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INHIBITION BY ANTITUMOUR AGENTS OF LABELING OF NUCLEAR PROTEINS *IN VIVO* WITH L-ARGININE-U-^{14}C

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Abstract—Arginine-U-^{14}C was injected into animals bearing the Walker tumor at various times after treatment with one of a series of antitumor agents, including Myleran®, Leukeran®, 5-fluorouracil, 6-mercaptopurine, podophyllotoxin, colchicine and HN2. The tissues were excised 1 hr later and the labeling of proteins in various cellular fractions was determined. In the Walker tumor, the inhibition of labeling of the acid-insoluble nuclear proteins was the only constant effect of these agents. Podophyllotoxin suppressed the labeling of the nuclear proteins to a greater extent than the labeling of proteins of other fractions of the Ehrlich ascites tumor; however, colchicine did not exert a selective effect on labeling of the nuclear proteins. The effects on labeling of the proteins of the liver and spleen varied from inhibitor to inhibitor. From these data it would appear that the inhibition of labeling of the acid-insoluble nuclear proteins is an intermediate stage of the inhibitory activity of these compounds.

EXPERIMENTS recently reported from this laboratory[1] have indicated that the mustard, 5-bis(2-chloroethyl)aminouracil exerted a marked suppressive effect on the incorporation of L-arginine-U-^{14}C into the nuclear proteins of the Walker 256 carcinosarcoma, particularly the acid-insoluble nuclear proteins. Remarkable inhibitory effects were also noted with respect to the labeling of the acid-insoluble nuclear proteins of the spleen. In both tumor and spleen the labeling of the histones was also suppressed, but the labeling of the cytoplasmic proteins was not affected. In recent studies with a number of mustards of amino acids, Nyhan and Pula[2] noted that the labeling of the proteins of a variety of cellular fractions was markedly suppressed and the specific effects found with the aminouracil mustard did not seem to be a general phenomenon for the mustards. The present experiments were designed to extend the studies previously reported to a variety of types of antitumor agents, including mustards, sulfonoxy compounds, and purine and pyrimidine analogues.

The doses of the agents used in these studies were those reported to exert suppressive effects on the growth of tumors *in vivo*. At intervals of 23 and 47 hr after treatment with the drug, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing animals. The tissues were treated as previously indicated,[3, 4] to ascertain the extent of inhibition of labeling of the proteins of various intracellular fractions. The drugs employed included 6-mercaptopurine, 5-fluorouracil, Myleran,® Leukeran® (Chlorambucil), HN2, colchicine and podophyllotoxin. The results obtained provide evidence that

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the suppression of incorporation of L-arginine-U-^{14}C into nuclear proteins is a common effect of a variety of effective antitumor agents. However, this effect is usually accompanied by simultaneous suppression of incorporation of this amino acid into other cellular fractions.

MATERIALS AND METHODS

For the most part, the procedures employed in the present study were those used previously.[1, 3, 4] The agents employed in this study and their doses were as follows: 5-fluorouracil, 25 mg/kg;[5] 6-mercaptopurine, 50 mg/kg and 100 mg/kg;[5] Leukeran® (chlorambucil) 15 mg/kg and 30 mg/kg;[7–9] Myleran®, 15 mg/kg and 30 mg/kg;[10] HN2, 2 mg/kg;[11] colchicine[12] and podophyllotoxin.[13] These agents were intraperitoneally administered, in dimethyl-formamide,[11] at intervals of 23 and 47 hr, respectively, prior to the administration of the labeled arginine. One hour after the injection of the arginine-U-^{14}C, the animals were exsanguinated and the tissues were treated as previously described.[3,4] Nuclear preparations of Ehrlich ascites cells were made by the method recently described.[4] However, twenty-five strokes of the pestle through the fluid were necessary for disrupting these cells. In those instances in which the results with higher doses were not significantly different from the results obtained with the lower doses, the data in the figures are for the lower doses. The tumors were implanted from 6 to 8 days prior to injection of the drug. The tumors ranged in diameter from 0·5–1·0 cm when Walker tumors were used. The total ascitic fluid ranged from 3 to 6 ml when ascites tumors were used.

RESULTS

Leukeran

The effects of Leukeran® on the uptake of amino acids into the various cellular proteins of the Walker 256 carcinosarcoma and the spleen are indicated in Figs. 1 and 2. As was found with the aminouracil mustard,[1] the specific activities of the proteins of the liver were not influenced by the drug. In doses of either 15 or 30 mk/kg, Leukeran® exerted a marked suppressive effect on the labeling of both nuclear and cytoplasmic fractions of the Walker tumor (Fig. 1). The specific activities of the nuclear proteins were approximately 30 per cent of control values at 24 hr after the administration of Leukeran.® The specific activities of the proteins were 34 per cent, 44 per cent and 44 per cent of control values in the microsomal fraction, mitochondrial fraction and the cytoplasmic sap, respectively. This drug did not produce the selective suppression of labeling of the nuclear proteins in the tumor which was found with the aminouracil mustard. On the other hand, the results for spleen (Fig. 2) were much more like those found with the Walker tumor in the previous studies with the aminouracil mustard.[1] The specific activities of the nuclear proteins were markedly suppressed at 24 hr after the administration of the drug, i.e. to approximately 21 per cent of control values. On the other hand, the specific activity of the proteins of the cytoplasmic fractions were reduced to 60 per cent, 75 per cent and 81 per cent, respectively, of control values for proteins of the cytoplasmic sap, mitochondrial proteins, and microsomal proteins.

HN2

Nyhan and Pula[2] and Nyhan[14] have reported the similarity of some aspects of their results with HN2 with the results reported from this laboratory on the effects of the

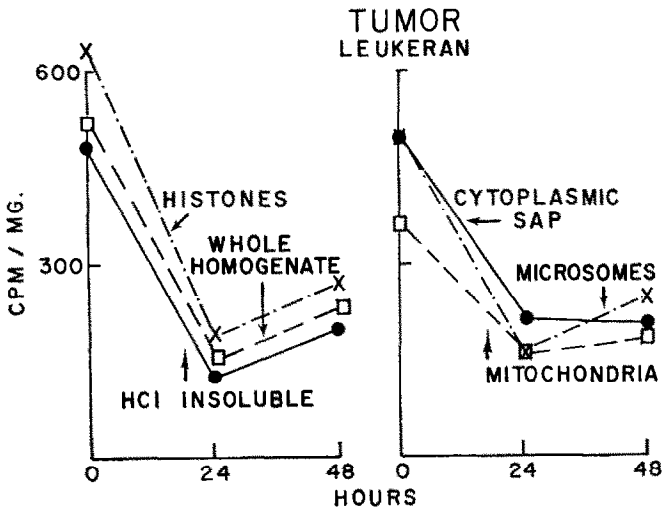

FIG. 1. Effect of Leukeran® on labeling of the proteins of the Walker tumor. In these experiments, 15 mg/kg of Leukeran® was administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected, into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

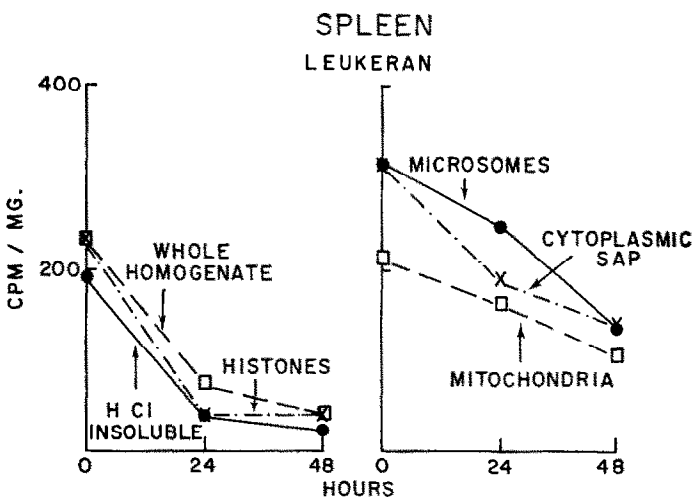

FIG. 2. Effect of Leukeran® on labeling of the proteins of the spleen. In these experiments, 15 mg of Leukeran® per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

aminouracil mustard on the uptake of labeled arginine into proteins of the Walker tumor. Similar results have obtained in the present experiments (Table 1) with the Walker tumor. Similarly, in the spleen, the decrease in the specific activity of the proteins of the cytoplasmic fractions was not marked at 24 hr after administration of the drug. However, the labeling of the histones was suppressed to 50 per cent of control values. These results are similar to those obtained with other mustards. As was found previously,[1] an increase was noted in the specific activity of proteins of the liver following treatment of the animal with nitrogen mustard.

Myleran

The third of the alkylating agents included in this study was Myleran®, a compound which did not markedly suppress the growth of the tumor in the time period studied.

TABLE 1. SPECIFIC ACTIVITIES OF CELLULAR PROTEINS AFTER TREATMENT OF THE ANIMAL WITH HN2

At 23 hr following injection of 2 mg of HN2 per kg, arginine-U-^{14}C was injected intraperitoneally and the tissues were treated as described in the text. The values presented are averages of two experiments. The data for individual experiments are in the parentheses. The values are counts/min per mg dry weight of protein.

Protein fractions	Tumor		Liver		Spleen	
	counts/min per mg	% of control	counts/min per mg	% of control	counts/min per mg	% of control
Whole homogenate	277 (242,312)	67	471 (452,489)	183	187 (164,209)	75
Mitochondria	268 (258,279)	74	542 (490,594)	204	366 (360,372)	166
Microsomes	398 (395,400)	81	739 (700,778)	170	342 (266,417)	100
Cytoplasmic sap	365 (350,380)	74	421 (360,482)	199	316 (302,329)	100
Histones	354 (320,387)	56	364 (348,380)	200	135 (130,139)	53
HCl insoluble nuclear proteins	322 (295,348)	67	360 (359,360)	188	179 (173,185)	95

The specific activities of the nuclear proteins of the Walker tumor (Fig. 3), spleen (Fig. 4), and liver (Fig. 5) were decreased to different extents following the administration of the drug. The specific activities of the HCl-insoluble nuclear proteins and the histones of the tumor (Fig. 3) were reduced to 41 per cent and 54 per cent of control values, respectively at 24 hr after administration of a dose of 15 mg/kg of Myleran®. The decreases in the specific activities of the cytoplasmic fractions were markedly less at 24 hr. These results for the tumor were consistent with those obtained previously with the aminouracil mustard.[1] However, the very marked effect of the mustards, i.e. HN2, the aminouracil mustard and Leukeran®, on the labeling of nuclear proteins of the spleen (Fig. 4) was not noted with Myleran®. In the first 24 hr

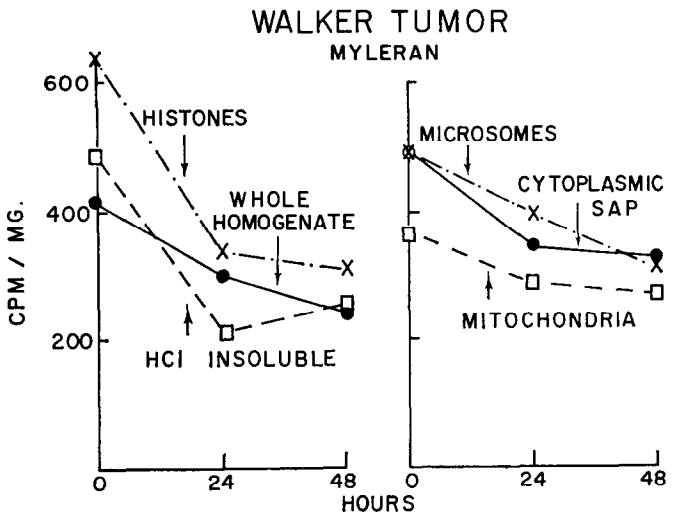

FIG. 3 Effect of Myleran[10] on labeling of the proteins of the Walker tumor. In these experiments, 15 mg of Myleran[10] per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

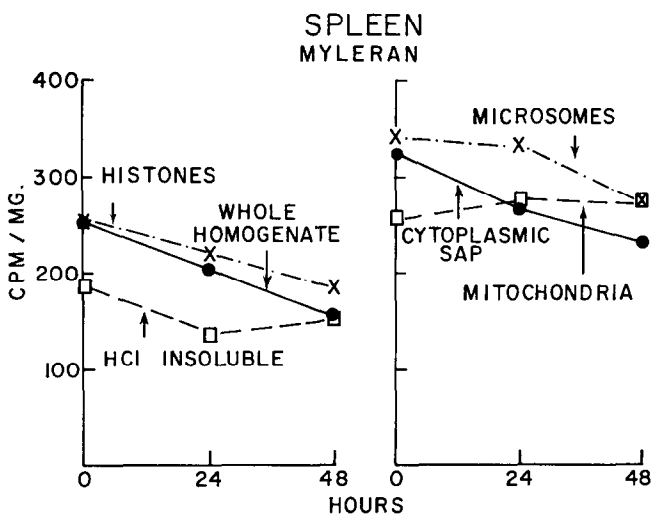

FIG. 4. Effect of Myleran[10] on labeling of the proteins of the spleen. In these experiments, 15 mg of Myleran[10] per kg were administered intrapertitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

after administration of Myleran®, there was a marked effect on the labeling of the proteins of the liver which was not found with the mustards. The specific activity of the HCl-insoluble nuclear proteins decreased to 45 per cent of control values and similar suppressive effects were noted on labeling of the proteins of the microsomal fraction. By 48 hr, some recovery of the incorporating reactions was evident in the tumor and liver.

5-Fluorouracil

Inasmuch as each of the alkylating agents exhibited some specificity of effect in suppression of the incorporation of arginine-U-^{14}C into the nuclear proteins of the Walker tumor, the corresponding studies were made on analog inhibitors, i.e. 5-fluorouracil and 6-mercaptopurine, In the Walker tumor (Fig. 6), 5-fluorouracil suppressed labeling of histones to 60 per cent of control values and suppressed labeling of acid-insoluble nuclear proteins to 70 per cent of control values at 24 hr after administration of the drug. At 48 hr after administration of the drug, the labeling of the histones was suppressed to 50 per cent of control values and the labeling of the HCl-insoluble nuclear proteins was suppressed to 32 per cent of control values. In contrast, the labeling of the mitochondria was virtually unaffected.

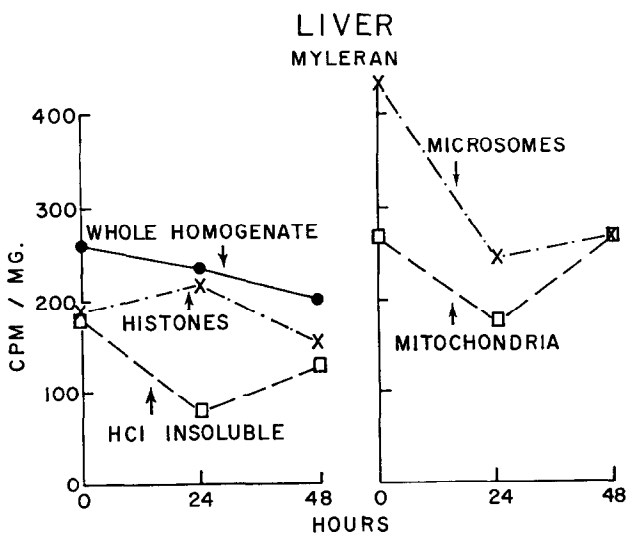

FIG. 5. Effect of Myleran[10] on labeling of the proteins of the liver. In these experiments, 15 mg of Myleran[10] per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment. 10 μc of L-arginine-U-^{14}C were injected into the tumor-bearing rat. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

The specific activities of the proteins of the microsomes and cytoplasmic sap were reduced to approximately 60 per cent of control values at 48 hr after administration of the drug. 5-Fluorouracil also suppressed the labeling of the nuclear proteins of the spleen and the liver of the tumor-bearing animal. The specific activity of the histones of the spleen (Fig. 7) was reduced to 40 per cent of control values by 24 hr following

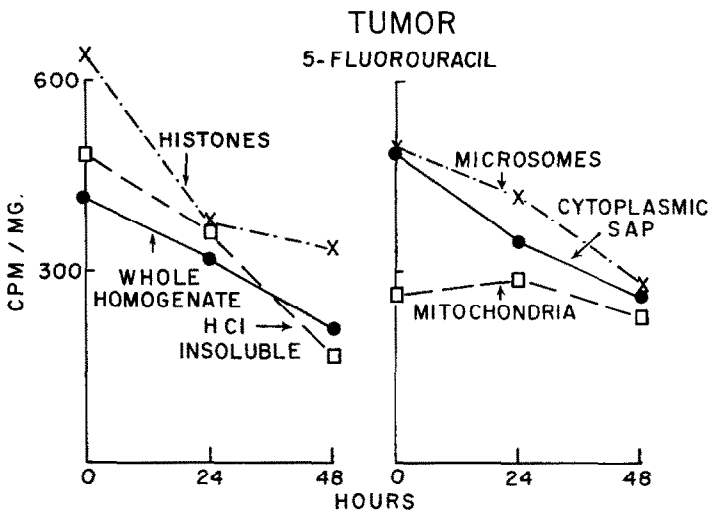

FIG. 6. Effect of 5-fluorouracil on labeling of the proteins of the Walker tumor. In these experiments, 25 mg of 5-fluorouracil per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

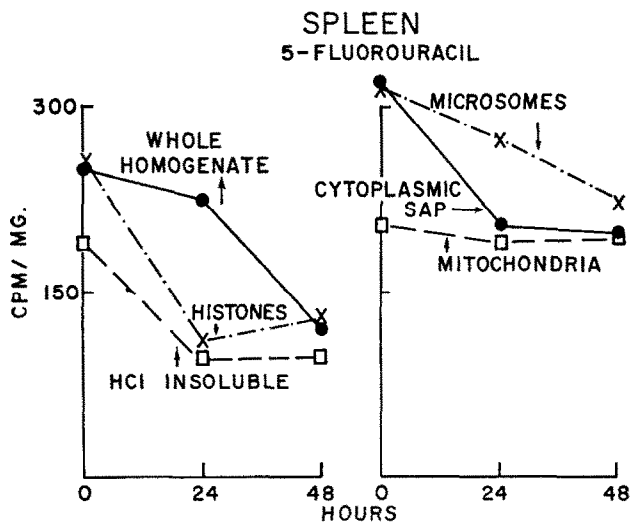

FIG. 7. Effect of 5-flourouracil on labeling of the proteins of the spleen. In these experiments, 25 mg of 5-fluorouracil per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

the administration of the fluorouracil and the specific activity of the acid-insoluble nuclear proteins was reduced to 50 per cent of control values at that time. The specific activities of the cytoplasmic proteins were also decreased but to a lesser extent. In the liver (Fig. 8), the specific activities of the proteins of each of the fractions were

suppressed following administration of the drug. The inhibitory effect was almost uniform in the fractions of the liver as contrasted to the other tissues studied.

6-Mercaptopurine

At 24 hr following the administration of 6-mercaptopurine, the most marked effect in the tumor (Fig. 9) was the suppression of labeling of the acid-insoluble

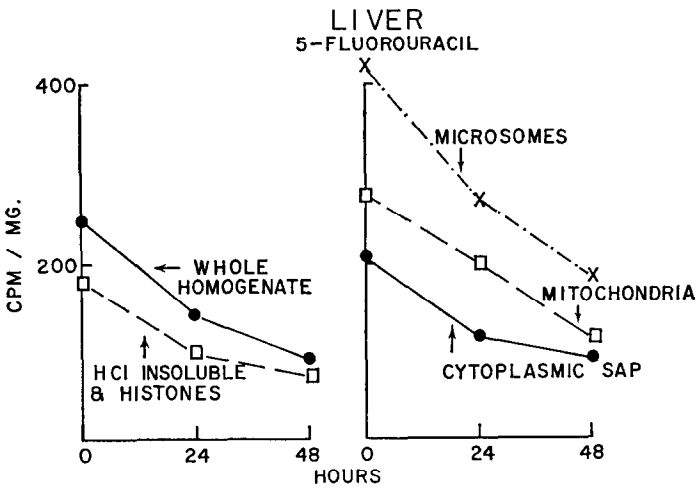

FIG. 8. Effect of 5-fluorouracil on labeling of the proteins of the liver. In these experiments, 25 mg of 5-fluorouracil per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. One hour prior to the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three or four determinations.

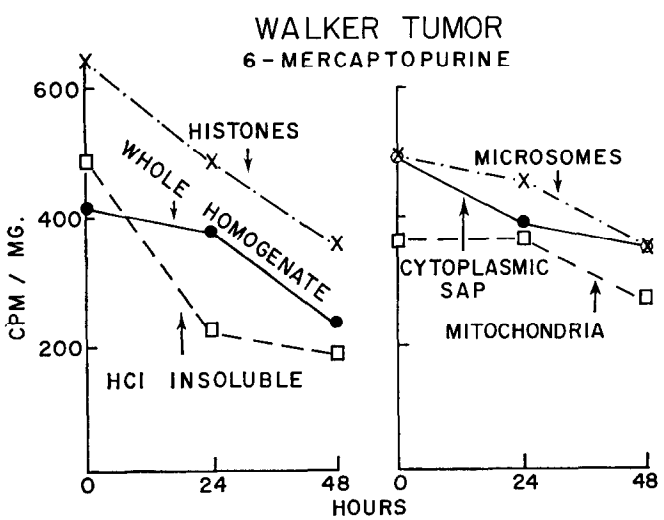

FIG. 9. Effect of 6-mercaptopurine on labeling of the proteins of the Walker tumor. In these experiments, 50 mg of 6-mercaptopurine per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three to four experiments.

nuclear proteins. The labeling of these proteins was suppressed to approximately 40 per cent of control values. Suppression of labeling of the cytoplasmic fractions was not marked at either 24 or 48 hr following the administration of this agent. The suppression of labeling of the histones continued during the second 24 hr; thus, the specific activity of the histones at 48 hr after administration of the drug was 55 per cent of control values. Significant changes were not found in the specific activities of the proteins of various fractions of the spleen (Fig. 10) at 24 hr after administration of the drug. In the second 24 hr, there was a suppression of labeling of each of the fractions of the spleen to 60–70 per cent of control values. In the liver (Fig. 11), the specific activity of the nuclear proteins was decreased to 70 per cent of control values at 24 hr after administration of 6-mercaptopurine. The uptake of the isotope into the proteins of the mitochondria and the cytoplasmic sap was also about 70–80 per cent of the control values at 24 hr after administration of the drug. The labeling of the microsomal proteins was decreased to about 60 per cent of control values at that time. In the second 24 hr, there was no marked change in the specific activity of these proteins.

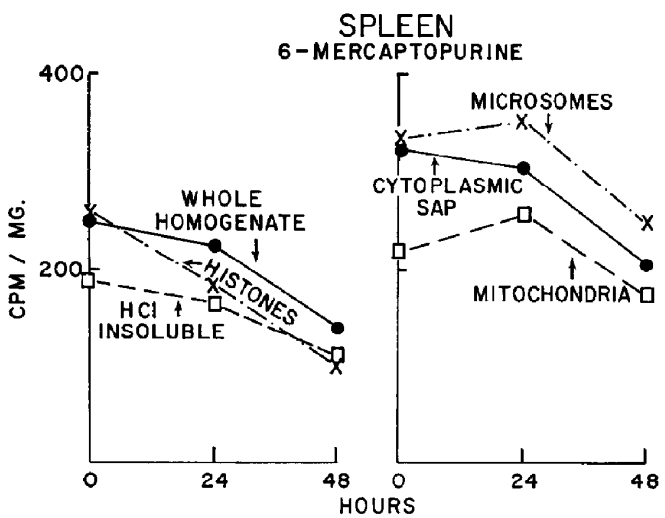

FIG. 10. Effect of 6-mercaptopurine on labeling of the proteins of the spleen. In these experiments, 50 mg of 6-mercaptopurine per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three to four experiments.

Colchicine

The data presented in Table 2 indicate that colchicine suppresses the uptake of isotope of L-arginine-U-^{14}C into proteins of the Ehrlich ascites tumor. The suppression of labeling of the nuclear proteins was equivalent to that of the proteins of the whole homogenate. With this compound, the relative suppression of labeling of the cytoplasmic proteins exceeded that of the nuclear proteins.

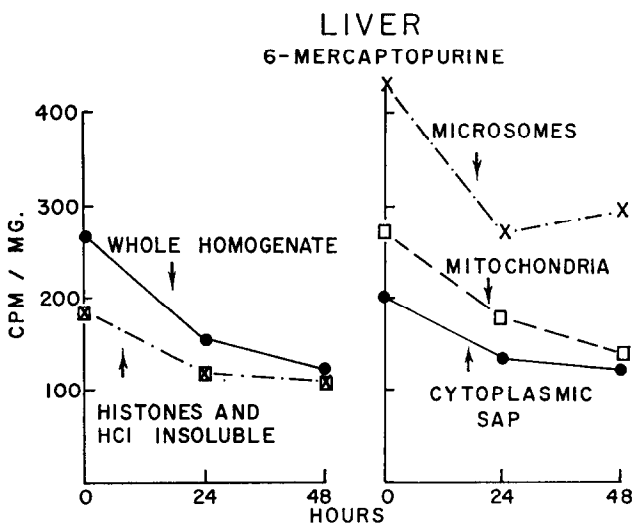

FIG. 11. Effect of 6-mercaptopurine on labeling of the proteins of the liver. In these experiments, 50 mg of 6-mercaptopurine per kg were administered intraperitoneally 24 or 48 hr prior to the termination of the experiment. At the termination of the experiment, the tissues were excised and the specific activities of the proteins were determined. Each point on the graph is the average of three to four experiments.

TABLE 2. EFFECTS OF COLCHICINE AND PODOPHYLLOTOXIN ON LABELING OF NUCLEAR PROTEINS OF EHRLICH ASCITES CELLS

Fractions	Controls[4] Average ± standard error	Colchicine[12] Average ± standard error	Podophyllotoxin[3] Average ± standard error
Whole homogenate	4160 ± 590	2630 ± 480	2370 ± 500
Acid insoluble	4260 ± 500	2895 ± 370	2660 ± 580
Histones	5700 ± 680	3850 ± 760	2630 ± 595
Microsomes	3090 ± 1000	1320 ± 320	1690 ± 300
Mitochondria	1860 ± 280	920 ± 140	1320 ± 360
Cytoplasmic	3230 ± 1340	1130 ± 310	1770 ± 440

In the experiments with colchicine, 2 μg of the drug were injected into the ascites fluid *in vivo* 24 hr prior to the injection of 2·5 μc of the L-arginine-U-^{14}C. After 1 hr, the cells were removed and treated as previously described[3, 4] for other tissues. In experiments with podophyllotoxin glucoside, 2 mg of the drug were injected into the Ehrlich ascites fluid *in vivo*, 18 hr prior to the experiment. The values for control experiments, experiments with colchicine and experiments with podophyllotoxin are averages of five, eight and four individual determinations, respectively. The *P*-value for depression of the uptake of isotope into mitochondria in the experiments with colchicine was less than 0·05. The *P*-value for depression of uptake of isotope into histones in the experiments with podophyllotoxin was also less than 0·05. The standard error was determined from the equation: $E = \sqrt{\{\Sigma d^2/[n(n-1)]\}}$.

Podophyllotoxin

Marked suppression of the labeling of proteins of the Ehrlich ascites tumor was also found with podophyllotoxin. With the conditions employed, the specific activities of the nuclear proteins were suppressed to 47 per cent of control values. The suppression of labeling of the proteins of the cytoplasmic fractions was not as marked as the suppression of labeling of the nuclear proteins.

DISCUSSION

Perhaps the most interesting feature of the present study is the consistent suppression of labeling of the acid-insoluble nuclear proteins by the effective antitumor agents studied. In addition to the animouracil mustard, Myleran®, Leukeran®, HN2, 5-fluorouracil, and 6-mercaptopurine and podophyllotoxin all produced suppression of labeling of this nuclear protein fraction. In most instances, this suppression was accompanied by decreased uptake of the tracer into other protein fractions. The data for 6-mercaptopurine and Myleran® most resembled those obtained with the aminouracil mustard in the apparent early specificity of effect on the acid-insoluble nuclear proteins without a marked suppression of amino acid incorporation into the cytoplasmic fractions of the tumor. The less selective agents in this respect included 5-fluorouracil, Leukeran®, HN2 and podophyllotoxin.

In other tissues, the effects were more varied than those found for the tumor. In the spleen, Leukeran® produced a marked inhibition of labeling of the nuclear proteins. 5-Fluorouracil suppressed labeling of the nuclear and cytoplasmic proteins of the spleen. On the other hand, marked effects on the labeling of the nuclear proteins were not produced in the spleen with either 6-mercaptopurine or Myleran® in these experiments.

In the liver, the aminouracil mustard produced essentially no change in the incorporation of L-arginine-U-^{14}C into cellular proteins. A similar result was found in these experiments for 6-mercaptopurine and for Leukeran®. However, 5-fluorouracil markedly suppressed the uptake of labeled arginine into all fractions of the liver and marked inhibition of labeling of the acid insoluble nuclear proteins and the microsomal proteins was found following injection of Myleran®.

The data presented in these studies suggest a relationship between antitumor activity and suppression of biosynthesis of the insoluble nuclear proteins. Fractionation of these proteins has been attempted with some success with the aid of DEAE columns.[13] Studies now in progress have been designed to overcome some of the problems of these chromatographic systems. Improvements have been made in technics for packing the columns and maintenance of constant flow rates for prolonged periods, which should provide improved resolution of the proteins. Investigations have recently been initiated on the lipoprotein components of the nuclear proteins.

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