analogue, 5-fluoroorotic acid, is incorporated into a fraction of RNA having many of the characteristics of messenger RNA. Little, if any, incorporation of the analogue into cytoplasmic ribosomal RNA was demonstrated. Earlier studies[4] demonstrated that the analogue is incorporated into transfer RNA and preliminary investigations would confirm these results. Thus, it becomes reasonable to assume that the effects of the administration of 5-fluoroorotic acid *in vivo* on enzyme levels may be attributed to its incorporation into different types of RNAs.

The results described in this paper demonstrate that two inducible enzymes in rat liver, serine dehydratase and tyrosine α-oxoglutarate transaminase are affected in quite different ways after the pyrimidine analogue is administered. In the case of cortisone induction, administration of the analogue simultaneously with the inducer causes a marked inhibition of serine dehydratase induction. If given 5 h after administration of the inducer, it has little effect. This finding is in support of our earlier studies[5]. In addition, it has been demonstrated that the initial induction of serine dehydratase depends on the synthesis of messenger RNA as judged by the effects of actinomycin D. Since it is possible to inhibit serine dehydratase induction, even when the base level of the enzyme is virtually zero, one may suggest that the initial induction of this enzyme always requires RNA synthesis and, as has been previously reported[31], the lifetime of the messenger RNA template for serine dehydratase is less than 8 h. On this basis, it is reasonable to ascribe the effects seen with this enzyme to the incorporation of the analogue into messenger RNA, which inhibits subsequent enzyme synthesis possible through the production of an abnormal message.

On the other hand, results with tyrosine α-oxoglutarate transaminase may be explained on the basis that the synthesis of this enzyme actually occurs on a stable template as has been earlier suggested[32]. Thus, regulation of tyrosine transaminase must occur at the translational level and therefore would probably involve transfer RNA rather than changes in messenger RNA in the context of our discussion here. The inhibition of the degradation of tyrosine α-oxoglutarate transaminase described here and earlier[6] would suggest, therefore, that the incorporation of the analogue into transfer RNA may be also involved in the regulation of the *in vivo* dynamic degradation of this enzyme. Experiments are presently underway to test this hypothesis.

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