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EFFECTS OF ANTIMITOTIC AGENTS ON EHRLICH ASCITES CELLS*

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Abstract—The biochemical effects on Ehrlich ascites cells of acriflavine, podophyllotoxin, 6-hydroxylaminopurine, and 8-ethoxycaffeine, compounds known to inhibit cell multiplication, were studied. Acriflavine was shown to inhibit DNA synthesis, and extracts of cells preincubated with acriflavine were less able to synthesize inosinate. Podophyllotoxin was a potent inhibitor of purine synthesis *in vitro* but relatively ineffective *in vivo*. It did block purine interconversion, and the general inhibitory effects of the agent were attributed to this action. The adenine analog, 6-hydroxylaminopurine, blocked the conversion of inosinate to adenylate and guanylate. This specific inhibition led to a generalized decrease in formation of nucleic acids. Purine synthesis *de novo* was blocked by 8-ethoxycaffeine in extracts of ascites cells, the effect was much greater when the cells had been preincubated with the inhibitor. The action of this compound was attributed to this specific block. The compounds each appeared to have distinct modes of action. It is suggested that some of these may mimic natural mechanisms of control of cell division.

ACRIFLAVINE, podophyllotoxin, 8-ethoxycaffeine, and 6-hydroxylaminopurine have in common the ability to disrupt normal mitoses. Acriflavine exerts such effects on mouse ascites tumors.[1] Ormsbee[2] showed that podophyllotoxin inhibited some tumors, and it has also been shown to impair mitotic figures in marine eggs.[3] The third agent, 8-ethoxycaffeine, inhibited mitotic activity in tissue cultures of human cells,[4] and 6-hydroxylaminopurine has been shown by Biesele *et al.* to inhibit mitoses in human epidermoid carcinoma no. 2.[5] The effect of these compounds on several synthetic systems in Ehrlich ascites cells has been evaluated to determine whether the similarity of these compounds in inhibiting mitoses is reflected biochemically.

EXPERIMENTAL

Preparation of cells

The hypotetraploid Ehrlich ascites tumor used in these studies was provided by Dr. John A. Jacques. It is a strain originally obtained from Dr. George Klein, Karolinska Institutet, Stockholm, Sweden. The cells were grown i.p. in HA/ICR mice. Five days after injection, cells were removed and mixed with an equal volume of Krebs–Ringer bicarbonate buffer (KRB). Red cells were lysed by the addition of 2 vol. distilled water.[6] The cells were washed and resuspended in KRB.

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Incubation of cells

Five-ml aliquants of the cell suspension were added to 25-ml Erlenmeyer flasks. The final volume was brought to 10 ml with KRB after addition of appropriate substrates and compounds under study. The flasks containing the cells were equilibrated for ½ hr in a metabolic shaker (90 to 100 oscillations/min) at 37° in an atmosphere of 5% carbon dioxide–95% oxygen prior to the addition of the compounds. Aliquots of the cell suspension were removed for dry-weight determinations; the samples were dried in vacuum to constant weight. At the end of the incubation period the flasks were chilled in ice. Cell counts were done in a Neubauer bright-line hemocytometer. The flasks contained an average of 35×10^6 cells/ml. About 3 per cent of the cells were damaged as determined with trypan blue.[7]

Materials

Glycine-1-^{14}C (sp. act. 4·5 mc/mmole) was obtained from U.S. Nuclear Corp., Burbank, Calif.; thymidine-methyl-^{3}H (1 mc/ml/0·1 mg) from New England Nuclear Corp., Boston, Mass. Hypoxanthine (10 μc/μmole) was prepared by the deamination of adenine-8-^{14}C (Isotopes Specialty Co., Burbank, Calif.) with sodium nitrite and hydrochloric acid.[8] The material was purified by the use of ion-exchange columns. Acriflavine hydrochloride was obtained from the National Aniline Division, Allied Chemical and Dye Corp., New York. It was diluted with buffer immediately before use and was protected from light by aluminum foil.[9] Podophyllotoxin was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif.; 8-ethoxycaffeine from G. D. Searle and Co., Chicago, Ill.; 6-hydroxylaminopurine from Dr. A. Giner-Sorolla of this division. These compounds were dissolved in buffer just prior to use. Potassium salt of ribose-5-phosphate was prepared from the barium salt obtained from Schwarz Bio-Research, Orangeburg, N.Y.,[10] and the stock solution stored at −20°.

Cytological preparations

Slides were prepared for radioautography according to the method of Fitzgerald.[11] Slides used to determine mitotic indices were prepared according to the methods of Diacumakos *et al.*[12] The index was calculated as the per cent of total cells in each mitotic stage. For radioautography, the slides were exposed to Kodak AR-10 stripping film for four weeks at 5°.

Isolation of cell constituents

Cells were resuspended and washed in KRB containing small amounts of non-radioactive precursors and then ground in a Teflon–glass Potter–Elvehjem homogenizer for 2 min. This was done primarily to obtain a uniform suspension of the cells. The suspended cells were transferred to other tubes and an equal volume of 20% trichloroacetic acid was added to each tube.[13] The soluble portion was removed, and the cellular residue was dehydrated with graded alcohol washings 80–100%. The lipids were then extracted with hot alcohol–ether and ether.

The soluble fraction was adjusted to pH 8–9, 1 ml saturated barium acetate solution and 2·5 vol. ethanol were added and the barium nucleotides collected by centrifugation. The supernatant solution was reduced in volume to about 10 ml by evaporation *in vacuo* and acidified with hydrochloric acid. The residue was dissolved in water and

aliquots plated on aluminum planchets for radioactivity assay. The barium nucleotides were dissolved in 1 N hydrochloric acid and the barium removed by the addition of a slight excess of sulfuric acid. The purines were liberated by hydrolysis at 100° for 1 hr, precipitated as silver salts, regenerated with hydrochloric acid, and separated by paper chromatography.[14]

Sodium nucleates were extracted from the cellular residue according to the method of Roll and Weliky,[15] and the RNA and DNA separated.[16] The RNA was dissolved in 1 N H_2SO_4 and heated at 100° for 1 hr. The silver purines were precipitated and treated as were those from the soluble nucleotides. The DNA was heated with 0·03 ml of 70% perchloric acid per gram of cells at 100° for 1 hr.[17] The solutions were diluted to 5 ml and any residue removed. The solution was treated twice with 1-ml portions of Dowex-1·Cl^-.[18] The Dowex residues were washed and the washing added to the treated solution. The solutions were taken to dryness and the purines separated chromatographically.

The protein residue left from the sodium chloride extraction was washed with 80% ethanol containing unlabeled glycine, then with 90%, 100% ethanol, ethanol–ether, and ether. Samples were dissolved in 88% formic acid and aliquots plated onto stainless steel planchets with a detergent (Triton X-100, Rohm & Haas Co., Philadelphia, Pa.) to ensure smooth plating.[19]

Portions of the protein were treated with 2,4-dinitrofluorobenzene to remove nonprotein glycine.[20] After removal of any amino acid derivatives the residue was hydrolyzed and 2,4-dinitrophenyl derivatives prepared.[21] The glycine and serine derivatives were isolated on Celite columns[22] and their identity confirmed by paper chromatography.[21]

Cell-free systems

Cells were washed with KRB containing 15 μmoles glutamine/ml* and then ground with three times their weight of alumina A-303 (Aluminum Corp. of America) in a mortar and pestle in the cold. Disruption of the cells was verified microscopically. The preparations were centrifuged at 1,600 *g* for 30 min and the precipitate discarded. The extract from about 35×10^8 cells was diluted to 10 ml with KRB containing 100 μmoles ribose-5-phosphate, 1 μmole ATP, 150 μmoles glutamine, 150 μmoles L-aspartate, 300 μmoles sodium bicarbonate, 0·63 μmole glycine-1-^{14}C, and 1 μmole folic acid.[23]

Solutions were incubated for 1 hr at 37° under an atmosphere of 5% carbon dioxide–95% oxygen. At the end of the incubations, 20 mg hypoxanthine in 1 N hydrochloric acid was added as carrier and the hypoxanthine silver picrate isolated and purified.[24] To the final solution of hypoxanthine, 10 mg nonradioactive glycine and 1 ml 0·1% aq. ninhydrin were added. The solutions were heated at 100° for 20 min and the ninhydrin and its derivatives extracted with *n*-butanol. The aqueous solution was evaporated to dryness, and the residue was dissolved in 0·01 M phosphate buffer, pH 7, measured spectrally and assayed for radioactivity.

Determination of thymidine-^{3}H utilization

The cells were incubated with 0·5 μc thymidine-methyl-^{3}H/ml. Samples were removed and the cells collected by centrifugation, washed three times with KRB containing a

* The presence of glutamine stabilizes the purine-synthesizing enzymes; J. M. Buchanan, personal communication.

small amount of nonradioactive thymidine, alcohol and ether and air dried. The residue was extracted twice with 2-ml portions of hot 7% perchloric acid at 100° for 30 min. The residual material was then washed with 1 ml perchloric acid and the washings added to the extracts. Aliquots of the perchloric acid solution were diluted 1 : 50 for assay in a scintillation counter. The amount of DNA was determined by the Burton[25] modification of the Dische[26] method.

RESULTS

Acriflavine

The radioactivity not precipitable by barium was considered a measure of the free glycine in the cells. This radioactivity was removed by treatment of the solution with 2,4-dinitrofluorobenzene and extraction with ether. Treatment of the cells with acriflavine resulted in a slight increase of radioactivity (Table 1). The purines and proteins

TABLE 1. ACTIVITY OF FREE GLYCINE*

Time (hr)	Control	Acriflavine	Podophyllotoxin	8-Ethoxycaffeine	6-Hydroxylaminopurine
1	77	94	85	150	155
2	50	66	47	140	74
3	29	37	29	101	54

* Data are reported as cpm/mμmole of the 2,4-dinitrophenylglycine; they are the average of two separate experiments. The cells were incubated as described in Experimental. The medium contained 0·20 μmole of undiluted glycine-^{14}C ml. The inhibitor concentrations were as follows: acriflavine 20 μg/ml, podophyllotoxin, 8-ethoxycaffeine, and 6-hydroxylaminopurine each 10^{-5} M.

synthesized from this precursor should be more radioactive as a result of this increased activity. A more valid presentation of relative synthesis results from a comparison of product activity to that of the precursor. In all tables in which the activities of substances synthesized from glycine have been listed an adjusted value has also been given, where

$$\text{adjusted value} = \frac{\text{treated}}{\text{control}} \times \frac{\text{control free glycine activity}}{\text{treated free glycine activity}}$$

The ability of the cells to synthesize purine-containing compounds and proteins from exogenously supplied radioactive glycine was determined. Results of these experiments are shown in Table 2. The synthesis of soluble purine nucleotides was reduced to about half that found in the control cells. The formation of DNA purines was reduced more extensively; DNA adenine was 30% and DNA guanine 16% of the control. The amount of protein synthesis when measured at 1, 2, or 3 hr was about half that of the control cells. Since the glycine in the cells was somewhat more highly labeled in the treated than in the control cells, the adjusted values were somewhat lower than those obtained directly.

The inhibition of guanine synthesis in the soluble nucleotides, RNA, and DNA was definitely greater than was the inhibition of adenine synthesis. Since the effect of acriflavine was greater on the DNA synthesis than in the other systems studied, this

was measured by other criteria. Cells were incubated with tritiated thymidine, and aliquants were removed at 1, 2, and 3 hr for examination by radioautography. The rest of the cells were collected at 3 hr, and the radioactivity of the isolated DNA determined. The data in Table 3 show that at all three time periods the number of cells with labeled nuclei was reduced to about 30% of the control values. The total

TABLE 2. EFFECT OF ACRIFLAVINE ON SYNTHESIS OF CELL CONSTITUENTS IN INTACT CELLS*

	Control	Treated / Control	Treated† / Control
Soluble nucleotides			
Adenine	420	0·68	0·54
Guanine	159	0·56	0·44
RNA			
Adenine	205	0·63	0·50
Guanine	67	0·48	0·38
DNA			
Adenine	38	0·30	0·24
Guanine	26	0·16	0·13
Total protein 1 hr	151	0·54	0·43
Total protein 2 hr	209	0·57	0·45
Total protein 3 hr	359	0·52	0·41

* Incubation mixture contained glycine-1-^{14}C, 0·20 μmole/ml, and the 'treated' cells, acriflavine 20 μg/ml. Values are given as cpm/μmole for purine and cpm/mg for protein. Purine incorporation was measured after 3 hr. They are the average of three separate experiments.

† Adjusted ratio $= \frac{\text{treated}}{\text{control}} \times \frac{\text{control free glycine activity}}{\text{treated free glycine activity}}$

TABLE 3. DNA SYNTHESIS BY TREATED AND UNTREATED CELLS*

	DNA	Labeled nuclei		
	3 hr	1 hr	2 hr	3 hr
Control	63	45	61	84
Acriflavine	0·29	0·24	0·39	0·34
Podophyllotoxin			0·89	0·76
8-Ethoxycaffeine		0·86	0·89	0·88
6-Hydroxylaminopurine	0·60	0·46	0·50	0·69

* Control values are cpm/μg for DNA and per cent labeled nuclei in 3,000 cells samples. Values for treated cells are treated values/control. Controls were from the same batch of cells and were analyzed concurrently with treated cells. Cells were incubated with the following concentration of inhibitors: acriflavine 20 μg/ml, the others 10^{-5} M.

activity in the isolated DNA was reduced to about the same extent. This value corresponds to the amount of reduction that was found in DNA adenine synthesis when glycine was used as a precursor.

The effect on purine synthesis was examined with cell-free extracts. The ability of these extracts to catalyze the synthesis of inosinate from glycine was determined. The

cells which were to be studied were incubated prior to extraction of the enzyme system. Half the cells were incubated with the inhibitor under study, in this case acriflavine, and the other half were incubated without the inhibitor. Cells were then homogenized and each extract was divided in half. The inhibitor was added to one half.

TABLE 4. INOSINATE SYNTHESIS BY CELL-FREE SYSTEMS*

Intact† cells	Cell-free‡ extract	Relative Synthesis‡			
		A	P	E	H
−	−	1·00	1·00	1·00	1·00
−	+	0·97	0·12	0·62	0·94
+	−	0·20	1·06	0·32	1·34
+	+	0·24	0·0	0·24	0·71

* The cells were incubated for 3 hr at 37° in KRB buffer containing 0·01 M glucose with or without inhibitor prior to grinding.

† Presence (+) and absence (−) of inhibitor in preincubation of cells and during actual assay of extract. Acriflavine was used at 20 μg/ml and the other inhibitors at 10^{-5} M.

‡ Values are those treated/control. Control values were about 900 cpm/μg cells (dry weight). All values are average of duplicate experiments. A = acriflavine, P = podophyllotoxin, E = 8-ethoxycaffeine, H = 6-hydroxylaminopurine.

The data in Table 4 show that the addition of acriflavine to the cell-free extract had no appreciable effect on the ability of the preparation to synthesize inosinate. However, cells that had been preincubated were only about 20% as efficient in the synthesis of this first purine-containing product.

Podophyllotoxin

The effect of podophyllotoxin on the synthesis of purines and proteins *in vivo* was examined, and it was found (Table 5) that the synthesis of both soluble purine nucleotides and proteins was reduced to about 60% in the presence of podophyllotoxin, as

TABLE 5. EFFECT OF PODOPHYLLOTOXIN ON UTILIZATION OF GLYCINE BY EHRLICH ASCITES CELLS*

		Control	Treated / Control	Treated† / Control
Soluble purines				
Adenine		765	0·67	0·66
Guanine		548	0·52	0·51
Protein	1 hr	82	0·67	0·66
	2 hr	112	0·61	0·60
	3 hr	283	0·70	0·69

* Incubation mixture contained 0·20 μmole glycine-1-^{14}C ml and the 'treated' cells 10^{-5} M podophyllotoxin. Control purine values are cpm/μmole at 3 hr, protein values are cpm/mg. The data are the average of two separate experiments.

† Adjusted ratio $= \frac{\text{treated}}{\text{control}} \times \frac{\text{control free glycine activity}}{\text{treated free glycine activity}}$

compared to the control value. The free glycine extracted from cells treated with podophyllotoxin had about the same activity as the control. The inhibition of protein synthesis was the same at 1, 2, or 3 hr. The effect of podophyllotoxin on DNA synthesis as determined by radioautography is shown in Table 3, and it can be seen that this agent did not greatly reduce the production of new DNA. The reduction in DNA synthesis was actually less than that observed on soluble nucleotide and protein synthesis.

The effects of podophyllotoxin on the cell-free system were then examined, and the results in Table 4 show that preincubation of the cells with podophyllotoxin did not affect inosinate synthesis. However, the addition of podophyllotoxin to the cell-free extracts almost completely inhibited the formation of inosinate; the addition of podophyllotoxin to extracts prepared from cells which had been preincubated with podophyllotoxin resulted in complete inhibition of the synthesis of inosinate. Since there was relatively little effect of podophyllotoxin on inosinate synthesis in the intact cells, it was felt that perhaps the reduction in synthesis of adenine- and guanine-containing nucleotides occurred at a later step. For this reason the effect of podophyllotoxin on the utilization of hypoxanthine for the synthesis of adenine and guanine of soluble nucleotides and RNA was examined. The data in Table 6 show that the formation of these purines was reduced to about one half the control values.

TABLE 6. EFFECT OF PODOPHYLLOTOXIN AND HYDROXYLAMINOPURINE ON HYPOXANTHINE UTILIZATION*

		P	H
Soluble nucleotides			
Adenine	2·73	0·41	0·45
Guanine	2·01	0·69	0·51
RNA			
Adenine	1·10	0·44	0·29
Guanine	0·30	0·52	0·49

* The data are the average of three separate experiments. Control values are cpm/mμmole at 3 hr. P is the ratio of activity of purine from podophyllotoxin-treated cells to the values from control cells; H is the corresponding values from 6-hydroxylaminopurine-treated cells. Cells were incubated as described under Experimental with the inclusion of 1 μmole hypoxanthine-8-^{14}C/ml (10 μc/μmole) and in the treated cells 10^{-5} inhibitor. Conversion to uric acid was less than 0·01%. Carrier urate was added and reisolated and purified by reprecipitation.

6-Hydroxylaminopurine

The synthesis of adenine- and guanine-containing soluble nucleotides and proteins in the presence and absence of 6-hydroxylaminopurine was examined (Table 7). The activity of the isolated nucleotides was lower than that of the control, the value for adenine being 72% and that of guanine 53%. The reduction in protein synthesis was evinced by the fact that the protein of treated cells had between 50 and 60% of the activity of the control protein at 1, 2, and 3 hr. However, with this agent, the activity of the free amino acid glycine was almost three times as great (Table 1) as that found

in the control experiment. When the amount incorporated was adjusted for the higher precursor activity, the apparent inhibition due to the presence of 6-hydroxylaminopurine was between 80 and 85%. The inhibition of guanine synthesis was again greater than the inhibition of adenine synthesis.

TABLE 7. GLYCINE UTILIZATION IN PRESENCE AND ABSENCE OF 6-HYDROXYLAMINOPURINE*

		Control	Treated / Control	Treated† / Control
Soluble nucleotides				
Adenine		1,728	0·72	0·20
Guanine		644	0·53	0·15
Protein	1 hr	119	0·52	0·15
	2 hr	153	0·62	0·17
	3 hr	297	0·61	0·17

* Cells were incubated as described under Experimental with the addition of 0·02 μmole glycine-1-^{14}C/ml and in the treated cells 10^{-5} M hydroxylaminopurine. Control purine values are cpm/μmole at 3 hr; protein values are cpm/μg. All results are average of duplicate experiments.

† Adjusted ratio $= \frac{\text{treated}}{\text{control}} \times \frac{\text{control free glycine activity}}{\text{treated free glycine activity}}$.

The effect of this agent on DNA synthesis was examined, and the data (Table 3) show that the amount of DNA formed in the presence of the agent was somewhere between 50 and 60% of that found in the control. This was true when one examined the number of labeled nuclei found at 1, 2, or 3 hr incubation, and the activity of the isolated DNA which was isolated after 3 hr incubation.

The synthesis of inosinate by cell-free extracts was not very much reduced by 6-hydroxylaminopurine (Table 4). There was actually a slight increase in the ability of the extract from preincubated cells to synthesize inosinate. When 6-hydroxylaminopurine was added to the extracts *in vitro* there was a slight inhibition. Since it was felt that the reduction in inosinate synthesis observed was not likely to explain the net inhibition in intact cells, the ability of cells to convert exogenous hypoxanthine to adenine and guanine was examined. The data (Table 6) show that the presence of 6-hydroxylaminopurine reduced the conversion of exogenous hypoxanthine to both adenine and guanine. The synthesis of adenine was reduced more than was that of guanine.

8-*Ethoxycaffeine*

Cells were incubated with radioactive glycine in the presence and absence of 8-ethoxycaffeine (Table 8). The radioactivity of soluble nucleotide and RNA adenine and guanine of the treated cells was about two thirds of the control values. There was a greater reduction in the synthesis of adenine. Protein synthesis was the same at all three time periods studied—about 70% of control. There was, however, a very great increase in the activity of the free glycine of these cells, and when the fractional incorporation was adjusted for this difference in precursor activity, it was found that

the reduction in synthesis of adenine and guanine and proteins was very extensive. The treated cells had about one fifth the value of the control cells.

8-Ethoxycaffeine treatment resulted in a very small reduction in the number of nuclei found after incubation of cells with thymidine-^{3}H (Table 3). 8-Ethoxycaffeine did have an inhibitory effect upon ability of cell-free preparations to synthesize inosinate (Table 4). If the compound was added to extracts of cells which had previously not been in

TABLE 8. EFFECT OF 8-ETHOXYCAFFEINE ON GLYCINE UTILIZATION*

		Control	Treated / Control	Treated† / Control
Soluble nucleotide				
Adenine		290	0·79	0·21
Guanine		190	0·64	0·17
RNA				
Adenine		51	0·73	0·20
Guanine		11	0·66	0·18
Protein	1 hr	73	0·73	0·20
	2 hr	108	0·79	0·21
	3 nr	135	0·72	0·20

* Cells were incubated as described under Experimental with addition of 0·2 μmole glycine-1-^{14}C/ml and in the treated cells 10^{-5} M 8-ethoxycaffeine. Control purine values are cpm/μmole at 3 hr, protein values are cpm/mg. Values are average of triplicate experiments.

† Adjusted ratio $= \frac{\text{treated}}{\text{control}} \times \frac{\text{control free glycine activity}}{\text{treated free glycine activity}}$.

contact with the inhibitor, the amount of synthesis was 60% of the control value. If the cells were preincubated, the cell extracts had only 30% of the control value. If the extracts of preincubated cells were further incubated *in vitro* with 8-ethoxycaffeine, the synthesis of inosinate was only 24% of the control value.

DISCUSSION

With glycine-^{14}C as the indicator, acriflavine did not completely block metabolic processes, since the intracellular glycine activity decreased at about the same rate as did that in the control cells. There was, however, a general reduction in protein, nucleotide, and nucleic acid synthesis. The reduction of protein synthesis was constant with time, and the most pronounced inhibition *in vivo* was that of DNA synthesis. The decrease in formation of DNA adenine from exogenous glycine and that of incorporation of thymidine-^{3}H were the same. This accords with the conclusion that the inhibition was due to some step common to both purine and pyrimidine nucleotides, possibly the polymerization step.

Acriflavine was, however, also capable of interfering with the function of the inosinate-forming systems of cells which had previously been incubated with the drug. On the other hand, the addition of the compound to the broken cell system produced no inhibition, and some aspect of the position or sequential metabolic relationships of cell components is involved in the fact that this agent acts *in vivo* but not *in vitro*.

It appears that in nondividing cells some function of DNA, other than DNA synthesis, is blocked by acriflavine.

The action of acriflavine on cells synthesizing DNA, and on those using the DNA as a template for the synthesis of other substances, might both be correlated with the known ability of acriflavine to bind to DNA. Kurnick and Radcliffe[27] have recently shown that some compounds that bind DNA prevent the action of DNAase. An analogous inactivation of DNA as a template could explain the observed reductions in metabolic activity; the unavailability of the DNA could prevent replication of a cell's DNA and hence its division; a masked DNA would not be available for the synthesis of active messenger RNA. In normal cells it is reasonable to believe that the control of metabolic activity of cell division is controlled by such masking of DNA as, for instance, that by histones, as has been suggested by Allfrey.[28] Thus, in this case, the exogenous inhibitor may mimic the natural control.

In contrast, podophyllotoxin is a potent inhibitor of purine synthesis *in vitro* but relatively ineffective *in vivo*; the observed reduction in biosynthesis *in vivo* could well be explained by these properties. Since podophyllotoxin is so potent *in vitro* and not *in vivo*, the possibility arises that it may not be able to enter the cell. However, the inhibitory effects noted on other systems would mean that either these are much more sensitive or else permeability of the cell is not a primary factor. It is possible that podophyllotoxin is unable to enter particular subcellular areas.

The adenine analog, 6-hydroxylaminopurine, caused a generalized reduction in the synthesis of purine derivatives and of protein. The synthesis *de novo* of the purine moiety as represented by inosinate (IMP) was not reduced, but the conversion of hypoxanthine to adenylate and guanylate was reduced. The possibility that this represents a blockage of pyrophosphorylase is not likely, since the sequence from glycine to IMP is not impaired, and that from glycine to adenylate and guanylate is. It would thus appear that the block is between inosinate and adenylate and guanylate. The inability of the cell to carry out these processes reduced the utilization of glycine, and the activity of the exogenous precursor remained higher than in the control. A reduction in the utilization of IMP would be reflected in lowered formation of RNA and DNA and consequently in protein synthesis, and from the available data it appears that the site of action of this inhibitor is in this conversion step.

8-Ethoxycaffeine caused a general reduction of biosynthesis of compounds derived from glycine, with the specific site being the inhibition of purine synthesis *de novo*. The effect was greater after incubation of intact cells, but was also real when the inhibitor was added to the cell-free preparation. The inhibition is of a magnitude to explain the observed effects.

The four compounds studied are capable of producing inhibitions of mitosis. However, they are not similar in their biochemical effects. Only in the case of acriflavine has a mode of inhibition been suggested that resembles a 'natural' means of regulation. However, all the other sites of action may be such points of control. Many different exogenous inhibitors are known; similarly, natural control of cell division may depend upon a variety of inhibitors, the action of some of which may be mimicked by these exogenous compounds.

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