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BIOTRANSFORMATION AND SOME METABOLIC EFFECTS OF 5-(4-AMINOPHENYL)-CYTOSINE

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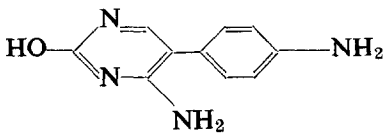
Abstract—5-(4-Aminophenyl)-cytosine possesses, according to results of clinical trials, good prophylactic activity against influenza A_2. This contribution presents a survey of transformations of the compound in the organism and of its distribution in the tissues of a variety of animals. In the urine of mice, after the administration of 5-(4-aminophenyl)-cytosine several metabolites were found, viz.: 5-(4-acetamidophenyl)-cytosine, 5-(3-hydroxy-4-aminophenyl)-cytosine, 5-(3-hydroxy-4-acetamidophenyl)-cytosine, and traces of 2-hydroxy-4-acetamido-5-(4-acetamidophenyl)-pyrimidine and 5-(3-hydroxy-4-aminophenyl)-cytosine-*O*-glucuronide. A considerable portion of unchanged 5-(4-aminophenyl)-cytosine, however, was rapidly excreted in the urine and the faeces.

When 5-(4-aminophenyl)-cytosine was administered to mice, a two-fold increase of incorporation of orotic-6-^{14}C acid, administered simultaneously, was observed. Other 5-arylpyrimidines enhanced the incorporation of orotic-6-^{14}C acid which wes utilized for the synthesis of liver ribonucleic acid as well. The incorporation of adenine-8-^{14}C or thymidine-7-^{14}C was, however, not influenced under identical conditions.

While studying the site of inhibitory action it was further observed that the presence of 5-(4-aminophenyl)-cytosine and some other 5-arylpyrimidines was accompanied by a decreased activity of succinate dehydrogenase. The inhibition of succinate dehydrogenase in a cell-free rat liver extract by 5-(4-aminophenyl)-cytosine is of competitive character, the ratio of the Michaelis aed inhibition constants being 1·84

IN SEVERAL compounds belonging to the group of 5-arylcytosines and 5-aryl-2-thiocytosines, an activity against the viruses of influenza[1, 2] (strain A PR-8), vaccinia, and WEE (*cit.*[3, 4]) was found. One of the most effective compounds against the influenza virus was 5-(4-aminophenyl)-cytosine, which exhibited simultaneously a relatively very low toxicity (LD_{50} i.v. 510 mg/kg, oral more than 10·0 g/kg). This compound, after a thorough pharmacological and toxicological investigation, was therefore employed for clinical trials as a prophylactic agent against influenza A_2 in the course of an epidemy in Czechoslovakia in the year 1962. Later on, it was employed in the treatment of tick encephalitis, being compared with some antibiotics,[5] and finally in some herpetic diseases.[6] Although these experiments have not been finished yet, the results obtained up to the present indicate that 5-(4-aminophenyl)-cytosine favourably influences the abovementioned virus diseases.



5-(4-Aminophenyl)-cytosine.

During the first phase of the investigation of the 5-(4-aminophenyl)-cytosine activity, the binding of this compound on the proteins of human blood serum was studied.[7] A considerable portion of 5-(4-aminophenyl)-cytosine remains free in the circulating blood. An investigation of its distribution showed that after a parenteral administration of the compound, a high level in the kidney was found very soon, indicating a rapid excretion.[7] Our experiments have been aimed at the investigation of the transformations of 5-(4-aminophenyl)-cytosine in animal organism and at the elucidation of the mechanism of its biological action.

EXPERIMENTAL

Materials

5-(4-Aminophenyl)-cytosine, 5-(4-acetamidophenyl)-cytosine, 5-(3-hydroxy-4-aminophenyl)-cytosine, 5-(3-hydroxy-4-acetamidophenyl)-cytosine, 5-(4-chlorophenyl)-2-carboxymethylthiocytosine, and 4-carboxymethylthio-5-phenylpyrimidine were prepared[1] in collaboration with J. Sluka and Z. Peřina in the Research Institute for Pharmacy and Biochemistry in Prague. Further, there were employed 1,3-dihydroxynaphthalene (British Drug House), sodium glucuronidate, monohydrate (L. Light), disodium succinate (Lachema), and potassium ferricyanide (VEB Laborchemie).

Valine-U-^{14}C (39 μCi/μmole), orotic-6-^{14}C acid (2·3 μCi/μmole), thymidine-7-^{14}C (25 μCi/μmole), and 5-(4-aminophenyl)-cytosine-2-^{14}C (12·5 μCi/μmole) were preparations of the Institute for Research, Production, and Utilization of Radioisotopes, Prague. Adenine-8-^{14}C (6·8 μCi/μmole) had been bought from Amersham. 5-(4-Acetamidophenyl)-cytosine-2-^{14}C was isolated from the tissues of mice which received 5-(4-aminophenyl)-cytosine-2-^{14}C. One hour after an i.p. injection (6μCi/μmole/mouse) the animals were sacrificed, the organs removed, homogenized and extracted with a 3-fold vol. of ice-cold 0·2 N-$HClO_4$. The supernatant was adjusted to pH 5·5 by 1N-KOH (with regard to a low solubility of both 5-(4-aminophenyl)-cytosine and its acetylated derivative in a neutral medium), and separated simultaneously with the collected urine on a Whatman paper No. 3 in a *n*-butanol–pyridine–acetic acid–water system (15:10:3:12). The zone corresponding to 5-(4-acetamidophenyl)-cytosine was eluated and further purified by chromatography.

Methods

Distribution and elimination of 5-(4-aminophenyl)-cytosine-2-^{14}C were investigated in female white mice (31–33 g) after an i.p. injection of the compound (0·3–1 μmole per animal). At selected time intervals the animals were sacrificed by decapitation, the tissues removed while cooling and extracted with a 3-fold vol. of ice-cold 0·2 N-$HClO_4$. The supernatants were centrifuged, adjusted to pH 5·5, and separated chromatographically in the following systems: *n*-butanol–pyridine–acetic

acid–water, *n*-butanol–acetic acid–water (10:1:3), and ethanol–acetic acid–water (20:1:14). The urine was collected and separated in the same way. Glucuronide was detected in eluates from chromatograms according to Fishman and Green.[8]

Deacetylation of 5-(4-acetamidophenyl)-cytosine-2-^{14}C was investigated in a cell-free rat liver extract. The livers were removed under cooling and homogenized with a 2·5-fold vol. of cold 1·15% KCl and homogenate was centrifuged (15 min, 5000 *g*, 3°). The incubation was performed at 37° in 5×10^{-2} M citrate buffer,[9] pH 7·4, in a total volume of 1 ml, after addition of 0·2 ml of a cell-free liver extract (5·2–5·4 mg of protein). The protein content was measured according to Lowry and collaborators.[10] The final concentration of 5-(4-acetamidophenyl)-cytosine-2-^{14}C was 2×10^{-4} M. Aliquots of the incubation mixture were separated in the system *n*-butanol–pyridine–acetic acid–water.

Investigation of the *in vivo* incorporation of nucleic acid precursors in mouse liver. Orotic-6-^{14}C acid, adenine-8-^{14}C, or thymidine-7-^{14}C were administered by i.p. injections of 1 μCi/μmole/animal to groups of three female white mice weighing 28–30 g. Simultaneously, either 5-(4-aminophenyl)-cytosine or another 5-aryl-pyrimidine was administered in a dose of 0·5–20 μmole per mouse. Control groups of animals received equal volumes of saline. After 2 hr the animals were sacrificed by decapitation; the livers were removed under cooling and homogenized in a Potter–Elvehjem homogenizer with a 3-fold vol. of ice-cold 0·2 N-$HClO_4$. After 10 min of standing in ice the extraction was repeated, and the sediment was hydrolyzed in 4 ml of 1 N-KOH for 18 hr at 20°C. Thereafter, 0·4 ml of 70% $HClO_4$ was added, the precipitate removed by centrifugation, and the pH of the supernatants was adjusted with 1 N-KOH to 7·5 (*cit.*[11]). In case thymidine-7-^{14}C incorporation, the sediments remaining after an alkaline hydrolysis of ribonucleic acids were heated[12] for 1 hr with 0·5 ml 70% $HClO_4$, and after centrifugation the supernatants were neutralized with 5 N-KOH. The supernatants obtained were subjected to chromatography using Whatman paper No. 3 in a system isobutyric acid–water–ammonia (66:33:1·5). The eluates of radioactive 3′(2′)-phosphates were further purified[11] in the following systems: isopropyl alcohol–hydrochloric acid–water (170:41:39), isopropyl alcohol–ammonia–water (7:1:2), and *n*-butanol–acetic acid–water. Absorbance and radioactivity were measured in aliquots of eluates of chromatographically and spectroscopically pure compounds. The incorporation is expressed as cpm/μmole.

Succinate dehydrogenase was assayed[13] in cell-free rat liver extract prepared by homogenization of the liver with a 6-fold vol. of ice-cold distilled water and by centrifugation of the homogenate (15 min, 10 000 *g*, 3°). The incubation was performed under moderate shaking at 37° in 0·2 M-borate buffer, pH 7·8, in a total volume of 2 ml, after addition of 0·2 ml cell-free liver extract (3·8–4·1 mg of protein). The concentration of succinate was 2×10^{-1} M, that of potassium ferricyanide 2×10^{-2} M, and those of 5-arylpyrimidines were $5–30 \times 10^{-3}$ M. After 60 min the incubation was stopped by the addition of 1 ml of 20% trichloroacetic acid, and after centrifugation 1 ml of the supernatant thus obtained was added to 0·5 ml of 1% $FeCl_3$. After 20 min the solution was diluted with distilled water to 10 ml, and the absorbance was measured at 650 nm on a Beckman DU spectrophotometer.

Radioactivity measurements. The zones of radioactive compounds were eluated with water on planchets. After drying the radioactivity was measured in an infinitely thin-layer using an automatic 2π scanner (Frieseke–Hoepfner).

RESULTS AND DISCUSSION

Metabolic transformations of 5-(4-*aminophenyl*)-*cytosine*

In our previous paper[7] we reported that 5-(4-aminophenyl)-cytosine is bound to blood serum proteins. The greater part of the compound, however, circulates in the free form and is easily excreted from the organism. For this reason we were interested in ascertaining how far the retention and the distribution of 5-(4-aminophenyl)-cytosine in the tissues depends on the route of administration. Table 1 indicates that

TABLE 1. DISTRIBUTION OF 5-(4-AMINOPHENYL)-CYTOSINE IN ANIMAL TISSUES AFTER ORAL AND SUBCUTANEOUS ADMINISTRATION

Tissue	Rat		Guinea pig		Rabbit	
	oral	s.c.	oral	s.c.	oral	s.c.
Blood	2·65	1·70	3·20	4·05	5·1	28·8
Liver	1·00	1·10	2·50	2·40	15·4	37·8
Kidney	0·85	13·50	1·30	28·10	6·4	43·2
Lungs	0·21	0·45	0·32	0·85	4·8	9·9
Spleen	0·16	0·36	0·26	1·40	3·2	4·8
Heart	0·16	0·09	0·28	0·45	2·4	3·6

5-(4-Aminophenyl)-cytosine was administered either in aqueous suspension (orally) or in aqueous solution, pH 5·5 (s.c.) in doses of 500 mg/kg body weight. The animals employed were female rats (170–180 g), guinea pigs (380–400 g) and rabbits (1800–2000 g). After 4 hr the animals were sacrificed and the tissues removed under cooling were homogenized in a 2·5-fold volume of 0·1 N-HCl. The homogenates were centrifugated, and aliquots of the supernatant were chromatographically partitioned[7] on Zerolite 225 by elution with 30% ethanol mixed with 1 N-NH_4OH. The figures indicate the contents of 5-(4-aminophenyl)-cytosine in mg per organ.

the oral administration is more favorable for an increased retention in the tissues. The data presented show also the existence of species differences in the distribution. While in rat and guinea pig the greatest part of 5-(4-aminophenyl)-cytosine is found in the kidney, in rabbit it is concentrated mostly in the liver. For clinical use, the oral administration of several doses daily seems suitable.

Further study of 5-(4-aminophenyl)-cytosine transformations was performed in mice using a radioactive compound. Fig. 1 shows the time-course distribution of

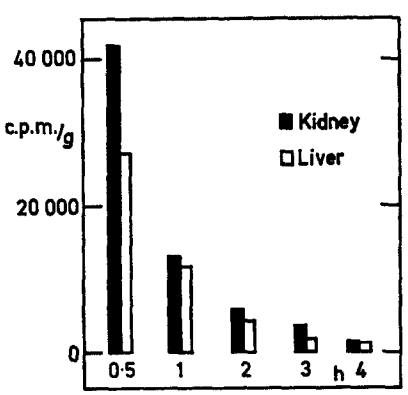


FIG. 1. 5-(4-Aminophenyl)-cytosine level in mice after i.p. administration. 5-(4-Aminophenyl)-cytosine-2-^{14}C was administered to a group of three animals. The results are expressed after calculation per 1 g wet tissue weight, h duration of action in hours.

radioactivity in the mouse liver and kidney after an i.p. administration of 5-(4-aminophenyl)-cytosine-2-^{14}C. It is evident that a considerable portion of the compound administered is rapidly eliminated from the organism. We were interested whether 5-(4-aminophenyl)-cytosine is excreted unchanged or whether it is subject to biotransformative changes that might possibly facilitate its excretion. We analyzed therefore the urine of mice that had received 5-(4-aminophenyl)-cytosine-2-^{14}C and found (Fig. 2) several radioactive compounds. One of these compounds (No. 4) was

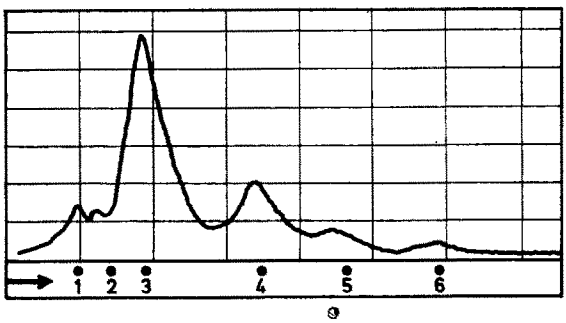


FIG. 2. Radioactive substance in the mouse urine after the administration of 5-(4-aminophenyl)-cytosine-2-^{14}C. 4 hr after the administration of 5-(4-aminophenyl)-cytosine-2-^{14}C (μmole/mouse) the urine was collected and chromatographed on Whatman paper No. 3 in the system *n*-butanol–pyridine–acetic acid–water. (1) 5-(3-Hydroxy-4-aminophenyl)-cytosine; (2) 5-(3-hydroxy-4-acetamidophenyl)-cytosine; (3) 5-(4-aminophenyl)-cytosine; (4) 5-(4-acetamidophenyl)-cytosine; (5) 2-hydroxy-4-acetamido-5-(4-acetamidophenyl)-pyrimidine; (6) supposed 5-(3-hydroxy-4-aminophenyl)-cytosine-*O*-glucuronide.

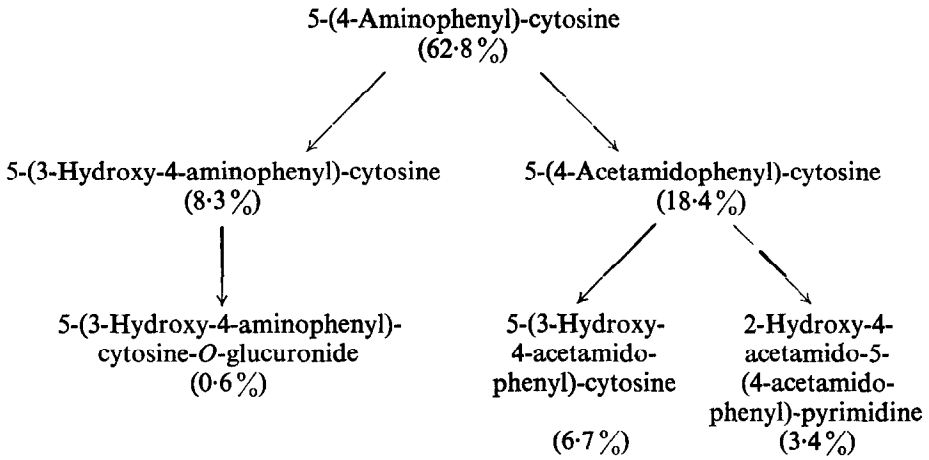
isolated by paper chromatography in pure state, analyzed, and identified as 5-(4-acetamidophenyl)-cytosine. In further three compounds (Nos. 1, 2, 5) we found the chromatographic mobility which corresponded to synthetically prepared 5-(3-hydroxy-4-aminophenyl)-cytosine, 5-(3-hydroxy-4-acetamidophenyl)-cytosine, and 2-hydroxy-4-acetamido-5-(4-acetamidophenyl)-pyrimidine, but the isolation of these compounds was not attempted. It was assumed, that the compound No. 6 was identical with 5-(3-hydroxy-4-aminophenyl)-cytosine-*O*-glucuronide.

Apart from urine, we analyzed also tissue extracts of mice after the administration of 5-(4-aminophenyl)-cytosine-2-^{14}C. We found after a brief exposure (30–45 min) a higher content of 5-(4-aminophenyl)-cytosine in the liver than in the kidney. Furthermore, in the kidney we found a compound that was assumed to be identical with 5-(3-hydroxy-4-aminophenyl)-cytosine-*O*-glucuronide. To verify this assumption, greater amounts of 5-(3-hydroxy-4-aminophenyl)-cytosine (5mg/mouse) were administered to mice, and the kidney extract subjected to chromatographic separation revealed the presence of glucuronic acid at the appropriate place.

Employing cell-free rat liver extract, deacetylation of 5-(4-acetamidophenyl)-cytosine-2-^{14}C was investigated. It is evident (Fig. 3) that this compound is easily deacetylated *in vitro*.

The results obtained indicate the following scheme of metabolites appearing

in mouse urine after the administration of 5-(4-aminophenyl)-cytosine:



The figures in parentheses show the percentual distribution of individual compounds in the urine in a typical experiment 1 hr after an i.p. administration of 5-(4-aminophenyl)-cytosine-2-^{14}C in a dose of 0·3 μmole/mouse.

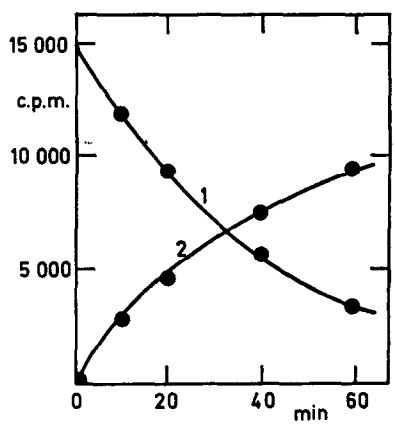


FIG. 3. Deacetylation of 5-(4-acetamidophenyl)-cytosine-2-^{14}C in the cell-free rat liver extract. 2×10^{-4} M 5-(4-acetamidophenyl)-cytosine-2-^{14}C was incubated with cell-free rat liver extract (5·1 mg of protein) in 5×10^{-2} M citrate buffer, pH 7·4, at 37°C in total volume of 1 ml. Chromatographic analysis of the incubation mixture in the system *n*-butanol–pyridine–acetic acid–water. (1) 5-(4-Acetamidophenyl)-cytosine-2-^{14}C; (2) 5-(4-aminophenyl)-cytosine-2-^{14}C.

Utilization of nucleic acid precursors in the presence of 5-(4-*aminophenyl*)-*cytosine*

Since 5-(4-aminophenyl)-cytosine is a substitution derivative of cytosine we were interested to ascertain whether it interferes in any way with the nucleic acid synthesis. We investigated therefore in mice the *in vivo* incorporation of radioactive precursors into liver ribonucleic and deoxyribonucleic acids, administering simultaneously with 5-(4-aminophenyl)-cytosine. While the incorporation of either adenine-8-^{14}C or thymidine-7-^{14}C was not influenced in any way by 5-(4-aminophenyl)-cytosine, an

increased incorporation of orotic-6-^{14}C acid was observed. The increase of orotic acid incorporation in relation to the amounts of 5-(4-aminophenyl)-cytosine administered is given in Fig. 4. This effect, however, is not specific for 5-(4-aminophenyl)-cytosine since the administration of other 5-arylpyrimidines increased the incorporation of orotic acid as well (Table 2).

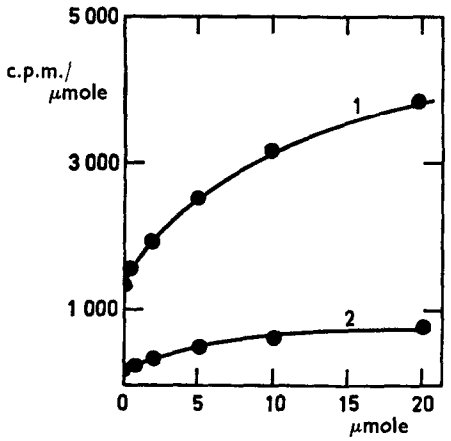


FIG. 4. Increased incorporation of orotic-6-^{14}C acid into liver ribonucleic acids in the presence of 5-(4-aminophenyl)-cytosine. Orotic-6-^{14}C acid (1 μCi/μmole/mouse) was administered to groups of 3 animals simultaneously with 5-(4-aminophenyl)-cytosine. After 2 hr the animals were sacrificed and the radioactivities of uridine-3'(2')-phosphate: (1) and of cytidine-3'(2')-phosphate; (2) of liver ribonucleic acid were measured. μmole, 5-(4-Aminophenyl)-cytosine administered per mouse; cpm/μmole, specific radioactivity of isolated nucleotides.

TABLE 2. UTILIZATION OF OROTIC-6-^{14}C ACID FOR THE SYNTHESIS OF LIVER RIBONUCLEIC ACID IN THE PRESENCE OF VARIOUS 5-ARYLPYRIMIDINES

5-Arylpyrimidine (10 μmole/mouse)	Uridine-3'(2')-phosphate		Cytidine-3'(2')-phosphate	
	Specific radioactivity (cpm/μmole)	Incorporation (%)	Specific radioactivity (cpm/μmole)	Incorporation (%)
Control	1333	100·0	207	100·0
5-(4-Aminophenyl)-cytosine	3100	232·5	629	303·1
5-(4-Chlorophenyl)-2-carboxymethylthiocytosine	2795	209·6	660	318·2
4-Carboxymethylthio-5-phenylpyrimidine	2761	207·1	614	296·1

Orotic-6-^{14}C acid was administered i.p. (1 μCi/μmole/mouse) simultaneously with 5-arylpyrimidines or with an equal volume of saline 2 hr before the animals were sacrificed.

Effect of 5-(4-aminophenyl)-cytosine on succinate dehydrogenase

The finding of the increased incorporation of orotic acid after the administration of 5-(4-aminophenyl)-cytosine led us to the investigation of the effect of this compound on the activity of dehydrogenases. It is known that a variety of compounds, containing 2,4-diaminopyrimidine moiety and thus resembling 5-(4-aminophenyl)-cytosine, behave as folic acid antagonists inhibiting the activity of folate reductase (see, e.g.[14, 15]).

In our paper succinate dehydrogenase of the cell-free rat liver extract was used as a dehydrogenase model.[13]

The inhibition of succinate dehydrogenase in dependence on the concentration of 5-(4-aminophenyl)-cytosine and of 5-(4-chlorophenyl)-2-carboxymethylthiocytosine is evident in Fig. 5 (50% inhibition of approximately 5×10^{-3} M 5-(4-aminophenyl)-cytosine, or 9×10^{-3} M 5-(4-chlorophenyl)-2-carboxymethylthiocytosine by

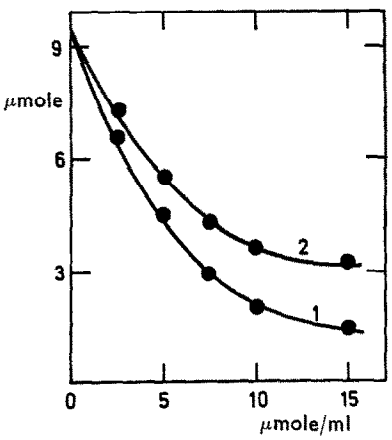


FIG. 5. Effect of 5-(4-aminophenyl)-cytosine and of 5-(4-chlorophenyl)-2-carboxymethylthiocytosine on succinate dehydrogenase. Incubated in 0·2 M-borate buffer, pH 7·8, for 60 min at 37·2° × 10^{-1} M disodium succinate, 2×10^{-2} M potassium ferricyanide, 3·84 mg of protein in the cell-free rat liver extract added. μmole, Potassium ferrocyanide formed; μmole/ml, concentration of 5-arylpyrimidine; (1) 5-(4-Aminophenyl)-cytosine; (2) 5-(4-chlorophenyl)-2-carboxymethylthiocytosine.

2×10^{-1} M succinate). Simultaneously with these compounds we investigated the effect of other 5-arylpyrimidines that in our experiments increased the incorporation of orotic acid. It seems (Table 3) that the increased incorporation of orotic acid is in relation to the degree of succinate dehydrogenase inhibition. It is still uncertain,

TABLE 3. EFFECT OF SOME 5-ARYLPYRIMIDINES ON SUCCINATE DEHYDROGENASE

5-Arylpyrimidine (20×10^{-3} M)	Inhibition (%)
5-(4-Aminophenyl)-cytosine	77·4
5-(4-Acetamidophenyl)-cytosine	77·4
5-(4-Chlorophenyl)-2-carboxymethylthiocytosine	60·2
4-Carboxymethylthio-5-phenylpyrimidine	53·8

Incubation in 0·2 M-borate buffer, pH 7·8, for 60 min at 37° in a total volume of 2 ml. 2×10^{-1} M Disodium succinate, 2×10^{-2} M potassium ferricyanide. 3·8 mg of protein in added cell-free rat liver extract.

however, whether the increased incorporation of orotic acid after the administration of 5-arylpyrimidines is due to an inhibition of dihydroorotic acid dehydrogenase.

To identify the inhibitory action of 5-(4-aminophenyl)-cytosine, we employed the method of Lineweaver and Burk.[16] From Fig. 6 the competitive character of the

succinate dehydrogenase inhibition by 5-(4-aminophenyl)-cytosine is evident. The value of Michaelis constant was calculated $K_m = 9{\cdot}2 \times 10^{-3}$ M, and the value of the inhibition constant $K_i = 5{\cdot}0 \times 10^{-3}$ M.

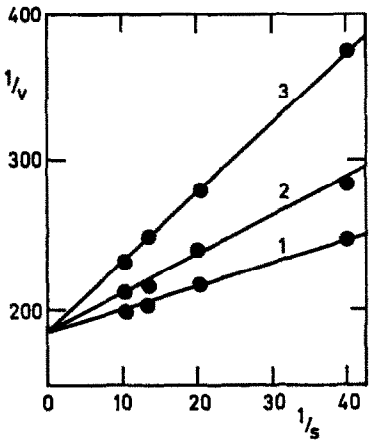


FIG. 6. The Lineweaver–Burk plot of the inhibition of succinate dehydrogenase by 5-(4-aminophenyl)-cytosine. Incubated in 0·2 M-borate buffer, pH 7·7, for 60 min at 37°. 2×10^{-2} M potassium ferricyanide, 3·81 mg of protein in cell-free rat liver extract, total volume 2 ml. (1) Control; (2) 2×10^{-3} M; (3) 8×10^{-3} M 5-(4-aminophenyl)-cytosine. s Disodium succinate in mole/.l, v potassium ferrocyanide in mole/l.

Because the biological effects of 5-(4-aminophenyl)-cytosine, for example its antibacterial action, could not be reversed by the addition of natural nucleic acids precursors (unpublished), and because its incorporation into nucleic acids could not be demonstrated, we suppose that this substance cannot be regarded as an antimetabolite of nucleic acids precursors. We assume that the biological effects of 5-(4-aminophenyl)-cytosine, and probably also the effects of other virostatically active 5-arylpyrimidines, are based on their interference with the dehydrogenase activity.

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