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Original Articles

Uptake and Distribution of Daunorubicin and Daunorubicin-DNA Complex in Mice as Studied by Whole-Body Autoradiography and Liquid Chromatography

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Summary. *The tissue distribution of daunorubicin (D) and daunorubicin-DNA complex (D-DNA) was studied in mice by means of whole-body autoradiography (WBA) and high-performance liquid chromatography (HPLC).*

A higher accumulation of radioactivity in the blood after 1 min and a lower initial accumulation in the cardiac muscle were found after administration of ^{3}H-D-DNA than after the injection of free drug.

Comparative studies of plasma levels of daunorubicin and daunorubicinol (DOH) in D- and D-DNA-treated animals by HPLC showed that the initial differences were negligible from 2 h onward.

A rapid accumulation of D in bone marrow occurred in both D- and D-DNA-treated mice. D reached its maximum level after 1 h and was almost constant for 12 h.

A new WBA finding was a rapid and specific accumulation of radioactivity in the pituitary gland, in the thyroid, and in the pancreatic islets, which might be of some interest in consideration of possible late endocrine side effects of anthraquinone glycoside therapy.

Introduction

The anthraquinone glycoside daunorubicin (D) has found widespread use in the treatment of acute leukemias. However, the use of the drug has been limited by its cardiotoxicity (e.g., Bonadonna et al., 1969; Jaenke, 1974). The mechanism responsible for the cardiac toxicity is unknown, although several hypotheses have been put forward (for review see Chabner et al., 1977). Chemical modification of the anthracycline molecule (Hurwitz et al., 1975; Israel et al., 1975; Lenaz et al., 1974) and an adjustment of the treatment schedule (Weiss et al., 1976) have been proposed to attenuate this severe side effect.

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Another approach is to link D to deoxyribonucleic acid (DNA) (Trouet et al., 1972). The D-DNA complex is thought to be preferentially taken up by cells capable of extensive endocytosis, thereby minimizing the localization of the drug in the cardiac muscle and increasing the uptake in the tumor tissue. The D-DNA complex has been shown to retain an antileukemic effect in mice (Trouet et al., 1972; Henry, 1974; Ohnuma et al., 1975) and has recently been tested in humans (Sokal et al., 1973; Cornu et al., 1974). The work on rats (Langslet et al., 1974) and rabbits (cited in Chabner et al., 1977) indicates that daunorubicin and adriamycin complexes with DNA are less cardiotoxic. However, the scarcity of data on the distribution of D-DNA in the body (Ohnuma et al., 1975) and the cardiotoxicity of the drug make it imperative to pursue further studies of this therapeutic approach.

In the present investigation the uptake and distribution of ^{3}H-D and ^{3}H-D-DNA in the heart and other tissues of mice have been studied with whole-body autoradiography (1) to obtain more detailed information on the specific sites of accumulation and to focus attention on any possible new site of action of the drugs in the body, and (2) to elucidate further the possible differences in the accumulation of D and D-DNA in heart muscle and its relation to other tissues. We used whole-body autoradiography for its superiority in detecting the drugs within organs composed of heterogenous tissues, such as pancreas or gastric wall. The concentration of D and its main reduced metabolite daunorubicinol (DOH) in plasma and bone marrow was studied by liquid chromatography (Eksborg, 1978).

Materials and Methods

Labeled Compounds

^{3}H-Daunorubicin (^{3}H-D) randomly labeled (specific activity 0.1 mCi/mg) was supplied by Pharma-Rhodia (Copenhagen). The radiochemical purity was checked by thin-layer chromatography with ra-

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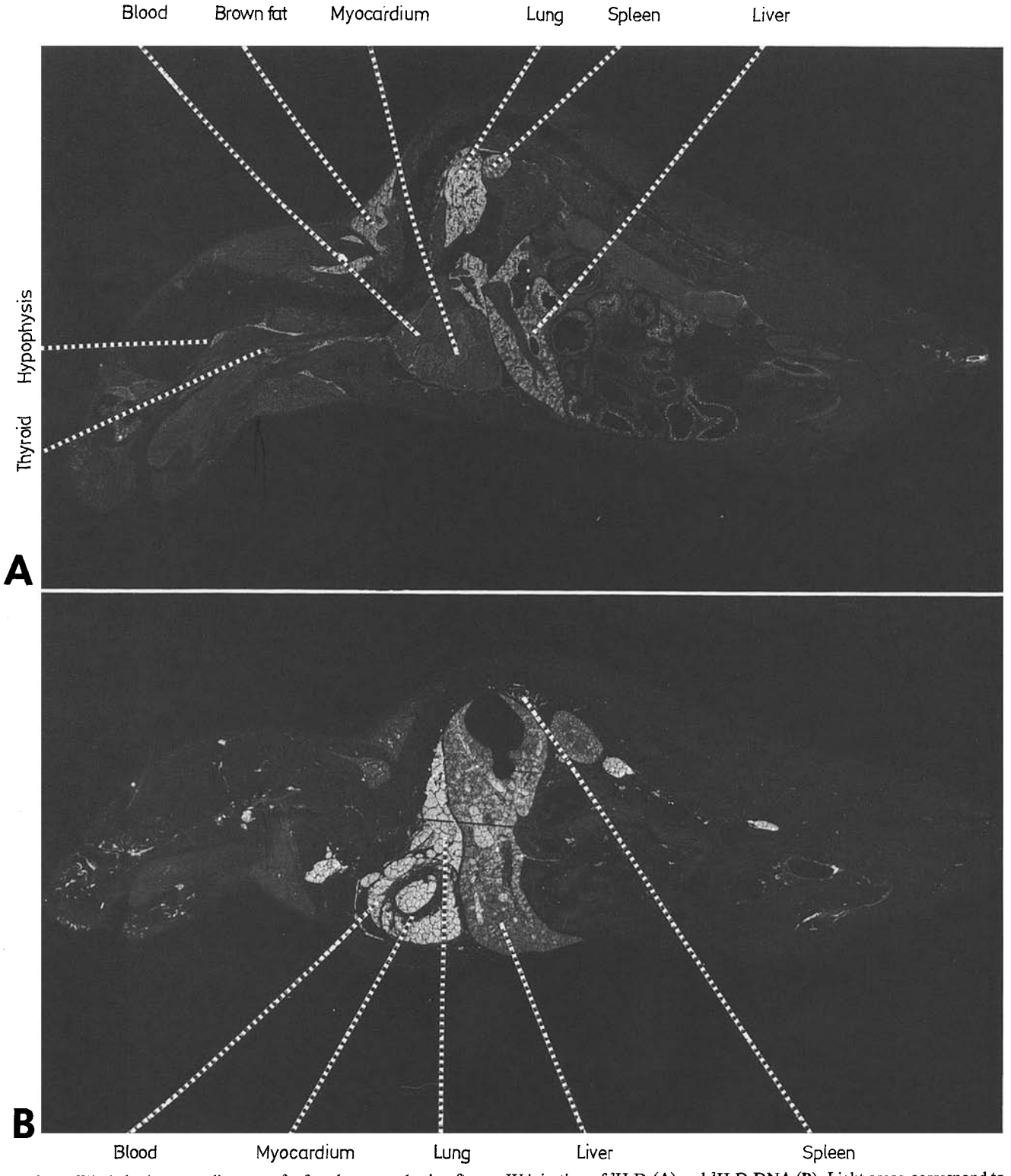

Fig. 1. Whole-body autoradiogram of a female mouse 1 min after an IV injection of ^{3}H-D (**A**) and ^{3}H-D-DNA (**B**). Light areas correspond to the high uptake of radioactivity. Note the difference in concentration of isotope in the blood. In the heart muscle a higher uptake of radioactivity can be seen after the injection of ^{3}H-D (A) than after ^{3}H-D-DNA (B). Exposure time: 29 days. Section thickness: 20 μm

diochromatogram scanning with chloroform-methanol-water (80 : 20 : 3 v/v/v) as solvent. DNA (herring sperm, grade VII) was obtained from Sigma Chem. Co. USA. ^{3}H-D was dissolved in physiological saline immediately before the experiment and injected into the tail vein of each mouse (3-month-old albino NMRI of both sexes). The ^{3}H-D-DNA complex was prepared according to Trouet et al. (1972) by mixing D with DNA in a ratio of 1 : 11.7 by weight, and was injected in the identical manner.

Whole-Body Autoradiography

Each of three mice received 50 μCi ^{3}H-D and each of three other mice 50 μCi ^{3}H-D-DNA complex. After prior anaesthesia with ether, one mouse in each group was killed by immersion in a mixture of hexane and solid CO_2 (−78° C) at 1 min, one at 2 h, and one at 24 h after injection. Further processing was performed according to Ullberg's autoradiographic technique (Ullberg, 1954). The tape-mounted sections were pressed against X-ray film (Kodirex, Kodak) and stored in a press at −20° C. After an exposure of 2 weeks to 3 months, the films were developed and the sections were stained with hematoxylin-eosin. For semiquantitative evaluation of whole-body autoradiograms the radioactivity in different organs was compared with autoradiograms of a simultaneously exposed ^{3}H-isotope standard (Berlin and Ullberg, 1963) by densitometry.

The chemical nature of the radioactivity in the blood and in the heart tissue at 1 min and 2 h after administration was studied by thin layer chromatography. The extraction of D (D-DNA complex) and its metabolites from plasma was carried out as described elsewhere (Eksborg et al., 1978). Heart muscle samples weighing approx. 200 mg were homogenized in five volumes of phosphate buffer pH 8.1 ($\mu = 0.1$). The homogenates were extracted with chloroform-1-heptanol (9 : 1 v/v) and the two phases were separated by centrifugation at 1500 *g*. The radioactivity obtained in different phases was determined in a Packard Tri-Carb liquid scintillation counter. The organic phase was subjected to thin-layer chromatography on silica-gel plates (Merck) with a chloroform-methanol-water (80 : 20 : 3 by volume) solvent system. The reference substances (^{3}H-D, ^{3}H-D-DNA) were always run on the same plate as the radioactive extracts. The radioactivity of the chromatograms was detected with the aid of a radiochromatogram scanner (Berthold LB 2723).

Quantitative Determination of Daunorubicin (D) and Daunorubicinol (DOH) in Bone Marrow and Blood

Concentrations of D and its main hydroxyl metabolite DOH were determined by reversed-phase liquid chromatography (Eksborg, 1978) in plasma and bone marrow. After injection of (10 mg/kg body weight IV) D or D-DNA, three mice were killed at each time point and their blood was pooled for plasma separation. After bleeding, both femurs of each mouse were split longitudinally and the bone marrow was scraped, pooled from the three mice, and weighed. Bone marrow was sonified by ultrasound (50 W, 30 s). D and DOH were then extracted and quantified according to Eksborg et al. (1978).

Results

Autoradiographic Studies

Daunorubicin (^{3}H-D). Following the IV administration of free ^{3}H-D the labeled material was rapidly taken up

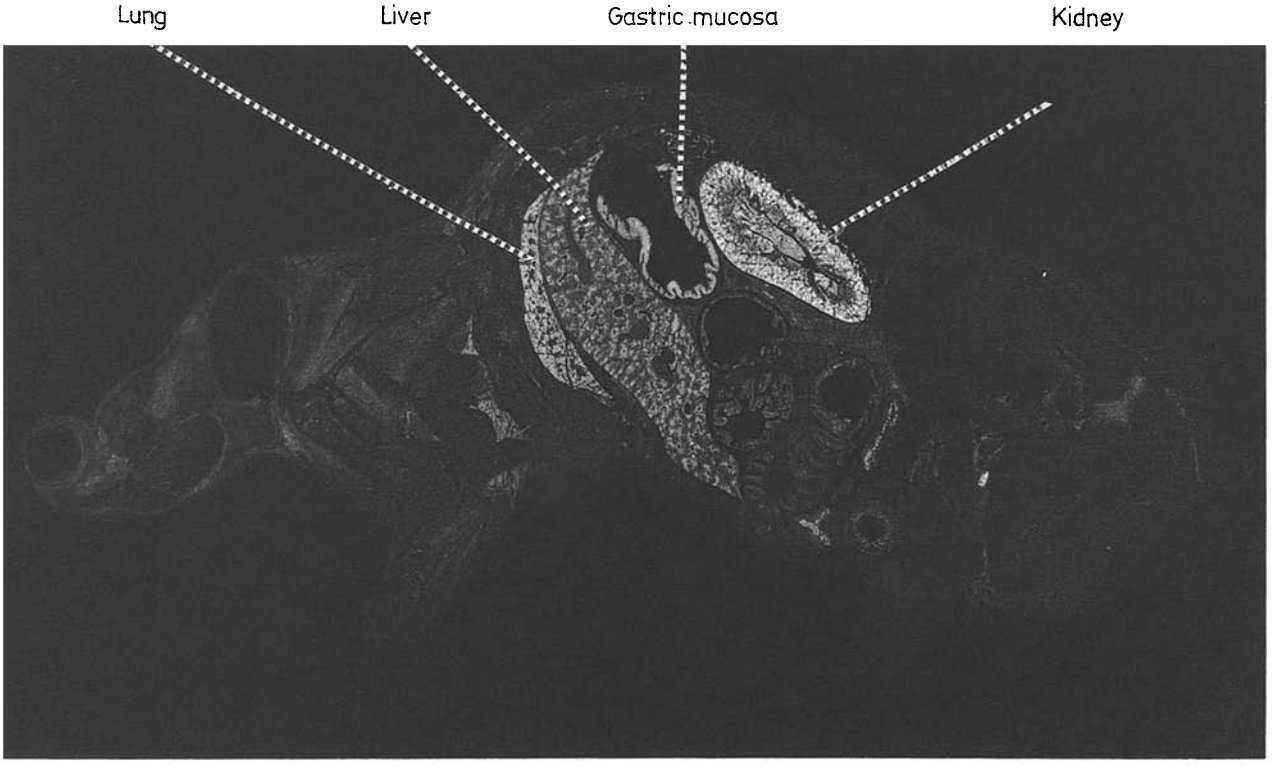

Fig. 2. Whole-body autoradiogram of a female mouse 1 min after an IV injection of ^{3}H-D. Gastric secretion of drug is indicated by a high concentration of radioactivity in stomach mucosa. An uptake of label is also seen in the lung, liver, and kidney. Exposure time 29 days. Section thickness: 20 μm

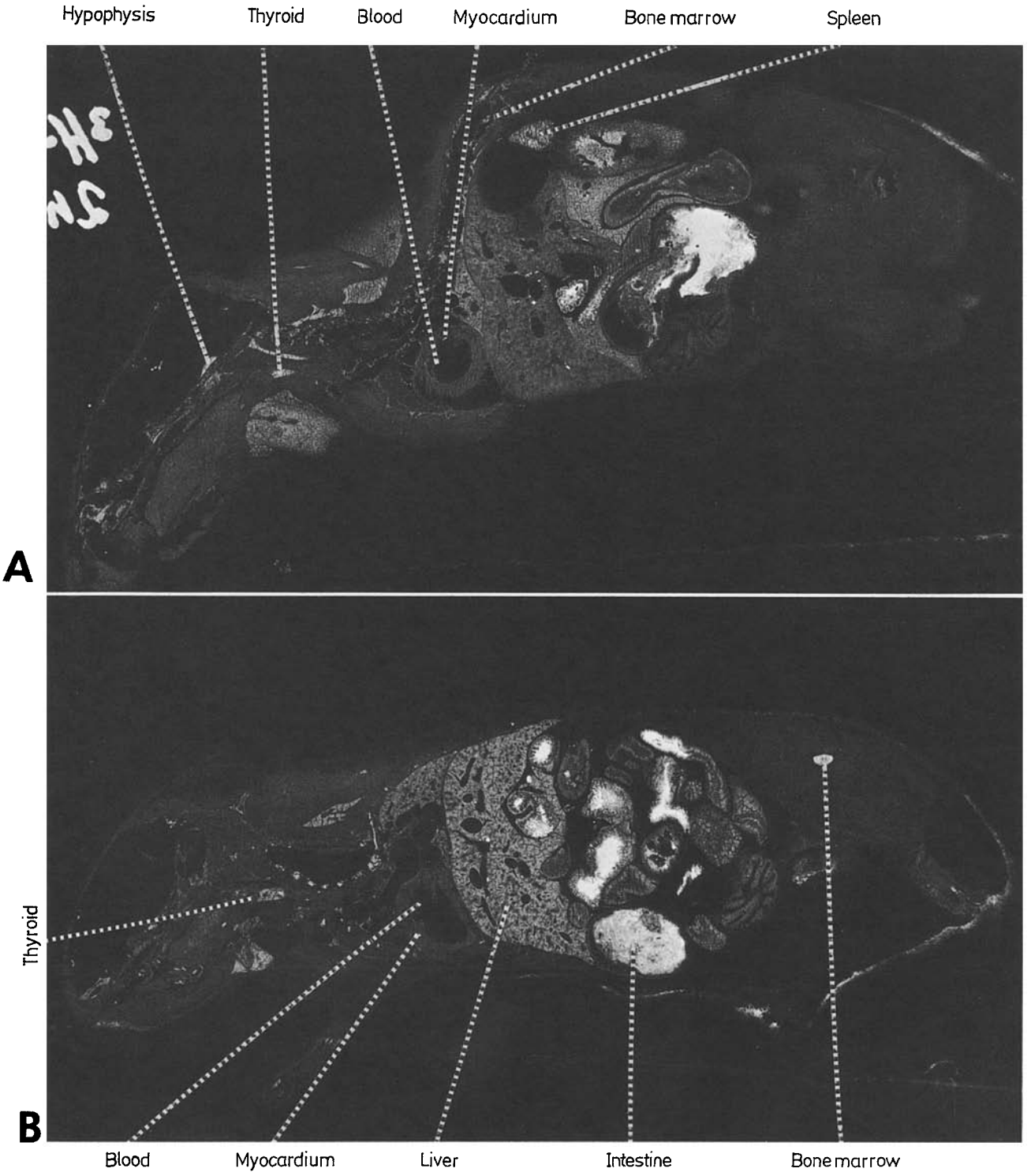

Fig. 3. Whole-body autoradiogram of a female mouse 2 h after an IV injection of ^{3}H-D (**A**) and ^{3}H-D-DNA (**B**). Light areas correspond to the high uptake of radioactivity. The general distribution picture is very similar for the two substances at this time. In the bone marrow, however, a significantly higher uptake is seen after the injection of ^{3}H-D-DNA complex. Exposure time: 95 days. Section thickness: 20 μm

from the blood and distributed in various tissues. As early as 1 min after the injection a significant accumulation of radioactivity could be seen in the lung, liver, brown fat, and kidneys, and in the secreting part of gastric mucosa (Figs. 1A and 2). The radioactivity in these tissues then decreased gradually during the period of observation, with very little label left 24 h after the injection (Figs. 1–4).

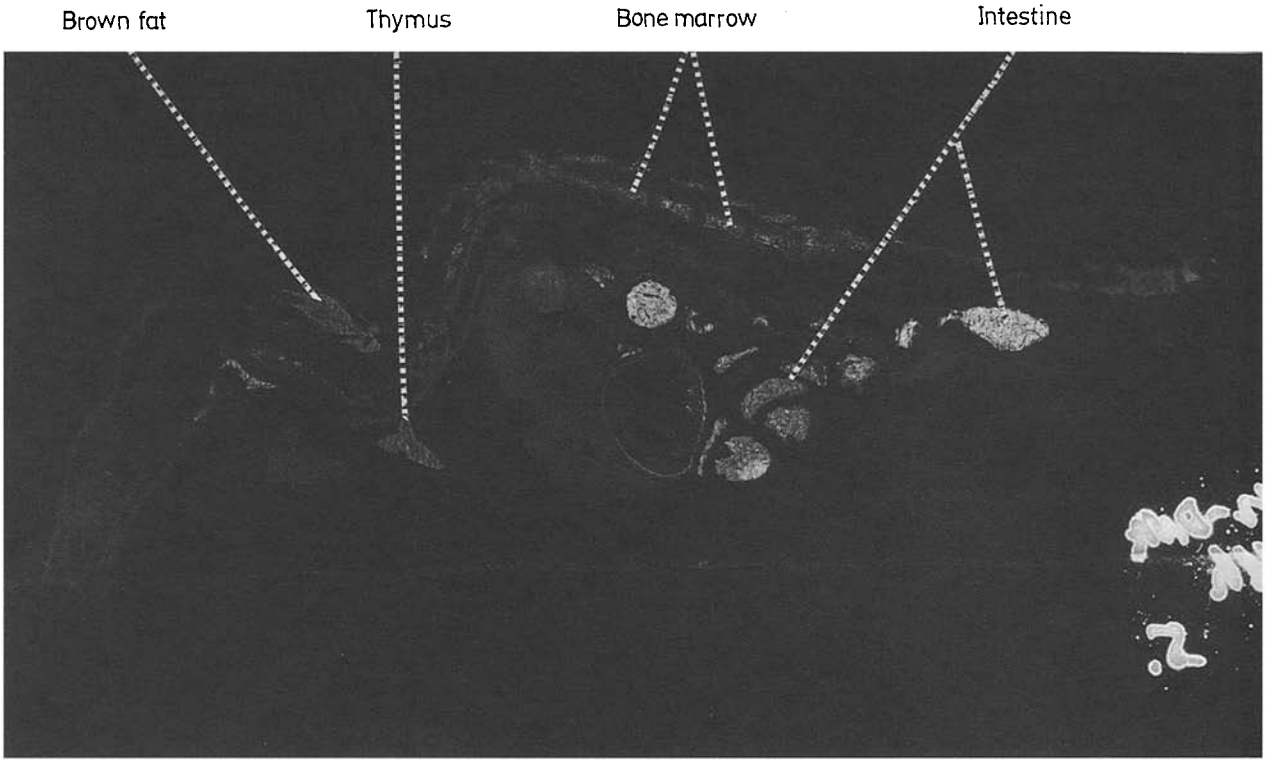

Fig. 4. Whole-body autoradiogram of a female mouse 24 h after an IV injection of ^{3}H-D-DNA. Light areas correspond to the high uptake of radioactivity. A retention of label can be seen in the bone marrow, thymus, brown fat, and intestinal contents. Exposure time 124 days. Section thickness: 20 μm

Table 1. Semiquantitative evaluation of the radioactivity of some organs on the whole-body autoradiograms after IV injection of ^{3}H-D and ^{3}H-D-DNA complex in mice[a]

	^{3}H-D			^{3}H-D-DNA		
	1 min	2 h	24 h	1 min	2 h	24 h
Heart	2048	1024	–	1024	512	–
Liver	2048	1024	256	2048	2048	256
Lung	4096	1024	256	4096	1024	256
Bone marrow	256	1024	1024	512	4096	1024
Thyroid	4096	4096	128	4096	4096	128
Pancreas	1024	2048	128	1024	2048	128
Pancreatic islets	2048	2048	512	1024	1024	512
CNS	128	128	–	128	128	–
Blood	1024	128	–	4096	128	–

[a] The radioactivity in different organs was compared with autoradiograms of simultaneously exposed ^{3}H-isotope staircases. These consisted of 12 steps of increasing isotope concentration in the geometric series. The radioactivity for the different localities is expressed as the relative isotope concentration of the staircase step with which it matched

The concentration of isotope in the cardiac muscle was initially the same as that in the liver, about twice that in blood, and higher than the concentration in the skeletal muscle by a factor of about 4 (Fig. 1A). After 2 h it had decreased and was still at about the same level as the radioactivity in the liver, still being about twice that in the striated muscle (Fig. 3A). Only traces of the labeling could be detected in the heart after 24 h (Figs. 1–3, Table 1). A specific and persistent accumulation of radioactivity was also located in some endocrine organs, such as the thyroid, the pancreatic islets, and the pituitary. The high labeling in these tissues persisted at the same level for 2 h (Figs. 1A, 3, 5 and 6) but became considerably lower at 24 h. Within the thyroid the label was localized around and between the follicles in the follicular cells. The resolution of the whole-body autoradiograms did not, however, permit any decision as to whether parafollicular calcitonin-producing cells were also taking up the substance. In the pituitary gland the intensity of labeling was similar in various parts of the gland.

The kinetics of the distribution pattern of isotope in the bone marrow, thymus, and spleen was somewhat different. After being initially very low, the concentration of radioactivity in these tissues increased gradually until 2 h after the injection, and remained high throughout the observation period (Fig. 3A). A similar pattern was seen in the exocrine pancreas, salivary glands, urine, and bile – the last two representing the excretory pathways of the drug and/or its metabolites (Fig. 3).

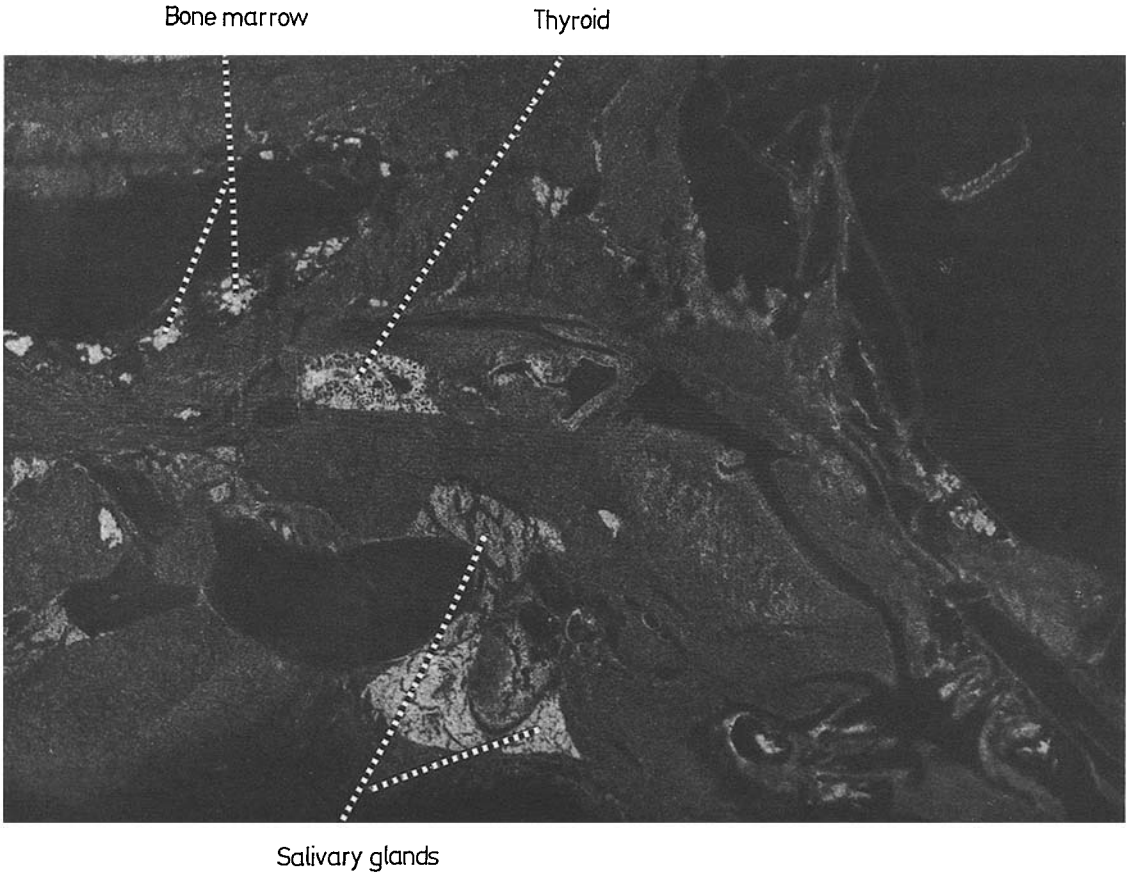

Fig. 5. Detail of Fig. 3B. A high accumulation of radioactivity (light areas) can be seen in the thyroid 2 h after the IV injection of ^{3}H-D-DNA complex. The label is localized mainly outside the colloids. A similar accumulation was noted after administration of ^{3}H-D

Daunorubicin-DNA Complex. With the exception of the high concentration of label in the blood at 1 min, the general distribution pattern of the labeled material in mice injected with ^{3}H-D-DNA complex was similar to that after free ^{3}H-D. Again, there was a rapid and vigorous uptake of radioactivity in the visceral tissues (Fig. 1B). The concentration of radioactivity in the liver exceeded that in mice injected with ^{3}H-D 2 h after administration.

The radioactivity in the bone marrow, thymus, and spleen showed a similar delayed and persisting pattern of accumulation to that seen with ^{3}H-D. At 2 h, however, there was a higher intensity of labeling in the bone marrow after ^{3}H-D-DNA injection than after the injection of free ^{3}H-D (Fig. 3, Table 1).

The concentration of radioactivity in the heart muscle was initially much lower (2—4 times) than that after ^{3}H-D injection (Fig. 1). Later on, the difference became less pronounced, but the labeling was still about half as intense as that seen with ^{3}H-D 2 h after the injection (Fig. 3B). As was the case with the free drug, very little activity could be detected in the heart muscle 24 h after the injection of ^{3}H-D-DNA (Fig. 4).

In the thyroid, pancreatic islets, and hypophysis there was also a specific and vigorous uptake of the isotope, distribution within the glands corresponding to that of ^{3}H-D. The intensity of labeling at the earliest check was somewhat lower than was observed with ^{3}H-D.

By 24 h after the injection of ^{3}H-D-DNA the distribution of the label on the autoradiograms did not differ significantly from that observed after the injection of ^{3}H-D in a free form. The retention of the isotope could be seen at this time in the intestinal contents, thymus, and bone marrow. The brown fat and the salivary glands also showed some labeling.

Analysis of cardiac tissue and blood 1 min after the administration of ^{3}H-D or ^{3}H-D-DNA revealed that the radioactivity originated almost exclusively from native drug (D). At 2 h the multiplicity of chromatographic peaks indicated the presence of metabolites.

Quantitative Determinations of Daunorubicin (D) and Daunorubicinol (DOH) by Reversed Phase Liquid Chromatography

Immediately after the IV injection, appreciably higher plasma levels of D were achieved with D-DNA complex

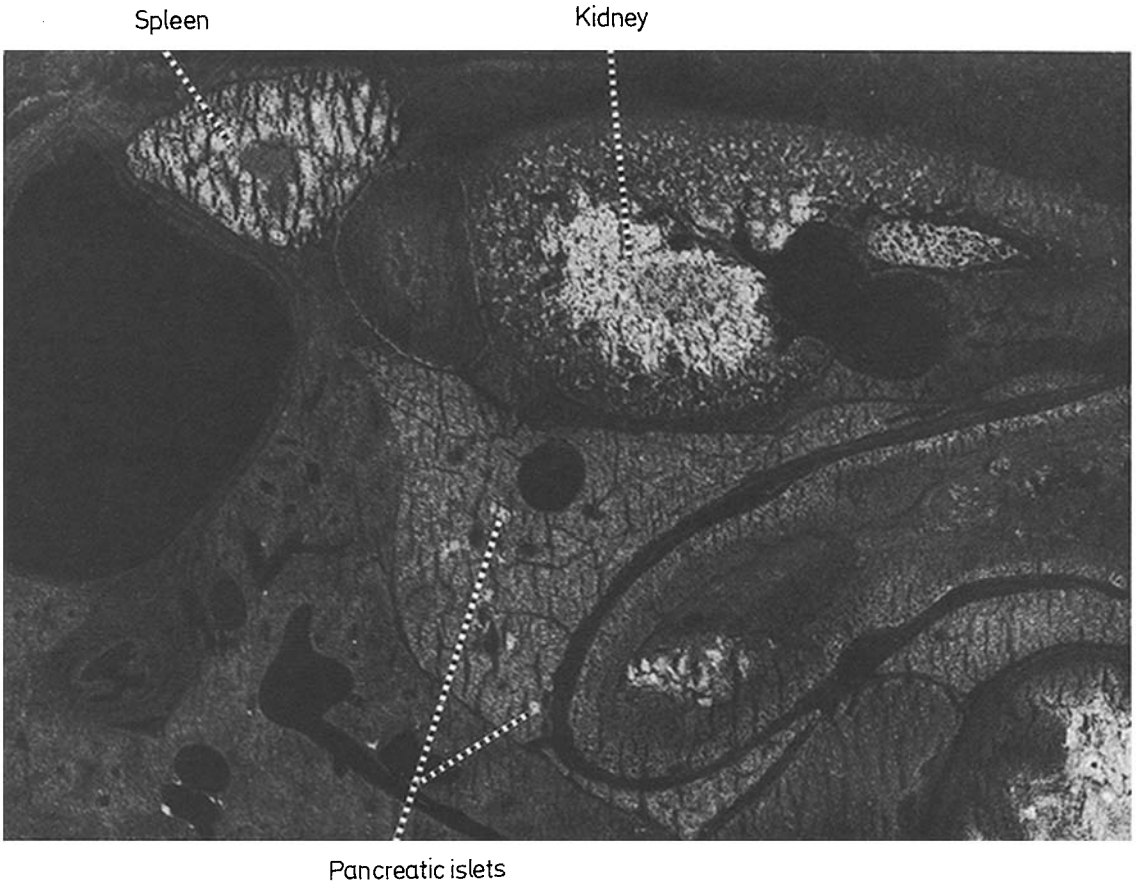

Fig. 6. Detail of a whole-body autoradiogram of a mouse 2 h after an IV injection of ^{3}H-D. Note accumulation of radioactivity (light areas) in the pancreatic islets

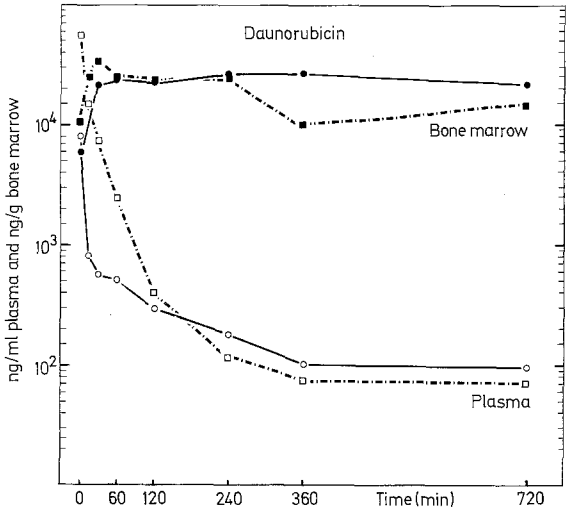

Fig. 7. Plasma and bone marrow levels of D after an IV injection of D at a dose of 10 μg/g, either as free drug (O—O) or as the DNA complex (□ – – – □)

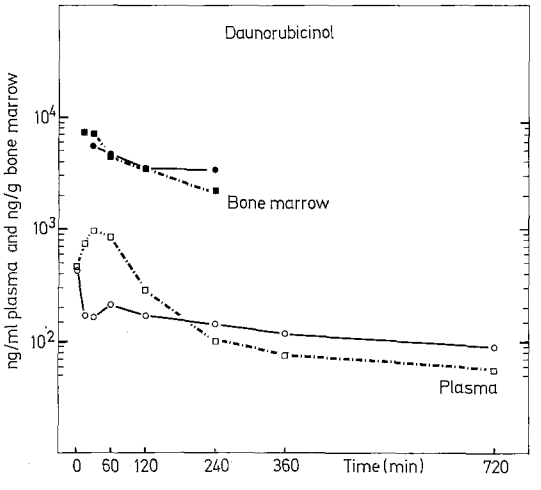

Fig. 8. Plasma and bone marrow levels of DOH after an IV injection of D at a dose of 10 μg/g, either as free drug (O—O) or as a complex with DNA (□ – – – □)

(Fig. 7) than with free drug. In both instances the plasma concentrations decreased rapidly and after 2 h reached comparable levels of 400–500 ng/ml in both free drug- and D-DNA-treated mice. The rapid decrease of plasma D during the first 2 h was followed by a slower decline with similar plasma concentrations in D- and D-DNA-treated animals between 2 and 12 h after administration (Fig. 7).

The plasma concentration patterns of DOH were also different during the initial 2-h period (Fig. 8). Being identical in the two groups 1 min after administration, the DOH concentration in D-treated mice decreased rapidly within 30 min, whereas in D-DNA-treated mice an initial increase in DOH level up to 1 h was followed by a rapid decline. At approximately 3 h the plasma levels were almost identical in both D- and D-DNA-treated animals and continued to decrease very slowly during the 12 h of observation.

After the initial 3-h period both D and DOH levels tended to be slightly lower in D-DNA treated animals (Figs. 7 and 8).

Bone Marrow

The kinetic pattern of the drugs was different from that in plasma. The D concentrations increased in bone marrow during the first hour and were slightly higher in D-DNA-treated animals. Hereafter they were maintained at approximately the same level during the 12-h observation period in D-treated animals. In mice treated with D-DNA the levels were slightly lower from 6 h onward (Fig. 7).

Concentrations of DOH in the marrow were identical in both D- and D-DNA-treated mice. One minute after administration they were approximately 15 times higher than in plasma, decreasing slowly and being 20 times higher than in plasma at 4 h.

Discussion

The tissue distribution of radioactivity after injection of ^{3}H-D-DNA and ^{3}H-D was similar in most tissues. The most significant differences were observed during the first 2 h and consisted of higher blood levels and lower accumulation of radioactivity in the cardiac muscle after injection of ^{3}H-D-DNA. The liquid chromatographic monitoring confirmed these findings and showed that differences in the plasma kinetics were due largely to D, but also DOH. Using the extraction procedure and total fluorescence assay of Bachur et al. (1970), Ohnuma et al. (1975) found higher levels in plasma of mice injected with D-DNA, but were not able to show any differences in the uptake in cardiac muscle. The differences might be explained by metabolites containing the radioactive label but not the fluorescent moiety.

The concentration of radioactivity in the cardiac muscle of ^{3}H-D-DNA treated mice was lower only during the first 2 h. These autoradiographic results were recently confirmed by quantitative liquid chromatography measurements of D and DOH concentrations in cardiac muscle as a function of time after administration. We found that the concentrations of D and DOH were almost identical 2 h and more, after administration (unpublished results). Moreover, the concentration of D was six to seven times that of DOH. This indicates that at this time most of the radioactivity in cardiac muscle might still come from native drug. Thus it is only during this short period that this organ might benefit from the lower concentration of native drug and its metabolites in ^{3}H-D-DNA treated mice: whether this leads to any protection remains to be determined. Langslet et al. (1974) showed some protection of the cardiac function in rats treated with DNA-linked drugs. The initially higher plasma/blood concentration of D in D-DNA-treated animals seems to have little influence on the accumulation of the drug in tissues, with the possible exception of the slightly higher uptake in the bone marrow. It appears that the excess of radioactivity in the blood of D-DNA-injected mice is excreted via the liver, as indicated by the higher accumulation of the radioactivity in the liver of these animals 2 h after administration.

The accumulation and long persistence of radioactive material in lymphomyeloid tissues found in our study after injection of both ^{3}H-D and ^{3}H-D-DNA confirmed the earlier observations of Rusconi et al. (1969) and Bachur et al. (1970) with the free drug. Using the quantitative measurements with liquid chromatography, we were able to show that the concentration of the main reduced metabolite, DOH, was less than 10% of the native substance in the marrow of both D- and D-DNA-injected mice. It seems that bone marrow handles D in a different way from other tissues. The rapid uptake of both D and D-DNA forms resulted in an early establishment of a steady state. The relatively constant levels of native substance and decreasing rather than increasing levels of DOH indicate a low activity of the metabolic pathway reducing D to DOH.

A new finding in this study was a specific and vigorous accumulation of radioactivity in the thyroid, pancreatic islets, and pituitary after administration of both ^{3}H-D and ^{3}H-D-DNA. The fact that it was observed as soon as 1 min after injection suggests that as in cardiac muscle, most of the radioactivity was due to unchanged drug. Accumulation of radioactivity was also noted in the secreting part of the gastric mucosa, a finding recently noted after administration of ^{14}C-D to rats (Liss et al., 1977). All these organs secrete peptide hormones and D has been shown to interact with proteosynthesis

via inhibition of RNA synthesis (Hartman et al., 1974). The finding might be of interest in relation to the hypothyroidism and the incidence of diabetic complications noted in some leukemic patients during treatment with D (unpublished observation). The probability of specific accumulation of D in endocrine tissues and the possibility of late endocrine side effects have to be born in mind when treating children and when more effective combinations of cytostatics will allow prolonged survival of patients with malignant diseases.

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