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EFFECT OF 5-BIS(2-CHLOROETHYL)AMINOURACIL ON INCORPORATION OF URACIL-2-^{14}C INTO RNA OF TUMOUR-BEARING RATS

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Abstract—At various time intervals following the administration, to rats bearing the Walker 256 carcinosarcoma, of 5-bis(2-chloroethyl)aminouracil in doses of 4 mg/kg, 20 μc of uracil-2-^{14}C were injected. The animals were sacrificed after 75 min, the tissues were excised, and the specific activities of the RNA of the intracellular fractions were determined.

The incorporation of uracil-2-^{14}C into the RNA of the various intracellular fractions of tumor and spleen was markedly inhibited. The effect on the spleen was more marked than was the effect on the tumor. In the liver, an increased incorporation of the labeled uracil into the RNA of all intracellular fractions was observed at 12 hr after the administration of the drug.

RECENT experiments carried out in this laboratory have indicated the extensive utilization of amino acids for the biosynthesis of nuclear proteins in transplantable rat tumors.[1] Other studies have demonstrated the inhibitory effect of 5-bis(2-chloroethyl) aminouracil, a mustard linked to a pyrimidine analog,[2] on the incorporation of L-arginine-U-^{14}C into nuclear proteins.[3] Kinetic studies revealed that a dose of 4 mg of the drug per kg suppressed the incorporation of L-arginine-U-^{14}C into the acid-soluble nuclear proteins within 6 hr after administration of the drug. The uptake of labeled arginine into the acid-soluble nuclear proteins was markedly inhibited after a lag period of 12 hr. The aminouracil mustard inhibited the incorporation of L-arginine-U-^{14}C into the nuclear proteins of this tumor before any impairment of growth could be detected.

Inasmuch as recent evidence indicates that RNA catalyzes protein synthesis, it seemed desirable to determine whether aminouracil mustard also suppressed the incorporation of uracil into the nuclear RNA, since it is conceivable that nuclear RNA might be a site of biosynthesis of the nuclear proteins. Several investigators have suggested that mustards might act directly on nucleic acids,[4, 5] and thereby interfere with cell metabolism and protein synthesis.[6] However, *in vivo* evidence for this possibility is still lacking.[7]

In the present studies, aminouracil mustard (4 mg/kg) was injected intraperitoneally into rats bearing the Walker 256 carcinosarcoma. At various intervals following the administration of the drug, uracil-2-^{14}C was injected intraperitoneally, and 75 min later the various intracellular fractions were isolated from tumor, spleen and liver from which RNA was extracted. These studies indicate that the aminouracil

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mustard inhibits the incorporation of uracil-2-^{14}C into the nuclear RNA of the Walker 256 carcinosarcoma and the spleen.

MATERIALS AND METHODS

The animals used in these experiments were male rats, obtained from the Holtzman Rat Company (Madison, Wisconsin), weighing 200–250 g and fed *ad libitum* on Purina laboratory chow. The Walker 256 carcinosarcoma was transplanted from 7 to 10 days prior to the experiment. The uracil-2-^{14}C used in these experiments had a specific activity of 3·2 mc/m-mole (Volk Company, Chicago).

In each experiment 20 μc of the tracer were injected intraperitoneally into the tumor-bearing rat. The rat was anesthetized 75 min later by the intraperitoneal injection of 50 mg of sodium pentobarbital per kg and exsanguinated by means of heart puncture. The tissues were rapidly excised and placed in beakers containing ice-cold saline, and these were immediately transferred to the cold room (4 °C) where they were homogenized in istonic sucrose. The homogenates were subjected to differential centrifugation for the separation of the various intracellular fractions.[1] The nuclei of the tumor and the liver were isolated as recently described.[8]

For the isolation of the RNA a slightly modified phenol extraction procedure was used, based on the methods of Kirby[9], Goldthwait[10], and Hall and Doty[11]. The nuclear and cytoplasmic fractions were dialysed overnight against distilled water at 4 °C, while the other fractions were stored at −15 °C during this time. The dialysed fractions were then made 0·2 M with respect to potassium phosphate buffer, pH 6·8. The other fractions were taken up in 6 ml of 0·2 M phosphate buffer, pH 6·8. The fractions were then shaken for 30 min at room temperature with an equal volume of phenol saturated with water and centrifuged at 600 **g** for 30 min. The aqueous layer was siphoned off, and the entire procedure was repeated once. The two combined aqueous extracts were then shaken twice with ether, and chilled in the cold room at 4 °C. All fractions were made 0·2 N with respect to HCl and centrifuged after 1-hr incubation in the cold room at 4 °C at 600 **g** for 30 min. The white precipitates were washed once with 95 per cent ethanol and then were dissolved in 0·03 N ammonium hydroxide. The concentration of RNA was determined by the orcinol method,[12] while the radioactivity was determined by plating samples at infinite thinness. Each point on the graphs is the average of three experiments and in each experiment one rat was used. The specific activity of the mitochondrial RNA was essentially the same as that of the microsomal RNA and hence these data were omitted from the graphs. The 5-bis(2-chloroethyl) aminouracil was generously supplied by the Upjohn Company through the courtesy of Dr. H. G. Petering. The drug was injected intraperitoneally in single doses of 4 mg/kg in a solution containing 4 mg of the drug per ml of dimethylformamide.*

RESULTS

Studies on untreated animals

To obtain baseline values for comparison of results obtained in treated animals, the content of RNA was determined in the intracellular fractions of tissues of untreated animals (Fig. 1). The tumor and spleen appear to be very similar with respect to RNA distribution. The nuclear fraction of the liver contained one-fourth the quantity of

* Dimethylformamide, if administered in the same quantities, but without aminouracil mustard, did not inhibit the incorporation of uracil-2-^{14}C into the RNA of the various intracellular fractions of tumor, liver or spleen.

RNA found in the tumor nuclei, while the microsomal fraction of the liver was richer in RNA than was that of the tumor. In the tissues studied, the microsomal fraction contained as much RNA as the remainder of the cell.

Kinetics of incorporation of uracil-2-^{14}C into RNA

The kinetics of the incorporation of radioactive uracil into RNA of the various intracellular fractions of the tumor, liver and spleen of the Walker tumor-bearing rat are shown in Figs. 2, 3 and 4, respectively. These data indicate a sharp initial rise in

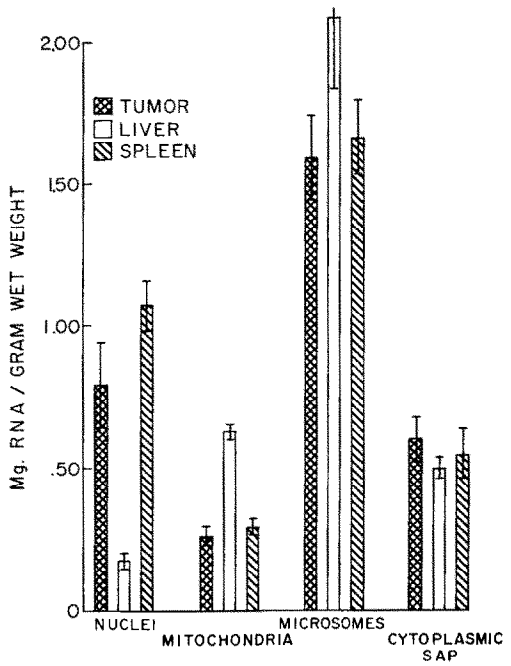

FIG. 1. Distribution of RNA in the various cellular fractions of tumor, liver and spleen in mg of RNA per g wet weight of tissue. The isolation of the RNA from the various cellular fractions was carried out according to the procedure described in the section on Materials and Methods. The results were obtained from nine experiments; the standard errors are indicated.

specific activity of nuclear RNA which tends to level off, or even decrease in the liver, after 1 hr.[13] In contrast, the specific activity of the RNA in the cytoplasmic fractions rises more slowly. In the tumor there was a lag period in the labeling of the RNA in the cytoplasmic fractions. The specific activity of the nuclear RNA of the tumor was the highest of the tissues studied at 180 min after injection of the tracer. The specific activity of the nuclear RNA of the liver reached a maximum at 75 min. As indicated in Fig. 5, the utilization of uracil is highest in the nuclear RNA of the tumor, as compared to the other tissues studied.[14, 15] At 75 min the RNA of the nuclear fractions of tumor, liver and spleen contained, respectively, 43, 57 and 33 per cent of the total counts per min per g of wet weight of tissue.

Kinetics of inhibition of labeling of RNA by aminouracil mustard

The kinetics of the inhibitory effects of the aminouracil mustard, in a dose of 4 mg/kg, on incorporation of uracil-2-^{14}C into the RNA of the Walker tumor, liver

and spleen, are presented in Figs. 6, 7 and 8, respectively. Fig. 6 indicates that there is a progressive suppression of the systems which incorporate the uracil into the nuclear RNA of the tumor. At 24 hr there was 50 per cent inhibition of labeling of nuclear RNA of the tumor. At this time, the labeling of the RNA of the cytoplasmic fractions was even more markedly inhibited than was the labeling of nuclear RNA. In the

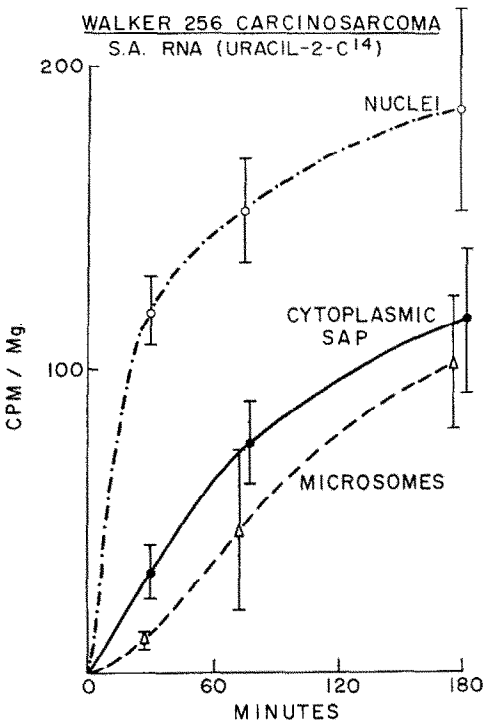

FIG. 2. Kinetics of the incorporation of uracil into the RNA of the various intracellular fractions of the Walker 256 carcinosarcoma. 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

spleen,* a progressive suppression of the labeling of the RNA in all fractions occurred until no radioactivity could be detected in any fraction after 24 hr. The rate of suppression of incorporation of uracil-2-^{14}C into the RNA of the spleen was greater than that of the tumor, and occurred at an earlier time. In the liver, the specific activity of the nuclear RNA increased to more than twice the control value at 12 hr after administration of the drug. The specific activity of the RNA in the cytoplasmic fractions rose slightly at this time point. None of the fractions studied showed a consistent change in the concentration of RNA as a result of the administration of aminouracil mustard.

DISCUSSION

Previously reported data concerning the inhibition by the aminouracil mustard of amino acid incorporation into the nuclear proteins of the Walker tumor and other

* As already reported,[3] the spleen of the animals shrink markedly in size after administration of aminouracil mustard. For this reason, the points at 12 hr after administration of the drug represent only two determinations, each of which was carried out on two to three pooled spleens in order to obtain a sufficient amount of tissue.

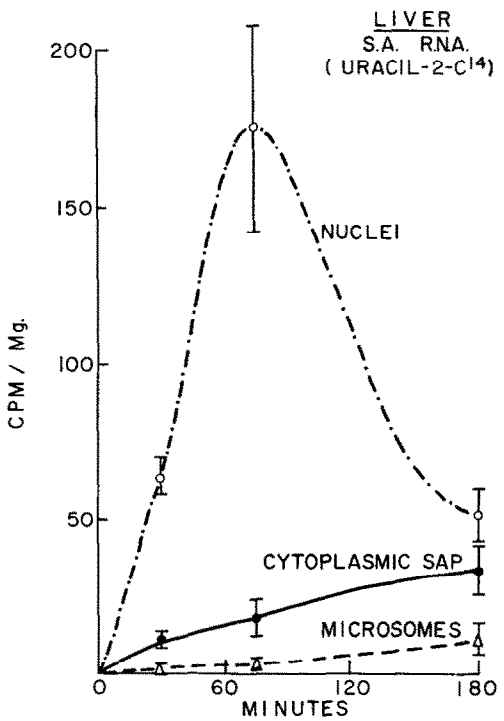

FIG. 3. Kinetics of the incorporation of uracil into the RNA of the various intracellular fractions of the liver. 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

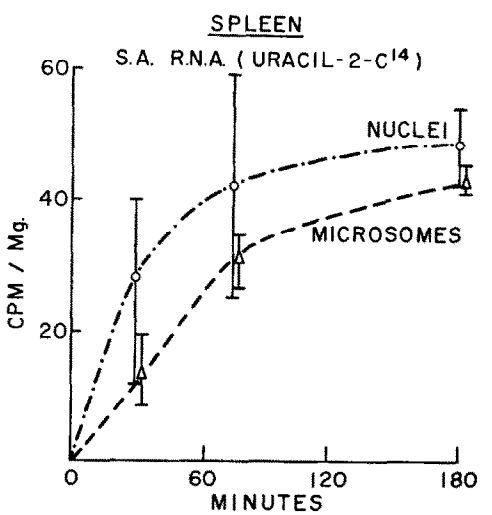

FIG. 4. Kinetics of the incorporation of uracil into the RNA of the nuclear and microsomal fractions of the spleen. 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

rat tissues, *in vivo*, suggested that the effects of the compound might occur selectively in the nucleus and particularly at sites involved in synthesis of acid-insoluble nuclear proteins.[3] Evidence for some intracellular specificity of action of the drug emerged from the finding that labeling of cytoplasmic proteins of tumor, liver and spleen was

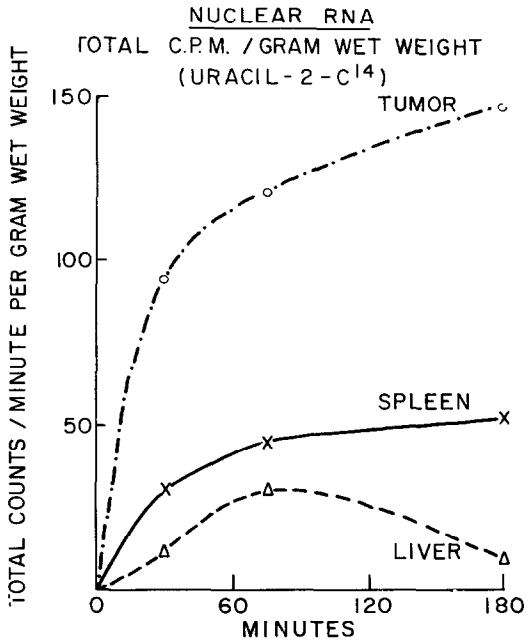

FIG. 5. Kinetics of the total labeling, per g wet weight of tissue, of the RNA of the nuclear fractions. The points were obtained by multiplying the average nuclear RNA content (Fig. 1) by the various specific activities.

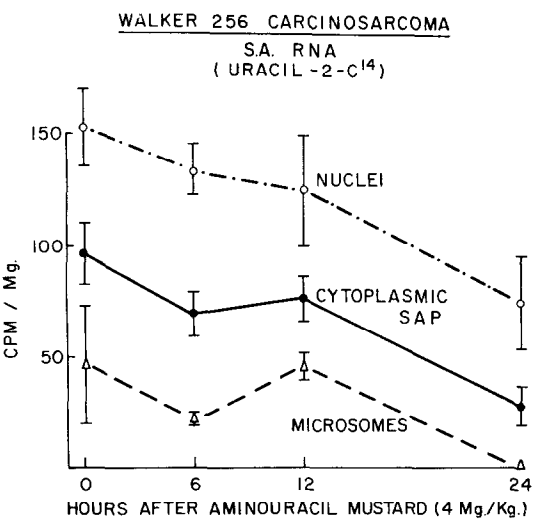

FIG. 6. Kinetics of the effect of aminouracil mustard on incorporation of uracil into the RNA of the various intracellular fractions of the Walker 256 carcinosarcoma; 75 min prior to the designated time, 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and treated as indicated in the section on Materials and Methods. The standard errors are indicated.

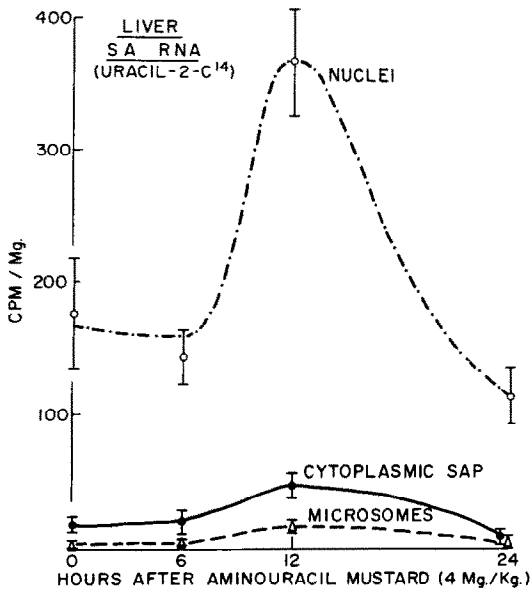

FIG. 7. Kinetics of the effect of aminouracil mustard on incorporation of uracil into the RNA of the various intracellular fractions of the liver; 75 min prior to the designated time, 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

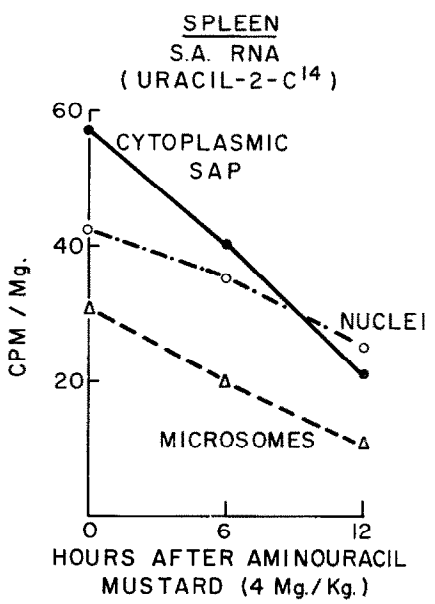

FIG. 8. Kinetics of the effect of aminouracil mustard on incorporation of uracil into the RNA of the various intracellular fractions of the spleen; 75 min prior to the designated time, 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The values and standard errors of these results at 0, 6 and 12 hr, respectively: nuclei, 42 $\pm$ 16·8, 25 $\pm$ 7, 25 $\pm$ 17; microsomes, 13 $\pm$ 5·5, 12 $\pm$ 9, 0; cytoplasmic sap, 40 $\pm$ 11·1, 21 $\pm$ 7, 0.

not inhibited, while the labeling of the nuclear proteins, particularly of the acid-insoluble nuclear proteins, was markedly decreased in tumor and spleen.

The present report indicates that the suppression of incorporation of uracil into the nuclear RNA of the Walker tumor by the aminouracil mustard was less marked than was the inhibition of incorporation of amino acids into the acid-insoluble nuclear proteins. It remains to be determined whether this suppression of uracil incorporation into RNA reflects a general suppression of incorporation of precursors into RNA or a more specific effect on uracil incorporation into RNA. However, the suppression of labeling of the nuclear RNA preceded the drop in labeling of the acid-soluble nuclear proteins. In the spleen, the incorporation of labeled arginine into the nuclear proteins was also inhibited to a greater extent in the first 6 hr after administration of the drug than was the incorporation of labeled uracil into the nuclear RNA. In contrast to the results obtained with L-arginine-U-^{14}C, the incorporation of uracil into the RNA of the *cytoplasmic* fractions was markedly suppressed. This result is consistent with the current concept that most if not all of the cytoplasmic RNA originates in the nucleus.[16, 17]

The intracellular selectivity of action of aminouracil mustard with regard to incorporation of labeled amino acids into the nuclear proteins of the Walker tumor, was not paralleled by a similar selectivity for the incorporation of uracil into the RNA of the various cellular fractions. As indicated previously, this result would be expected if the nucleus is the site of synthesis of most or all of the cellular RNA. This concept would imply that the primary site of action of the aminouracil mustard on biosynthesis of RNA is also in the nucleus. The results obtained do not rule out a role for nuclear RNA in the biosynthesis of acid-insoluble nuclear proteins, since a number of alternative explanations exist for these results. For example, the drug may attack existing molecules of nuclear RNA and destroy their catalytic activity; alternatively, the mustard may induce formation of abnormal RNA or suppress the formation of special RNA necessary for biosynthesis of the acid-insoluble nuclear proteins. It is evident that more knowledge needs to be obtained regarding nuclear RNA and its functions. With the aid of chromatographic methods, now becoming available, progress in this field may be accelerated.[10]

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