The Uptake of Guanine, Uracil, Lysine and Glycine by Ascites Cells Pretreated with Azaserine and 6-Thioguanine

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Synthetic purine and pyrimidine analogues have been widely studied as inhibitors of bacterial growth and as cancer chemotherapeutic agents. A number of these compounds, e.g. fluorouracil (Chaudhuri, Montag & Heidelberger, 1958) and 8-azaguanine (Mandel, Carlo & Smith, 1954), are incorporated to a greater or less extent into cellular RNA. Others, e.g. bromo- and iodo-deoxyuridine, have been shown to be incorporated into DNA (Hakala, 1959; Prusoff, 1959), and in small amounts 6-mercaptopurine appeared to be incorporated into both RNA and DNA (Bieber, Dietrich, Elion, Hitchings & Martin, 1961). In addition to their ability to become incorporated into nucleic acids, such substances may interfere with specific steps in nucleic acid synthesis. Thus iododeoxyuridine inhibits the incorporation of either [^{14}C]formate or [^{14}C]orotate into DNA thymine (Prusoff, 1960). Fluorodeoxyuridine, which is interconvertible with fluorouracil, acted as a powerful inhibitor of thymidylate synthetase in addition to being incorporated (as fluorouracil) into RNA (Cohen, Flaks, Barner, Loeb & Lichenstein, 1958; Habers, Chaudhuri & Heidelberger, 1959). Many similar examples appear in the literature. There have also been many suggestions that RNA containing abnormal bases has a diminished capacity for the synthesis of specific proteins or produces proteins with altered amino acid sequences (Creaser, 1956; Chantrenne, 1958; Mandel & Markham, 1958; Chantrenne & Devreux, 1960; Kvam & Parks, 1960; Mandel & Altman, 1960; Naono & Gros, 1960; Aaronson, 1961).

So far, however, no studies appear to have been made on the metabolism of cells that have incorporated 6-thioguanine. 6-Thioguanine has a pronounced inhibitory effect on many tumours, and a combination of thioguanine with pretreatment with azaserine increased both the 6-thioguanine incorporation and tumour inhibition (Sartorelli & LePage, 1958*b*). According to LePage (1960) the incorporation of 6-thioguanine into DNA was the only property that correlated with its tumour-inhibitory action. However, since incorporation into DNA

was always accompanied by incorporation into RNA, the evidence equally supports the view that this simultaneous dual incorporation is a significant factor.

It was decided therefore to investigate the ability of cells treated with 6-thioguanine to carry out protein and nucleic acid synthesis at a point after treatment when free 6-thioguanine is no longer present in the system. This has been attempted by giving cells a course of therapy with 6-thioguanine, or with 6-thioguanine in combination with azaserine, and subsequently studying the ability of such cells to take up label from [^{14}C]guanine or [^{14}C]uracil into their nucleic acids, and [^{14}C]lysine into their proteins. The system used has been a 6-thioguanine-sensitive S180 ascites tumour growing in Swiss mice. Because of the effects produced by azaserine alone, groups treated with azaserine alone have been included in all experiments.

MATERIALS AND METHODS

Animals and tumours. Adult Swiss female mice (weight 25–30 g.) were used as hosts for a 6-thioguanine-sensitive Sarcoma 180 ascites tumour. The tumour is one carried in this Laboratory, and has been maintained over a prolonged period by serial passage.

Drugs. Azaserine (*O*-diazooacetyl-L-serine) was obtained from the Cancer Chemotherapy National Service Center, National Institutes of Health, and 6-thioguanine from the Nutritional Biochemicals Corp., Cleveland, Ohio. Azaserine was dissolved in 0.9% NaCl solution and three doses of 1 mg./kg. were injected intraperitoneally as described below. 6-Thioguanine was dissolved in 0.9% NaCl with the addition of minimal quantities of NaOH, and three doses of 10 mg./kg. were injected intraperitoneally. When animals were given azaserine plus 6-thioguanine, the 6-thioguanine dose was halved. [^{14}C]Uracil, [^{14}C]guanine, L-[^{14}C]lysine and [^{14}C]glycine were obtained from New England Nuclear Corp., Boston, Mass. The labelled compounds were diluted with unlabelled material to give a specific radioactivity of 1–2 $\mu\text{C}/\mu\text{mole}$, dissolved in 0.9% NaCl, with the addition of minimal quantities of NaOH where necessary, and injected intraperitoneally in the amounts stated (usually about 1 $\mu\text{mole}/\text{mouse}$).

Schedule of drug administration. The use of cells pretreated with 6-thioguanine presents certain inherent difficulties. Since ascites-cell populations are asynchronous with respect to mitosis, and therefore DNA synthesis, a single injection of 6-thioguanine will allow incorporation into the

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DNA of only a proportion of the cells. In fact a minimum of three suitably spaced injections is necessary to produce maximum incorporation into DNA (LePage & Jones, 1961). However, the adoption of this procedure must mean that individual cells will have had 6-thioguanine in their nucleic acids for various periods before receiving the labelled compounds under investigation. Also, 6-thioguanine exerts a direct competitive inhibition of guanine uptake for at least 2 hr. after injection (Sartorelli & LePage, 1958*b*). This complication can be avoided by allowing a time-interval to elapse between the injection of 6-thioguanine and the administration of the labelled compound, but if the interval is too long there will be an increasing proportion of dead or dying cells. Further, when azaserine is used in combination with 6-thioguanine it must be given some time before the 6-thioguanine to produce a maximum increase in incorporation. In an attempt to take these factors into account the following procedure was adopted for each experiment. On day 0, each of a group of 60 mice was inoculated intraperitoneally with approx. 2×10^6 S180 ascites cells. Early on day 4, four groups of 12 mice each were selected from those inoculated. Any mice that appeared by inspection to have less or more ascites-cell growth than average were rejected. One group was set aside as controls, and the other three groups were treated as follows: (1) 6-thioguanine (10 mg./kg.) injected at 8 a.m. and 8 p.m. on day 4 and at 8 a.m. on day 5; (2) azaserine (1 mg./kg.) injected at 8 p.m. on day 4 and at 8 a.m. and 8 p.m. on day 5; (3) azaserine (1 mg./kg.) injected at 8 p.m. on day 4 and at 8 a.m. and 8 p.m. on day 5, and also 6-thioguanine (5 mg./kg.) injected at 8 a.m. and 8 p.m. on day 5 and at 8 a.m. on day 6. At 11 a.m. on day 5 the controls were injected with labelled compound, and at 11.15 a.m. the mice treated with 6-thioguanine were similarly injected; this was repeated on day 6 with the mice treated with azaserine and azaserine plus 6-thioguanine respectively. The staggering by 1 day of the control group and that treated with 6-thioguanine on the one hand and the groups treated with azaserine and azaserine plus 6-thioguanine on the other partly compensated for the greater decrease in cell population resulting from the latter treatments. Thus, when harvested, about 0.5 g. of packed cells was obtained from each control mouse, and 0.3–0.35 g. from each treated mouse.

Preparation of homogenates and subcellular fractions. The procedure therefore involved waiting for just over 3 hr. after the last injection of 6-thioguanine and about 15 hr. after the last injection of azaserine before giving the labelled compounds. Three mice from each group were killed at 0.5, 2.5, 5 and 21 hr., after the injection of labelled compound, their peritoneal contents removed with a Pasteur pipette and the peritoneal cavities each washed three times with 2 ml. of glucose–NaCl–tris buffer, pH 8.4. The cells from each group of three mice were pooled in a tared centrifuge tube immersed in ice. All subsequent operations were carried out at 0–5° unless otherwise stated. The pooled cells were diluted to about 15 ml. with buffer and centrifuged at 1000*g* for 10 min. The supernatants were discarded and the cells resuspended in 15 ml. of fresh buffer and centrifuged at 1000*g* for 10 min. The supernatants were again discarded, the tubes drained and the inner surfaces dried with filter paper. The tubes were rapidly reweighed and the net weight of cells was recorded in each case. Subcellular fractions were then prepared by the

following method. At time 0 min. the packed cells were stirred with 8 ml. of ice-cold water, and the suspension was transferred to the barrel of a Ten Broeck grinder (see Adams & Burgess, 1957). The suspension was dispersed by three up-and-down strokes of the pestle. At time 1 min. 45 sec. homogenization was completed by ten additional strokes of the pestle, and 1 vol. of 34% (w/v) sucrose and 2 vol. of double-strength 'medium A' (Littlefield & Keller, 1957) were rapidly added. The solutions were mixed by inversion. Microscopic examination showed few intact cells, many apparently intact nuclei and a few fragmented nuclei. Fractions were collected by centrifuging as follows: (1) 1000*g* for 10 min. in the International PR₂ refrigerated centrifuge to give the nuclear fraction (nuclei and debris); (2) 10000*g* for 10 min. in the International PR₂ refrigerated centrifuge to give the large-granule fraction; (3) 144000*g* for 90 min. in the Spinco model L centrifuge, with the type 40 angle-head, to give the microsomal fraction; (4) the supernatant from (3) was the soluble fraction. The pellets were drained, resuspended in a few millilitres of medium A and stored at –20° until they could be processed (usually about 48 hr.).

Extraction of nucleic acids. DNA and RNA were extracted by the method of Tyner, Heidelberger & LePage (1953). Samples of solutions containing DNA or RNA, when appropriate, were plated and counted by standard procedures in a Nuclear–Chicago gas-flow end-window counter (30% efficiency) and corrected for self-absorption.

Determination of nucleic acids. RNA was determined by the orcinol method (Hurlbert, Schmitz, Brum & Potter, 1954), and DNA by a modified diphenylamine reaction (Dische, 1955).

Protein fractions. Specific radioactivities of protein fractions were measured as follows. The residues remaining after nucleic acid extraction were washed three times with ethanol at room temperature and once with ethanol–ether at 45°. The product was dried *in vacuo* and weighed as protein. Each dry protein fraction was soaked for 30 min. in 0.1 N-NaOH (4 ml.). Then 4 ml. of water was added, and the mixture was homogenized in a Ten Broeck grinder, transferred to 10 ml. volumetric flasks and made up to volume. After standing for 48 hr. most of the material had gone into solution, and the residue was sufficiently finely divided to allow the mixture to be pipetted, plated and counted as described for nucleic acid.

Determination of acid-soluble uracil and cytosine pools. The total uracil (i.e. uracil plus uridine and uridylic acid) and total cytosine pools were determined as follows. Ascites cells (0.5–1 g. wet wt.) were homogenized in 5 ml. of water at 0° in a rotating-glass-pestle homogenizer. Then 1 ml. of 50% (w/v) trichloroacetic acid was added, the precipitate was centrifuged and washed with 3 ml. of 5% (w/v) trichloroacetic acid, and the supernatants were combined. The combined supernatants were passed through a small column (5 mm. $\times$ 20 mm.) of Dowex 50 (H⁺ form).

The eluate was collected, and remaining pyrimidines were washed off with 10 ml. of 0.01 N-HCl. At this stage 0.2 μ g. of [¹⁴C]uracil (8000 counts/min.) was added to the combined solutions to enable a correction to be made for ensuing losses. The solutions were dried in a vacuum desiccator over NaOH. To the solid residue 1.5 ml. of 70% (w/v) perchloric acid was added, and the mixture heated at 100° for 1 hr. to convert any pyrimidine nucleotides and nucleosides into free bases. The resulting mixture

Table 1. *Nucleic acid content of S180 ascites cells from untreated mice and from mice treated with 6-thioguanine, azaserine, or azaserine plus 6-thioguanine, and subsequently given [¹⁴C]guanine, [¹⁴C]uracil, or L-[¹⁴C]lysine*

Experimental details are given in the text. The doses given were: 6-thioguanine alone, 10 mg./kg.; azaserine alone, 1 mg./kg.; azaserine plus 6-thioguanine, 1 mg./kg. and 5 mg./kg. respectively. The mice received 100 μ g. (1 μ Ci) of [¹⁴C]guanine, 125 μ g. (2 μ Ci) of [¹⁴C]uracil, or 100 μ g. (1 μ Ci) of L-[¹⁴C]lysine, as indicated.

Time after injection of labelled compound (hr.)	Labelled compound											
	¹⁴ C[Guanine]				¹⁴ C[Uracil]				L- ¹⁴ C[Lysine]			
	Control	6-Thio- guanine	Azaserine	Azaserine + 6-thio- guanine	Control	6-Thio- guanine	Azaserine	Azaserine + 6-thio- guanine	Control	6-Thio- guanine	Azaserine	Azaserine + 6-thio- guanine
Nucleic acid content (mg./g. wet wt.)												
(a) RNA content	Nuclear fraction											
	0.5	3.6	3.2	3.9	4.0	2.5	2.5	2.7	2.5	2.4	2.7	2.1
	2.5	3.5	3.3	4.2	3.9	2.8	2.6	2.7	2.2	2.6	2.3	1.7
	5.0	3.5	3.5	4.0	3.6	2.7	2.6	2.8	1.9	2.5	2.3	1.5
	21	3.3	3.1	3.6	3.3	2.8	2.6	2.8	1.7	2.9	2.2	1.2
	Microsome fraction											
	0.5	6.3	6.3	6.1	5.5	6.0	5.6	4.8	4.5	6.6	6.5	5.8
	2.5	6.9	5.7	6.2	5.2	5.9	5.3	4.1	4.2	6.1	6.0	5.4
	5.0	6.0	5.8	6.4	5.3	6.0	4.8	4.8	3.9	6.3	5.7	4.3
	21	6.2	5.7	5.7	4.8	5.8	4.0	4.6	3.6	6.5	5.2	3.3
Soluble fraction	0.5	1.4	1.3	1.5	1.5	1.3	1.4	1.1	1.2	1.3	1.2	1.0
	2.5	1.2	1.6	1.3	1.1	1.3	1.3	1.2	1.1	1.1	1.4	1.2
	5.0	1.3	1.6	1.1	1.3	1.4	1.3	1.4	1.4	1.2	1.1	1.2
	21	1.2	1.3	1.1	1.2	1.4	1.5	1.2	1.3	1.3	1.2	1.2
	Large- granule fraction											
	0.5	0.28	0.20	0.21	0.21	0.27	0.22	0.28	0.20	0.18	0.25	0.22
	2.5	0.28	0.24	0.23	0.21	0.30	0.26	0.35	0.27	0.25	0.21	0.20
	5.0	0.30	0.22	0.25	0.22	0.24	0.20	0.25	0.27	0.30	0.22	0.29
	21	0.20	0.33	0.22	0.31	0.23	0.27	0.24	0.4	0.22	0.20	0.21
	(b) DNA content	Nuclear fraction										
0.7		11.0	11.0	11.3	11.0	11.4	11.3	10.6	10.5	10.1	10.5	10.5
2.7		12.0	11.1	12.2	11.5	11.5	10.7	11.3	11.3	10.6	10.2	9.2
5.0		11.2	11.8	12.5	11.7	10.9	11.0	10.1	11.5	10.0	10.7	9.8
21		11.0	10.9	12.0	11.4	10.8	10.7	10.6	11.2	10.7	10.6	10.0

was neutralized with 50% (w/v) KOH and kept at 0° for 30 min., and the precipitated KClO_4 and charred debris were removed by centrifuging at 0°. A column (5 mm. $\times$ 20 mm.) of Dowex 1 (formatted form) was prepared by washing with conc. formic acid, followed by water until the eluate was neutral. The supernatant from the KClO_4 precipitate was placed on this column, followed by 10 ml. of 0.01N-HCl. The combined eluates were evaporated to dryness in a vacuum desiccator. The residue was dissolved in 2 ml. of 0.1N-HCl, and again placed on a column of Dowex 50 (H^+ form). The column was washed with 10 ml. of water and the combined eluates were evaporated to dryness. The residue was dissolved in 0.5 ml. of 0.05N-HCl, and run on paper with 70% (v/v) 2-methylpropan-2-ol made 0.8N with respect to HCl. Uracil, cytosine and thymine were run as markers. Circles were cut from the paper at the appropriate places, and the free bases were eluted with 3 ml. of N-HCl and determined by measuring the extinction at 260 m μ with a Beckman DU spectrophotometer. About 30% of the radioactivity of the added [^{14}C]uracil was recovered, and the amounts of uracil and cytosine found were therefore corrected by this factor in estimating the total amount present originally.

RESULTS

Groups of S180 ascites mice were treated with azaserine, 6-thioguanine, or azaserine plus 6-thioguanine as described above, and together with a control group were injected with 100 μg . (1 μC) of [^{14}C]guanine. Fig. 1 shows the subsequent specific radioactivities of the nucleic acids obtained from the various subcellular fractions, and Table 1 the DNA and RNA contents of each fraction. In the untreated cells there was a very rapid appearance of label in the nuclear RNA followed by a rapid disappearance. The label appeared more slowly in the soluble RNA (s-RNA), and at the slowest rate in the microsomal RNA. The pattern of labelling in the large granule fraction resembled that in the microsomal fraction. In the cells treated with 6-thioguanine alone, the appearance of label in the nuclear, large-granule and s-RNA fractions was very similar to the pattern in untreated cells. However, there appeared to be a decreased rate of appearance of label in the microsomal RNA. Azaserine alone increased the specific radioactivity of all RNA fractions, despite the long time-interval of 15 hr. between the last azaserine injection and the injection of labelled compound. In the cells treated with azaserine plus 6-thioguanine this stimulation was largely abolished, and in the microsomal fraction the label appeared at only about one-half of the normal rate over the first 5 hr. The results with DNA resembled those with s-RNA. On a tissue-wet-weight basis the contents of DNA, nuclear, large-granule and s-RNA fractions appeared to be unchanged, but in the cells treated with azaserine plus 6-thioguanine there was a decrease in microsomal RNA. The experiment was

repeated with 125 μg . (2 μC) of [^{14}C]uracil/mouse (Fig. 2). In the nuclear, large-granule and s-RNA fractions the pattern of labelling was similar to that in the preceding experiment. In the microsomal fraction treatment with 6-thioguanine decreased the rate of labelling to about 50% of that in the

Fig. 1. Specific radioactivities of nucleic acids from S180 ascites cells at various times after the injection of 100 μg . (1 μC) of [^{14}C]guanine into each host mouse. Each point represents the pooled cells from three mice. The host mice were either untreated ($\bullet$), or were pretreated according to the text schedule with 6-thioguanine ($\circ$), azaserine (Δ), or azaserine + 6-thioguanine ($\blacktriangle$). (a) Nuclear RNA; (b) s-RNA; (c) microsomal RNA; (d) large-granule RNA; (e) DNA.

Fig. 2. Specific radioactivities of nucleic acids from S180 ascites cells at various times after the injection of 125 μg . (2 μC) of [^{14}C]uracil into each host mouse. Each point represents the pooled cells from three mice. The host mice were either untreated ($\bullet$), or were pretreated according to the text schedule with 6-thioguanine ($\circ$), azaserine (Δ), or azaserine + 6-thioguanine ($\blacktriangle$). (a) Nuclear RNA; (b) s-RNA; (c) microsomal RNA; (d) large-granule RNA; (e) DNA.

control, as large a decrease as that produced by treatment with azaserine plus 6-thioguanine. In the DNA, azaserine produced a 30% inhibition of label uptake, 6-thioguanine a 60% inhibition, and almost no label appeared for at least 5 hr. in the cells treated with azaserine plus 6-thioguanine. As in the preceding experiment the DNA content of the cells was unaltered, but the cellular RNA contents were affected to a greater extent (Table 1). The nuclear RNA content fell to about 65% of the normal value in cells treated with azaserine plus 6-thioguanine and the microsomal RNA content to about 60% of the normal value. In cells treated with azaserine alone or 6-thioguanine alone the microsomal RNA content was also below normal. No change was observed in the large-granule and s-RNA contents.

In further experiments treated and untreated cells were given 100 μ g. (1 μ c) of [14 C]lysine (Fig. 3). Compared with untreated cells, in the nuclear fraction 6-thioguanine alone caused a decrease in uptake, azaserine alone a greater decrease and azaserine plus 6-thioguanine a still greater decrease. A similar result was obtained in the soluble protein fraction, except that no inhibition was seen in cells

treated with 6-thioguanine alone. In the microsomal protein fraction 6-thioguanine alone inhibited label uptake by about 50%, azaserine alone by 65% and azaserine plus 6-thioguanine by about 85%. All treatments also diminished the uptake in the large-granule fraction. No change was observed in cellular DNA content, and the results with RNA resembled those obtained after the administration of [14 C]uracil in the preceding experiment. The protein content of the various fractions are shown in Table 2, and although there were no marked changes in the treated cells there was some evidence of a decreased protein content in the nuclear and microsomal fractions of cells treated with 6-thioguanine alone or with azaserine plus 6-thioguanine.

In a further experiment, groups of 12 mice were injected with three doses of 0.2, 0.5, 2.0 and 8.0 mg. of azaserine/kg. beginning on the evening of day 4 after the inoculation with S180 cells. At 15 hr. after the last azaserine injection the animals were divided into subgroups of six, and each subgroup was injected with 120 μ g. (1 μ c) of L-[14 C]lysine/mouse and 100 μ g. (1.3 μ c) of [14 C]glycine/mouse respectively. Groups of untreated controls were given the labelled compounds at the same time. At 30 min. after the injection of the labelled compounds, three mice from each subgroup and three control mice were killed, their cells were harvested, and DNA and RNA ([14 C]glycine-injected groups only) and protein were isolated. In this experiment the cellular fractions were not separated. The remaining mice were killed 5 hr. after receiving the labelled compounds. The results are given in Table 3. As reported by Fernandes, LePage & Lindner (1956), [14 C]glycine uptake into both nucleic acid fractions was strongly inhibited, and maximum inhibition was produced by low doses of azaserine. However, the uptake of both [14 C]glycine and [14 C]lysine into protein was also inhibited. The effect was greater 5 hr. after the injection of the labelled compounds than after 30 min.

The results show that the uptake of [14 C]uracil into RNA of cells treated with azaserine alone was greater than in controls. Since this result was somewhat unexpected, experiments were carried out in an attempt to provide an explanation. The stimulation of [14 C]guanine uptake by cells treated with azaserine alone is usually considered to result from a decrease in the guanine pool due to the inhibition of the synthesis of guanine. It seemed necessary therefore to see if the cellular uracil pool was also reduced. Sixteen mice were inoculated with S180 ascites cells, and divided into two groups. On the evening of day 3 after inoculation one group was injected with 1 mg. of azaserine/kg., followed by two further injections at 12 hr. intervals. On the morning of day 5 the mice were killed in groups

Fig. 3. Specific radioactivities of protein fractions from S180 ascites cells at various times after the injection of 100 μ g. (1 μ c) of L-[14 C]lysine into each host mouse. Each point represents the pooled cells from three mice. The host mice were either untreated (●), or were pretreated according to the text schedule with 6-thioguanine (○), azaserine (△), or azaserine + 6-thioguanine (▲). (a) Microsome fraction; (b) soluble fraction; (c) nuclear fraction; (d) large-granule fraction.

of four, and the acid-soluble pyrimidine pools determined (Table 4).

Since the results of Table 4 suggested that the total uracil pool was increased in animals treated with azaserine, an experiment was carried out to determine whether the stimulation of [^{14}C]uracil uptake could result from the experimental conditions. As stated above, the cell weight in animals treated with azaserine alone was about 30% below that in untreated animals, and since the same dosage of labelled compounds was given in each case this might result in an apparently larger uptake.

A group of 12 mice was inoculated with S180 ascites cells. In the afternoon of day 3 three mice were injected with 120 μg . (2 μc) of [^{14}C]uracil and three mice with half this quantity. After 2½ hr. the mice were killed, and the cells, after weighing, were resuspended in water and stored at -20° . On the evening of day 3 the remaining mice were injected with 1 mg. of azaserine/kg., the dose being repeated twice at 12 hr. intervals. At 8 a.m. on day 5, these mice were injected with [^{14}C]uracil in the same way as the controls. This staggering of the groups by about 36 hr. resulted in closely

Table 2. *Protein content of S180 ascites cell fractions from untreated mice and from mice treated with 6-thioguanine, azaserine, or azaserine plus 6-thioguanine*

Experimental details are given in the text. The doses of azaserine and 6-thioguanine given were as indicated in Table 1. All mice received 100 μg . (1 μc) of L-[^{14}C]lysine.

Time after injection of [^{14}C]lysine (hr.)	Protein content (mg./g. wet wt.)			
	Control	6-Thioguanine	Azaserine	Azaserine + 6-thioguanine
Nuclear fraction				
0.5	31	23	32	33
2.5	29	22	33	26
5	31	24	36	22
21	33	29	30	20
Large-granule fraction				
0.5	4.5	3.2	6.3	5.3
2.5	4.0	4.2	5.8	5.8
5	4.7	5.0	6.3	6.5
21	4.9	2.5	6.5	6.5
Ribosome fraction				
0.5	10.0	9.5	11.6	11.5
2.5	10.1	9.0	11.4	10.3
5	10.0	7.4	11.2	9.6
21	10.2	6.7	9.8	8.8
Soluble fraction				
0.5	39	35	39	44
2.5	35	33	38	38
5	34	30	40	38
21	36	29	37	37

Table 3. *Effect of different doses of azaserine on the uptake of [^{14}C]glycine into nucleic acids and protein, and of L-[^{14}C]lysine into protein, of S180 ascites cells*

Experimental details are given in the text. Three doses of azaserine were given to the host mice at 12 hr. intervals, the last one 15 hr. before the injection of the labelled compound. All mice received either (a) 100 μg . (1.3 μc) of [^{14}C]glycine, or (b) 100 μg . (1.0 μc) of [^{14}C]lysine.

		Time after injection of labelled compound (hr.)	Dose of azaserine ($\mu\text{g.}$) (given three times)				
			0	5	12.5	50	200
			Specific radioactivity (counts/min./mg.)				
(a)	Glycine DNA	0.5	300	235	180	190	200
		5	820	210	200	190	210
	RNA	0.5	1120	560	320	360	330
		5	2450	550	460	410	400
	Protein	0.5	800	700	450	600	730
		5	1100	750	730	650	750
(b)	L-Lysine Protein	0.5	1250	1275	1050	1070	850
		5	2800	1550	1500	1350	830

similar cell weights in the normal animals and those treated with azaserine. Total RNA and DNA were extracted and the specific radioactivities compared (Table 5).

DISCUSSION

It was not the purpose of the present study to investigate the action of azaserine itself, but only to make use of this compound to increase the incorporation of 6-thioguanine. However, the inhibition of [^{14}C]lysine uptake into protein and the increased [^{14}C]uracil uptake into the RNA of cells treated with azaserine alone was unexpected. According to Handschumacher & Welch (1960), 'the sites of enzymic inhibition established by AS [i.e., azaserine] have been clearly indicated as those which utilise glutamine'. However, very high concentrations of azaserine were required *in vitro* to inhibit glutamine utilization in the formation of phosphoribosylamine from phosphoribosyl pyrophosphate (Goldthwait, 1956) or of the conversion of uridylic acid into cytidylic acid (Kammer & Hurlbert, 1959). On the other hand, LePage and his group have clearly shown that azaserine irreversibly inhibits the enzyme concerned in the production of (α -N-formyl)glycinamide ribotide from (α -N-formyl)glycinamide, and an inhibition of

about 75 % was produced by the addition of about 0.1 μmole of azaserine *in vitro* (Greenlees & LePage, 1956; LePage, Greenlees & Fernandes, 1956). Glutamine was able to prevent this inhibition only partly when added before the azaserine and at about 1000 times its concentration.

The results of Table 4 showed that the [^{14}C]uracil uptake, in both normal cells and those treated with azaserine, was greatly affected by the amount of [^{14}C]uracil injected. However, even with closely similar wet weights of cells, the specific radioactivity of the RNA was higher in cells treated with azaserine. The results of Fig. 2 show that, 2.5 hr. after the administration of [^{14}C]uracil, the radioactivity present in nuclear and s-RNA fractions was at least twice the normal, whereas at this point the increase in microsomal-RNA radioactivity was much less. Since two-thirds of the cellular RNA resides in the microsomes it is not surprising that the increase in specific radioactivity shown in Table 4 was relatively small. The results in Fig. 2 were obtained with about 30 % fewer cells present in the preparations of cells treated with azaserine than in the normal cells. Whereas, from the results of Table 5, this will obviously be a factor in causing a higher uptake in the cells treated with azaserine that are given [^{14}C]uracil, it does not seem capable of accounting for the whole effect.

The determination of the uracil pool also provided no explanation, since the total pool in cells treated with azaserine was about 25 % greater than normal. The associated increase in cytosine pool suggests that azaserine does not inhibit the conversion of uridylic acid into cytidylic acid under the conditions studied.

The present results also show that the uptake of both [^{14}C]glycine and [^{14}C]lysine into cellular protein was inhibited by azaserine. The small dosage of azaserine (totalling about 0.5 μmole /mouse in the main experiments) and the long time-interval of 15 hr. between the last azaserine injection

Table 4. *Total acid-soluble uracil and cytosine in normal S180 ascites cells and cells after three doses of azaserine to the host mice*

Experimental details are given in the text. The dose given was 1 mg. of azaserine/kg. Pooled cells from four mice/group were examined.

	Base content ($\mu\text{g.}/\text{g. wet wt.}$)			
	Controls		Treated with azaserine	
Uracil	87	90	120	128
Cytosine	1-2	1-2	15	20

Table 5. *Effect of dosage to the host mouse of [^{14}C]uracil on the specific radioactivity of nucleic acids of S180 ascites-tumour cells*

Experimental details are given in the text. Pooled cells from three mice/group were examined.

	Dose of [^{14}C]uracil/mouse			
	120 $\mu\text{g.}$ (2 μC)		60 $\mu\text{g.}$ (1 μC)	
	Control	Treated with azaserine	Control	Treated with azaserine
Wet wt. of cells (g.)	0.55	0.60	0.57	0.54
RNA				
Total weight (mg.)	6.6	6.2	6.3	5.8
Specific radioactivity (counts/min./mg.)	1030	1230	550	720
DNA				
Total weight (mg.)	6.3	6.3	6.4	6.3
Specific radioactivity (counts/min./mg.)	570	420	300	240

tion and the injection of the labelled compound make it most unlikely that any competitive inhibitory effect of azaserine was responsible. It has been reported that, though incorporation of glycine into nucleic acid was strongly inhibited, incorporation into protein proceeded normally in cells treated with azaserine (Fernandes *et al.* 1956; Henderson, LePage & McIver, 1957). However, these authors used only one dose of azaserine and waited 3–6 hr. before giving labelled glycine, and their results were obtained with an Ehrlich ascites tumour. Although (Table 2) three injections of 0.2 mg. of azaserine/kg. produced about a 50% inhibition of the uptake of [^{14}C]glycine or [^{14}C]lysine 5 hr. after giving the labelled compound, there was evidence with [^{14}C]lysine that the inhibition slowly increased with increasing dose, suggesting that there are secondary effects of azaserine at higher dose levels.

There is evidence that 6-thioguanine exerts inhibitory effects on purine synthesis (Handschumacher & Welch, 1960; Sartorelli & LePage, 1958*a*) and competitively inhibits guanine incorporation into nucleic acid (Sartorelli & LePage, 1958*b*), but according to more recent work (LePage, 1960; LePage & Jones, 1961) the incorporation of 6-thioguanine into DNA was the only effect correlating with the tumour response. The present results have shown that, when cells that had received a course of 6-thioguanine therapy were given [^{14}C]uracil or [^{14}C]guanine, the pattern of labelling in the nuclear and s-RNA fractions was similar to that in normal cells. However, particularly in the cells given [^{14}C]uracil, there was a decrease in the rate of labelling of microsomal RNA. The labelling of this fraction was also abnormal in cells treated with 6-thioguanine that were given [^{14}C]lysine. The specific radioactivity of the protein 3–5 hr. after the injection of the labelled compound was only about 60% of that of untreated cells.

The effects were considerably increased by treatment with both drugs. Less [^{14}C]lysine label appeared in all cell fractions; in the microsome fraction it was decreased to 15% of the control. In cells treated with azaserine plus 6-thioguanine that were given [^{14}C]uracil or [^{14}C]guanine the increased uptake resulting from azaserine alone was largely abolished and the uptake of [^{14}C]guanine label into the microsome fraction further diminished.

The results also show that the microsomal RNA content was decreased in cells treated with azaserine alone or 6-thioguanine alone, and progressively decreased in cells treated with both compounds to about 60% of normal. As in the incorporation studies, the effects were less in cells given [^{14}C]guanine after treatment than in cells given [^{14}C]uracil or [^{14}C]lysine. Nuclear RNA

content was also considerably diminished in cells treated with azaserine plus 6-thioguanine that were given [^{14}C]uracil or [^{14}C]lysine, but appeared normal in cells given [^{14}C]guanine. Possibly the subsequent administration of [^{14}C]guanine allowed some cellular repair, or other reversal of the effect of 6-thioguanine. Analogous effects have been seen in bacteria, in that guanosine reversed the growth-inhibitory action of 8-azaguanine on *Bacillus cereus* (Mandel & Markham, 1958); in this organism the incorporation of 8-azaguanine produced a compensatory increase in RNA content so that the total guanine content approximated to that in a normal cell (Mandel & Altman, 1960).

There seem to be two main ways in which 6-thioguanine could affect the s-RNA-microsomal RNA-transfer system: either by interfering with the GTP cofactor, or by altering the properties of RNA by incorporation.

So far as the first alternative is concerned, it is probable that some of the injected 6-thioguanine will enter the GTP-cofactor pool as 6-thioguanine triphosphate. However, the findings of LePage & Jones (1961) suggested that only about 1% of nucleic acid guanine is replaced by 6-thioguanine, and even if the proportion in GTP is much higher it seems reasonable to believe that fresh GTP would be produced after a short interval. Sartorelli & LePage (1958*b*) found that, although 6-thioguanine completely inhibited guanine uptake into nucleic acid, this inhibition had almost completely disappeared 2 hr. after the administration of 6-thioguanine. Further, Roy, Kvam, Dahl & Parks (1961) found *in vitro* that 6-thioguanine triphosphate would substitute efficiently for GTP as cofactor for amino acid incorporation into liver microsomal protein. So far as nucleotide transfer is concerned, there now seems to be some doubt that GTP is necessary (Bosch, Bloemendal & Sluyser, 1960). Thus, although such a mechanism of 6-thioguanine action cannot be ruled out, such evidence as there is indicates that it is not the explanation of the present results.

Because of the very small amounts of RNA present in the large-granule fraction, and the near certainty of contamination, the results are less reliable than those obtained with other fractions. However, there appeared to be no decrease in the RNA content of this fraction in cells treated with azaserine plus 6-thioguanine, and 6-thioguanine alone produced no inhibition of the appearance of [^{14}C]uracil label. Thus the behaviour of the large-granule fraction differed in some respects from that of both the nuclear and microsomal fractions. The uptake of [^{14}C]lysine was inhibited to some extent after treatment with 6-thioguanine alone or azaserine alone.

So far as DNA is concerned, treatment with 6-thioguanine had no effect on the uptake of label from [^{14}C]guanine, except that it largely abolished the stimulation in uptake resulting from treatment with azaserine. However, 6-thioguanine alone produced a 50 % inhibition in the rate of appearance of label from [^{14}C]uracil, and azaserine plus 6-thioguanine produced an almost complete inhibition for at least 5 hr. These results with [^{14}C]uracil suggest that cells treated with 6-thioguanine have a decreased rate of DNA synthesis, and that this approached zero in cells treated with azaserine plus 6-thioguanine. If this is correct the apparently unaffected uptake of [^{14}C]guanine may again be a result of attempts at cellular repair.

From present ideas on the interdependence of the synthesis of DNA, RNA and protein, it seems reasonable to consider that the observed interference with RNA function and the synthesis of protein and of DNA would seriously interfere with cellular function and the ability to effect repair. It is tentatively suggested therefore that the lethal action of the therapy with azaserine plus 6-thioguanine on S180 ascites cells results from a combination of these processes.

SUMMARY

1. In S180 ascites cells pretreated with 6-thioguanine, uptake of label from [^{14}C]guanine or [^{14}C]uracil into nuclear RNA and soluble RNA (s-RNA) proceeds at the normal rate. However, particularly with [^{14}C]uracil, the label appears at a decreased rate in microsomal RNA.

2. The uptake of [^{14}C]uracil label into DNA in such cells is decreased to 50 % of normal.

3. Pretreatment of cells with azaserine results in a decreased uptake of L-[^{14}C]lysine, and in increased uptake of [^{14}C]guanine and [^{14}C]uracil.

4. Pretreatment of cells with a combination of both drugs results in an almost complete inhibition of the appearance of [^{14}C]uracil label in DNA, and an 85 % decrease in the uptake of L-[^{14}C]lysine into microsomal protein.

5. It is suggested that in cells treated with 6-thioguanine (either alone or in combination with azaserine) there is a defect in the ability of microsomal RNA to accept amino acids and nucleotides transported by s-RNA.

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