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SUPPRESSION OF UPTAKE OF L-ARGININE-U-^{14}C INTO CHROMATOGRAPHICALLY SEPARATED CATIONIC NUCLEAR PROTEINS OF TISSUES OF TUMOUR-BEARING RATS BY 5-BIS (2-CHLOROETHYL) AMINOURACIL*

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Abstract—At varying time periods ranging from 2 to 24 hr after pre-treatment with 4 mg of 5-bis (2-chloroethyl) aminouracil per kg, rats bearing the Walker 256 carcinosarcoma were injected intraperitoneally with 10 μc of L-arginine-U-^{14}C. One hour after injection of the tracer, the acid-soluble nuclear proteins of tumor, liver and spleen were isolated and chromatographed on carboxymethylcellulose columns. Comparison of the control values with the results obtained 4 hr following administration of the drug indicates that, in the spleen, the specific activities of the proteins of those radioactive protein peaks which have been termed RP1-A, RP2-A, RP3-A, RP4-A and RP5-A, were reduced to 14, 28, 22, 0 and 36 per cent, respectively, of the control values. In the tumor, 24 hr after administration of the drug, the specific activities of the proteins of radioactive protein peaks RP3-A and RP4-A were reduced to 24 and 38 per cent of the control values, respectively. The specific activities of the proteins of the liver were not significantly changed at any of the times they were examined. These data provide evidence that the effects of aminouracil mustard on the incorporation of this amino acid into nuclear proteins are, in part, specific. The data also provide an indication of the marked sensitivity of metabolism of nuclear proteins of the spleen to the effects of this drug.

RECENT studies from this laboratory[1] have indicated that the nitrogen mustard of uracil, 5-bis (2-chloroethyl) aminouracil markedly suppresses the incorporation of L-arginine-U-^{14}C into the nuclear proteins of the Walker tumor. Although the inhibition of uptake of the isotope was greatest in the acid-insoluble nuclear proteins, the inhibition of incorporation of the L-arginine-U-^{14}C into the cationic nuclear proteins (histones) was also very marked. The latter proteins have recently been found to contain a variety of chromatographically separable components,[2] some of which are different in tumors from those found in other tissues. In view of the availability of reproducible methods for chromatographic analysis of the cationic nuclear proteins, the question of specificity was examined in the attack of the mustard as an inhibitor of amino acid incorporation into the individual components of the cationic nuclear

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proteins. In the present studies, rats bearing the Walker 256 carcinosarcoma were treated with 5-bis(2-chloroethyl) aminouracil[1] and 1 hr before the termination of the experiment, L-arginine-U-^{14}C was injected intraperitoneally. The liver, spleen and tumor were treated as previously described for extraction and chromatographic analysis of the cationic nuclear proteins.[3] The data obtained indicate that although the drug exerts a suppressive effect on the incorporation of L-arginine-U-^{14}C into the cationic nuclear proteins, the effects on the incorporation of this amino acid into cationic nuclear proteins are in part specific.

MATERIALS AND METHODS

The animals used in these experiments were male rats weighing 180–220 g, obtained from the Holtzman Rat Company (Madison, Wisconsin). The Walker 256 carcinosarcoma was implanted from 7 to 10 days prior to the experiment.* In the present studies, 5-bis (2-chloroethyl) aminouracil,† dissolved in dimethylformamide, was administered intraperitoneally as a single dose of 4 mg/kg, to rats bearing the Walker 256 carcinosarcoma.[1] At a specified time interval following the administration of the drug, 10 μc of L-arginine-U-^{14}C (Nuclear-Chicago) with a specific activity of 5·3 mc/mmole was injected intraperitoneally. One hour after the administration of the labeled arginine the rat was anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg), and exsanguinated by aspiration of blood from the aorta. The tissues were rapidly excised, placed in ice-cold 0·25 M sucrose, and homogenized in isotonic sucrose (1:9 w/v) in the cold room (4 °C). The nuclei of tumor and liver were isolated by differential centrifugation.[3] For the nuclear preparation of the spleen the precipitate collected at 600 *g* was utilized. The cationic nuclear proteins were extracted from the nuclei with 0·25 N HCl for 30 min. The acid extract was separated from the insoluble residue by centrifugation at 600 *g* and clarified by centrifugation at 100,000 *g* for 30 min. The supernatant solution was dialyzed overnight against 0·5 N acetic acid at 4 °C and chromatographed in the cold room (4 °C) on carboxymethylcellulose columns with 1 N and 8 N formic acid as the eluting agents.[2] The protein concentration in the effluent fractions was measured by determination of the optical density of the solutions at 280 mμ in a Beckman DU spectrophotometer. Aliquots of each effluent fraction were pipetted onto stainless steel planchets, dried by warm air on a rotating turntable and assayed for radioactivity in a Nuclear-Chicago automatic counting system.

Two types of control experiments were carried out. In one group the animals were injected with dimethylformamide (1 ml/kg). Animals in the other group were untreated. Since the results were not significantly different between these two groups, the data have been pooled and designed as the "control".

RESULTS

Specific activity of protein of unfractionated acid extract of nuclei

Fig. 1 presents the specific activities (counts/min per E_{280}) of the protein of the whole acid-extract of the nuclear preparations of the Walker tumor and spleen. In the spleen the incorporation of labeled arginine into the acid-soluble nuclear proteins

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was inhibited by 72 per cent with 4 hr after administration of the drug. Progressively greater inhibition was noted at succeeding time intervals. In the tumor no inhibition of labeling was found up to 12 hr after administration of the drug; however, by 18 and 24 hr the incorporation of label was reduced by 26 and 49 per cent, respectively, of control values. The incorporation of radioactive arginine into the proteins of the

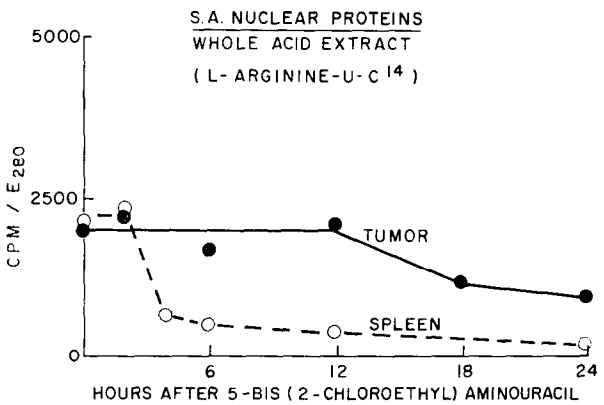

FIG. 1. Kinetics of the effects of the aminouracil mustard on incorporation of L-arginine-U-^{14}C into the whole acid-soluble nuclear protein extracts of Walker tumor and spleen. Each point represents the average of from two to four experiments. The values expressed as counts/min per E_{280} ± the standard error for the control and after 2 hr, 6 hr, 12 hr, 18 hr and 24 hr, were 1980 ± 30, 2270 ± 670, 1660 ± 320, 2120 ± 430, 1450 ± 310 and 1000 ± 190, respectively, for the tumor. The values for the control and after 2 hr, 4 hr, 6 hr and 12 hr, were 1700 ± 280, 2320 ± 480, 650 ± 130, 560 ± 160 and 440 ± 80, respectively, for the spleen.

whole acid extract of the liver did not differ significantly from the control values at 24 hr following administration of the drug.

Chromatographic patterns

The patterns for protein distribution in the chromatograms of the cationic nuclear proteins were the same in these studies as those reported previously.[2] The pattern for elution of the radioactive protein was different when arginine-U-^{14}C was used as the radioactive precursor, as compared to the pattern when lysine-U-^{14}C was employed.[2] With samples from spleen, five radioactive peaks were eluted from the column; these have been designated* as RP1-A, RP2-A, RP3-A, RP4-A and RP5-A (Fig. 2). Of these, RP1-A, RP4-A and RP5-A were eluted simultaneously with protein peaks *A*, *E* and *F*, respectively, while RP2-A was eluted with the first peak of the split peak labeled protein peak *B*. In samples of the Walker tumor, only RP3-A and RP5-A were present (Fig. 3). In samples of liver, RP2-A, RP3-A, and RP5-A were found; RP2-A

* The abbreviation used for the various radioactive peaks is RP1-A, etc. (first radioactive peak obtained with L-arginine-U-^{14}C as the tracer). Inasmuch as the positions of the peak tubes varied ± 3 fractions from the mean positions indicated in the chromatograms,[2] it is apparent that many criteria are required for adequate comparisons of the proteins of different tissues other than volume of acid required for elution. Studies are now in progress on the structures of the proteins of the various chromatographic peaks which will provide evidence as to their identity and differences.

and RP3-A were eluted just before and just after the peak of protein peak *B* (Fig. 4). RP5-A was eluted with protein peak *E*.

Kinetics of the effects of the aminouracil mustard on incorporation of L-*arginine-U-*^{14}C *into various chromatographic fractions*

The kinetics of the inhibitory effects of the aminouracil mustard on the incorporation of labeled arginine into various chromatographic fractions of the Walker tumor and

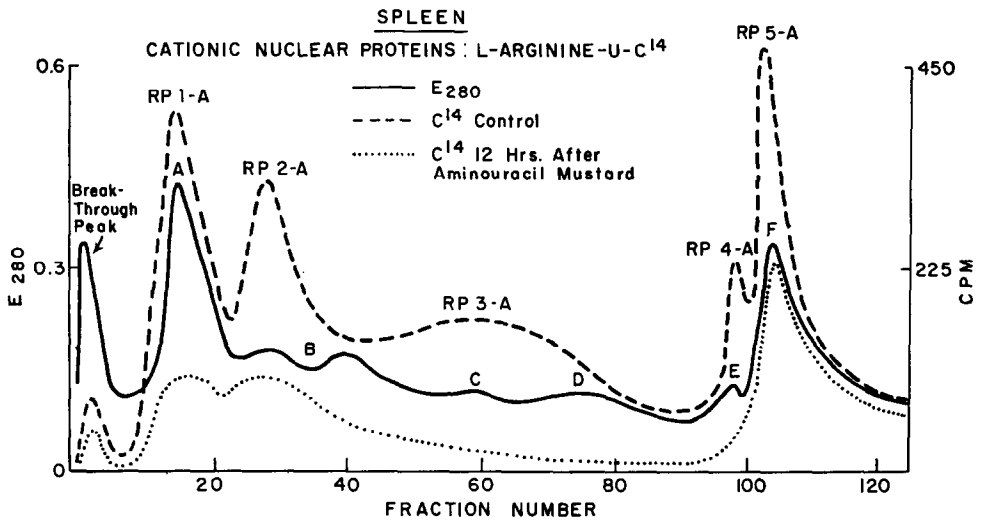

FIG. 2. Chromatographic patterns of protein concentration and radioactivity for the acid-soluble nuclear proteins of spleen. The radioactivity patterns represent both the untreated animal and the animal 12 hr after administration of the drug. Radioactivity and protein concentration were assayed in each effluent fraction and hence points are omitted from the chromatograms. The data are averages of from two to four experiments.

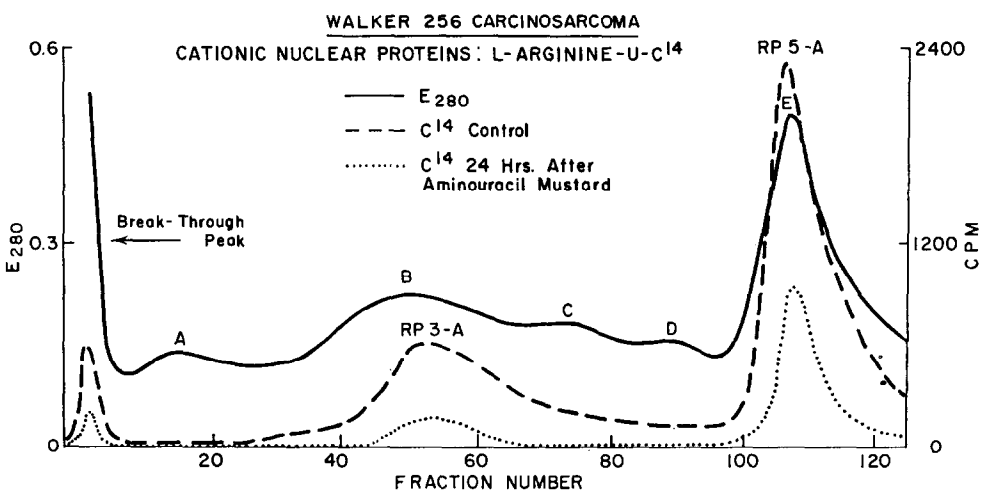

FIG. 3. Chromatographic patterns of protein concentration and radioactivity for the acid-soluble nuclear proteins of the Walker 256 carcinosarcoma. The radioactivity patterns represent both the untreated animal and the animal 24 hr after administration of the drug. Also see legend for Fig. 2.

spleen are presented in Figs. 5 and 6. In the *Walker tumor* the inhibition of incorporation of label* into fractions RP3-A and RP5-A follow a parallel course. Within 2 hr after administration of the drug, the specific activities of RP3-A and RP5-A were 67 and 73 per cent of the control values, respectively. By 24 hr after administration of the drug the specific activities were 24 and 38 per cent of control values of RP3-A

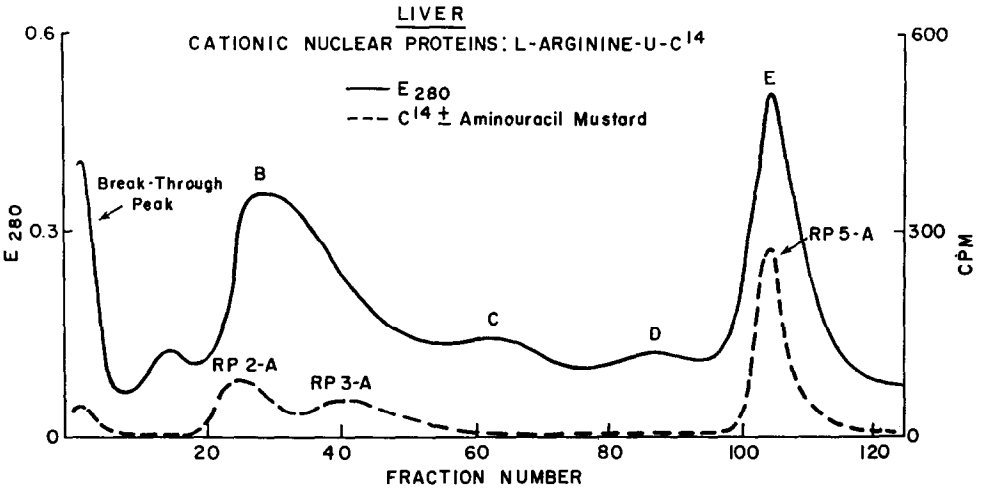

FIG. 4. Chromatographic patterns of protein concentration and radioactivity for the acid-solubel nuclear proteins of liver. Also see legend for Fig. 2.

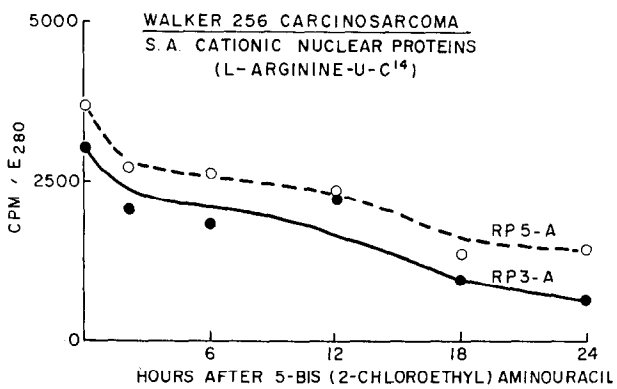

FIG. 5. Kinetics of the effects of the aminouracil mustard on incorporation of L-arginine-U-^{14}C into chromatographically isolated peaks of the acid-soluble nuclear proteins of the Walker tumor. Each point represents the average of from two to four experiments. The values expressed as counts/min per $E_{280} \pm$ the standard error for the control and after 2 hr, 6 hr, 12 hr, 18 hr and 24 hr, were 3020 $\pm$ 500, 2030 $\pm$ 790, 1820 $\pm$ 340, 2200 $\pm$ 60, 970 $\pm$ 520 and 610 $\pm$ 70, respectively, for peak RP3-A, and 3710 $\pm$ 450, 2700 $\pm$ 720, 2630 $\pm$ 670, 2320 $\pm$ 290, 1360 $\pm$ 540 and 1450 $\pm$ 340, respectively, for peak RP5-A.

* The specific activity of each radioactive protein fraction studied is expressed as counts/min per E_{280}. In order that these values be readily convertible to conventional specific activity values of counts/min per mg of protein, the eluted fractions comprising each defined protein peak eluted from a single column were pooled, and the E_{280} values and total mg of protein determined. Each value represents the average of two or three experiments and is presented as mg dry protein/$E_{280} \pm$ the standard error. The values found for the tumor were 1·53 $\pm$ 0·22 and 1·31 $\pm$ 0·12, for peaks *B* and *E*, respectively; for the spleen, 0·18 $\pm$ 0·03, 1·35 $\pm$ 0·15, and 1·11 $\pm$ 0·22, for peaks *A*, *B*, and *F*, respectively; for the liver, 2·00 $\pm$ 0·06, and 2·13 $\pm$ 0·03 for peaks *B* and *E*, respectively.

and RP5-A, respectively (Table 1). In the *spleen* a marked inhibition of labeling of the various peaks was found by 4 hr following the administration of the drug (Fig. 6). The evidence for intracellular selectivity of action of this mustard is found in Table 1. The specific activities of proteins in RP1-A, RP2-A, RP3-A and RP5-A were 14, 28, 22 and 36 per cent of control values, respectively. The fraction termed RP4-A was

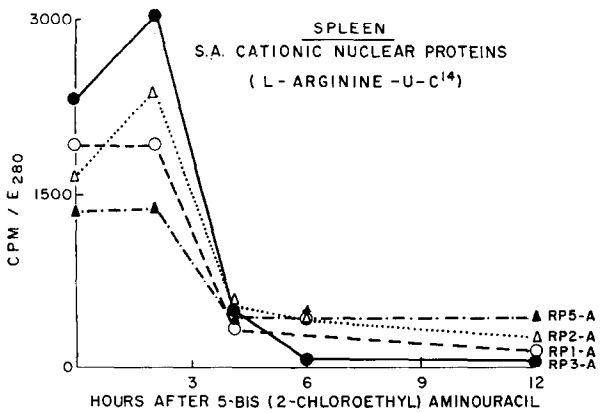

FIG. 6. Kinetics of the effects of the aminouracil mustard on incorporation of L-arginine-U-^{14}C into chromatographically isolated peaks of the acid-soluble nuclear proteins of spleen. Each point represents the average of two to three experiments. The values expressed as counts/min per E_{280} ± the standard error for the control and after 2 hr, 4 hr, 6 hr and 12 hr, were 1920 ± 130, 1920 ± 160, 330 ± 70, 410 ± 30, and 130 ± 1, respectively, for peak RP1-A, 1630 ± 600, 2380 ± 60, 540 ± 190, 420 ± 40 and 240 ± 50, respectively, for peak RP2-A, and 1350 ± 230, 1380 ± 200, 480 ± 100, 500 ± 70 and 430 ± 70, respectively, for peak RP5-A. Values for peak RP3-A for the control and after 2 hr and 4 hr were 2300 ± 580, 3050 ± 200 and 470 ± 90, respectively. The control value for peak RP4-A was 1890 ± 500.

TABLE 1. RELATIVE INCORPORATION OF L-ARGININE-U-^{14}C INTO CHROMATOGRAPHIC PEAKS OF ACID-SOLUBLE NUCLEAR PROTEINS OF TUMOR AND SPLEEN AFTER ADMINISTRATION OF 4 MG OF THE AMINOURACIL MUSTARD PER KG, AS COMPARED TO CONTROL VALUES

(The data are expressed in terms of percentage of control values ± the standard error. The number of experiments is shown in parentheses. The standard error, E, is $\sqrt{[\Sigma d^2/n(n-1)]}$.

Fraction	Spleen 4 hr after administration of the drug	Tumor 24 hr after administration of the drug
	% control	% control
RP1-A	14·2 ± 5·0 (3)	
RP2-A	27·5 ± 12·4 (3)	
RP3-A	21·9 ± 2·7 (3)	24·0 ± 2·9 (3)
RP4-A	0 (3)	
RP5-A	36·3 ± 1·7 (3)	38·4 ± 1·9 (3)

not found in samples of spleen at periods of 2 hr or more following the administration of the drug (Table 1).

Table 2 presents the comparative specific activities of the radioactive protein peaks obtained in chromatograms of the acid-soluble nuclear proteins of livers of control animals and livers from animals 24 hr after administration of the 5-bis (2-chlorethyl) aminouracil. The specific activities of the proteins were somewhat increased in the whole acid-extract and in RP5-A in comparison with control values.*

TABLE 2. THE INCORPORATION OF L-ARGININE-U-^{14}C INTO ACID-SOLUBLE NUCLEAR PROTEINS OF LIVER AFTER ADMINISTRATION OF 4 MG OF THE AMINOURACIL MUSTARD PER KG

(The data are presented as counts/min per $E_{280} \pm$ the standard error. The number of experiments is shown in parentheses. The standard error, E, is $\sqrt{[\Sigma d^2/n(n-1)]}$.

Fraction	Control		24 hr after administration of the drug	
Whole acid extract	475 ± 90	(3)	681 ± 108	(2)
RP2-A	676 ± 168	(3)	630 ± 203	(2)
RP3-A	642 ± 89	(3)	656 ± 188	(2)
RP5-A	840 ± 157	(3)	1088 ± 174	(2)

DISCUSSION

The data obtained in this study indicate partial intracellular specificity of action of the agent, 5-bis (2-chloroethyl) aminouracil. Such a result represents an advance in chemotherapy, since intra-tissue specificity of effect has been sought[4, 5] both from empirical and from theoretical considerations.[4-7] While the aminouracil mustard has been very effective against certain neoplasms of animals, it has not been therapeutically superior to other alkylating agents in human cancer chemotherapy.[8-11] However, therapeutic trials have not yet been completed. Nonetheless, any indication of intracellular specificity of action is particularly important from the standpoint of the endeavors of biochemists to establish the key reactions which characterize the neoplastic transformation in terms of alterations in the structure or activities of nucleoproteins.[2, 12] The nitrogen mustard of aminouracil has been used in these studies because the data on kinetics of inhibition of nuclear protein biosynthesis indicated a separation of biochemical events with respect to the time sequence of inhibition of labeling of various intracellular proteins.[1] Studies now in progress with a variety of antitumor agents have shown that this time sequence of inhibition is best demonstrated with the aminouracil mustard, which is therefore a useful biochemical tool.

The availability of methodology for the separation of components of the nuclear proteins has provided some evidence for differential effects of the aminouracil mustard on the anabolism of specific cationic nuclear proteins. However, these data are at present preliminary in the sense that as yet the task remains of isolation, purification and characterization of the proteins in the fractions obtained. Such studies are now in

progress with particular emphasis on those proteins in the area of that fraction of the proteins of the Walker tumor which has been termed RP2-L. If the proteins of this peak prove to be unique to the tumor, it might be possible to devise drugs which exert as marked an effect on this fraction as was found with aminouracil mustard and the fraction termed RP4-A of the spleen.

The data obtained in this study emphasize the remarkable effects of aminouracil mustard on the metabolism of the nuclear proteins of the spleen. Both the rate and extent of the inhibition of amino acid incorporation into the cationic nuclear proteins of the spleen were greater than that found for the tumor. One possible explanation for the marked effect of the aminouracil mustard on the proteins of the spleen is a selective affinity for the lymphocytes or their precursors in the spleen. Although greater suppression was observed for the chromatographic peaks eluted earliest in both tumor and spleen, it is apparent that specificity of site of action has not yet been achieved by the aminouracil mustard. This result, together with other data now being obtained for other antitumor agents, supports the possibility that the rate of amino acid incorporation into nuclear proteins of the spleen might be used in examinations of new carcinotoxic agents for general cytotoxicity.

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