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# INTRODUCTION

In higher organisms cell division is preceded by DNA synthesis and DNA leaves the cell only at the time of its death (excluding certain types of differentiated cells such as RBC). Therefore, a radioactive precursor specific for DNA may be used to measure processes of cellular proliferation and turnover. Although thymidine labeled with either $C^{14}$ or $H^3$ has proven very useful in such studies, quantitative assay is made difficult by the weak energy of the β radiation from these isotopes requiring digestion or solubilization of the tissue for assay. The present report describes simple procedures using the thymidine analogue IUDR labeled with gamma emitting isotopes of iodine. Not only does this obviate sample preparation but even permits the location and evaluation of regions of DNA synthesis in the intact mammal. In addition, the disappearance of radioactivy provides a quantitativelmeasure of cell death, uncomplicated by the reutilization observed when cells labeled with $H^3$-thymidine die.

This paper describes the preparation of radioactive IUDR, its fate upon injection and the distribution in various tissues at subsequent times (both as observed by sacrifice and by scanning techniques). Methods of maximizing incorporation and minimizing complications from catabolic products are described. Following papers document its specificity as a label for DNA (  ) and describe its use in following some of the changes observed in synthesis and turnover of DNA produced by radiation and radiomimetic drugs (  ) and by cortisone (  ) Techniques involving a second isotope and of iodine for the measurement of the catabolic iodide pool are described.

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## EXPERIMENTAL PROCEDURES

### Preparation of IDU

The iodination of deoxyuridine was carried out basically as described by Prusoff ( ) but several modifications were necessary to obtain the high specific activities desired. The detailed procedure was as follows: 10 to 20 mg of deoxyuridine (Schwarz Bioresearch, Inc.) were dissolved in 1 ml of 2N nitric acid and added to an equal volume of $I^{131}$ carrier-free radioiodide (Oak Ridge). The mixture was then heated at 60° for 1 hour in a hood to protect the worker from volatile radioactivity. The solution was then cooled, neutralized with ammonium hydroxide and absorbed on a column of Dowex 1 resin on the chloride cycle (about 10 cc in a 15 cm long column). After washing with 0. ammonium nitrate to remove the unreacted deoxyuridine and then with water, the IDU was eluted with 0.01N acetic acid, the emergence of the peak being noted by its radioactivity. Immediately after IDU follows a small peak of iodouracil, but with care this can be easily intercepted so that the radioactivity in the IDU peak is at least 95% in IDU (measured by co-crystallization with carrier IDU (Cal. Found. Biochem. Res.). The unreacted iodide remains on the column. The amount of this seems to depend upon impurities, presumably reductants, in the radioactive iodide.

Solutions of IDU containing 100 microcuries or more of radioactivity per ml are sensitive to their own radiation and deteriorate to a point where less than 2/3 is co-crystallizable in the carrier IDU in the course of several weeks. Agents most successful in limiting this destruction include iodouracil and IDU itself. They have not been utilized because of their possible effects on IDU metabolism.

The groups of Swiss albino female mice, 59 to 10 weeks old used in these studies were maintained on a standard diet and starting 1 to 2

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days before IUDR injection their drinking water was supplemented with 0.1% sodium iodide to prevent thyroidal accumulation of radioiodide. The radioactivity was injected either intravenously (in the tail) or intraperitoneally in an amount varying from 1/20 to 5 microcuries depending upon the needs of the experiment. After injection they were distributed in groups of not more that 10 per cage (floor area ) and the litter changed at least two times in the first few hours to minimize urinary contamination.

Individual mice were counted at the times indicated after emptying their bladders by finger pressure on the abdomen. This procedure was usually successful in females, but much less so in males. Females sacrificed after this treatment usually had completely contracted bladders or at ;most, only 0.1 to 0.2 ml of retained urine, whereas no procedure has been found equally effective in males.

The distribution of radioactivity among the various organs was determined on organs removed immediately after death by ether anesthesia or by cervical dislocation. Organs were weighed for estimation of the concentration in various tissues. The activity in the bones was determined by careful removal of the meat. However, for a more accurate determination of the rate of turnover of bone marrow, the lower half of the skeleton was isolated by dislocating the spine at the last rib, removing the feet and tail and most of the meat. The remaining meat was then removed by heating this portion of carcass in 10 ml of 1M oxalic acid at 100°C for 15 minutes to inactivate nucleases followed by cooling and addition of 2 ml of 5% pepsin and incubation at 45°C for 10 hours to digest muscle and tendon, The bones were then separated by decanting the digest and washed. This preparation contained over half of the activity found in the whole skeleton.

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During the first few hours after injection, when catabolic products still contributed significantly to organ radioactivity, their contribution was estimated by homogenization in isotonic sodium chloride followed by precipitation of the DNA (together with most of the tissue proteins) by the addition of trichloracetic acid to a final concentration of 5 to 10%. The distribution of this soluble activity was still further characterized as iodide, iodouracil or iododeoxyuridine by the following procedures: Iodide: $I^{131}$ -- to 1 or more milliliters of acidified deproteinized supernatant were added two drops of a saturated aqueous solution of methyl mercuric bromide and an equal volume of toluene. After shaking to equilibrate the phases, the toluene layer was sucked off (centrifugation was sometimes necessary to break emulsions) and counted. Activity in the toluene layer represents only iodide and most of the iodide, since $CH_3H_9I$ is less dissociated than $CH_3H_9$ and the distribution coefficient of $CH_3H_9I$ between toluene and water is about 100 to1 ( ). The aqueous layer may be re-extracted to ascertain that extraction was complete. Iodine in carbon tetrachloride may be used similarly for extraction of radioiodide with which it exchanges rapidly.

Iodouracil: $I^{131}$ - To a 1 ml aliquot of the aqueous phase after iodide extraction was added 10 ;mg of iodouracil and the mixture was heated to boiling to dissolve. The iodouracil which recrystallized in a yield of about 90% upon cooling to $0^{o}C$ was separated and the percent of the radioactivity in the crystals determined. Recovery of carrier in the crystals was lmeasured by resolution and measurement of optical absorption at 280 mμ), and from these data the percent of iodouracil was calculated assuming the crystals contained no occluded impurities. However, recrystallization of these crystals usually gave a lower specific activity indicating occlusion of other radioactive components.

1176460 Iododeoxyuridine: $I^{131}$ - This was similarly assayed by co-

crystallization from 1 ml of solution to which was added 10 mg of carrier, heating and cooling. These crystals formed in amount comparable to iodouracil, but more slowly, and this analysis did not seem to suffer from occlusion of impurities.

## RESULTS

### Organ Distribution

The kinetics of the distribution of radioactivity in the various organs is shown in Figure I, and the relative concentrations of incorporated activities (T.C.A. insoluble activity) in Table I. The incorporated activity is seen to correspond to known proliferative activities of the various tissues with bone marrow showing the largest concentration of activity; spleen is second, and thymus is third. The intestine is only fourth in specific activity but because of its mass, easily accounts for a major portion (up to half) of the total incorporated activity, while the skin is second. Activity in the spleen shows the greatest variation, and while part of this variation is due to the variation in size of this organ, a two-fold variation still exists when activities are expressed per gram of tissue. In contrast, the activity of the bone marrow, when measured as the total activity of the decapitated, skinned and completely eviscerated carcass, is surprisingly constant, varying only 20%.

The first part of the curves in Figure I include activity as iodide and it is only when these plateau, as radioiodide is eliminated, that total organ counts can be used as a measure of proliferative activity. Since ;iodide rapidly distributes interstitially, the initial portion of the curves have been drawn assuming a fast component in equilibrium with the blood (i.e. parallel to the blood curve). Since the iodide concentration

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in all tissues is fairly uniform, its fractional contribution to total activity is inversely related to proliferative activity. Thus, organs like the thymus plateau in the first few hours (Figure I), but by 15 hours the residual activity in even muscle and liver is a fair measure of labeled DNA. The curve for skeleton has been drawn to include the more precise data for bones isolated by pepsin digestion (Table ) and normalized between the 8 and 21 hour points. The curve for spleen was obtained on other mice and has been drawn to indicate the time of DNA turnover. However, individual spleens vary widely in uptake limiting the precision of data for this organ. The skin and hair present a special problem due to urinary contamination and therefore have not been analyzed very precisely.

After a plateau of duration varying with the organ, activities again decline (Figure I) due either to cell migration, enucleation, or death. This phenomenon can be seen most clearly in the intestine, where it begins quite abruptly between the first and second day, and thus is compatible with the known migration of mucosal cells out to the tips of the villi where they are sloughed into the lumen. An earlier decline is observed in the spleen and bone marrow beginning at 13 hours and even the liver shows a slow decline in activity. While the decline in liver has not been measured precisely because of the low level of incorporation, the data suggest a turnover time of about a week, indicating that labeling has involved the reticulo-endothelial rather than the hepatic cells. (Similar turnover was observed with $H^3$-thymidine ( )).

Site of IDU Catabolism. appears to be largely in the liver. This

We have recently found that contamination in skin and hair can be largely eliminated by extraction with 0.2% NaI in 75% n-propanol at 100°C. Two 1/2 hour extractions removed more than 80% of the iodide remaining in the skin 24 hours after an injection of radioiodide. A similar treatment of labeled DNA (in the intestine) resulted in only 10% loss of label)

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was demonstrated by comparing the incorporation of IDU in mice injected via the portal vein versus the vena cava. Table shows that the uptake in both skeleton and intestine was decreased to less than half via portal administration. This suggests that over half of the IDU passing through the liver is catabolized there. Conversely, uptake by the liver is only slightly increased by the intraportal route. This also suggests that the liver is the major site of IDU metabolism. (If it were the exclusive site, then this route of administration should not increase uptake at all).

The kinetics of incorporation have been followed by measurements on the blood and urinary compartments. Figure 2 shows the changes in blood activity observed in the first few minutes after intravenous injection. Blood samples were obtained from the tip of the tail at the times indicated and the distribution of total activity, as iodide, IDU and iodouracil (total minus the other two) was determined. For comparison, the disappearance of $I^{125}$ radioiodide simultaneously injected is also shown. IDU appears to first distribute extra-vascularly, like iodide and at least as rapidly, and then it disappears largely by catabolism to iodide. The half-time for metabolism (when circulating IDU equals its catabolic products) is about 3 minutes. This can be compared with the half-time for incorporation of 5 minutes shown in the succeeding paper and thus suggests that the rate of cellular uptake may limit utilization.

The kinetics of excretion of catabolic products hinge largely on the kidneys' ability to excrete iodide. In our mice drinking 0.1% NaI the half-time for iodide excretion is less than one hour, and as indicated by the rate of disappearance of the blood activity in Figure 1, the rate of disappearance decreases with time. The slower rate probably results from a trapping of a portion of the iodide in pools such as the stomach contents which are in slow equilibrium with the blood. The rate of excretion can be

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appreciably accelerated by the ingestion of large amounts of dilute saline solution, but no method of increasing the rate by a factor of two or more has been discovered. Radiation may markedly slow the rate of excretion by initiating the "delayed stomach emptying syndrome" described by Conard (  ) and others.

Because of the difficulty in obtaining cumulative urine samples, urinary kinetics have been examined by the double tracer technique using $I^{125}$ iodide and $I^{131}$ deoxyuridine. Urine samples obtained at varying intervals after the simultaneous injection of the mixed labels were extracted with iodine in carbon tetrachloride and the ratio of activities in the organic layer determined (Fig.  ). It can be seen that after the first hour, when metabolism of IDU is completed, the ratio of activities in the urine remains constant for ten hours, indicating that no measurable catabolism of labeled DNA to iodide has occurred during this interval. (To illustrate the sensitivity of this technique the dotted line shows the sudden change in the ratio caused by the death of a few per cent of labeled cells following x-radiation.)

Increased labeling efficiency which would be desirable for many purposes has been achieved to a moderate degree by either increasing the time of availability br by decreasing the size of the competing thymidine pool. To effect the former the portal vein has been clamped during and for ten minutes after injection. This slowed the catabolic rate sufficiently to increase the half-time for the conversion of blood activity to iodide to over 10 minutes and approximately doubled incorporation into bone marrow and thymus. However, this technique obviously interfered with blood flow through the intestine and spleen, causing their engorgement, and in fact, these organs showed less than normal incorporation.

The size of the thymidine (thymidylate) pool can be decreased by

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small amounts of fluorodeoxyuridine or amethopterin which block de novo thymidylate synthesis. FDU prevents normal thymidylate synthesis from deoxyuridylate by competitively inhibiting the enzyme to which it binds as fluorodeoxyuridylate ( ). Fig. shows the increased incorporation observed when the indicated amounts of FDU were injected a few minutes before the radioactivity. This potentiation lasted for several hours and since it required less than 1/1000 of a toxic dose of FDU, it appears to be a relatively practical and innocuous approach.

## DISCUSSION

1. Utility of technic - cell turnover.

Since DNA does not turn over, the disappearance of radioactivity from an organ or tissue, once the initial catabolic products have been eliminated, may be safely taken as an indication of the disappearance of labeled cells so that if the degree of labeling is known, an estimate of the number of cells disappearing may be made. Of course, it should be ascertained that the label is, in fact, in DNA, but this appears to be true at least for the intestine and for the liver, as demonstrated in the following paper. Therefore, it is probably also true for less metabolically active tissues, although the possibility of iodide accumulation, as in the thyroid and in saliva and gastric juice must be kept in mind.

Breakdown of DNA presumably proceeds through the mononucleotides with subsequent splitting to the nucleosides by phosphotases. In the case of the iodinated analogue, it has been possible to demonstrate the presence of IDU as an intermediate by flooding the mouse with thymidine two days after labeling. By giving the mice milk containing thymidine to drink over half of the radioactivity recovered in the urine at the time

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of DNA turnover in the intestine was identified as IDU by co-crystallization with added carrier.

The poor initial utilization of IDU is an advantage in turnover studies since it should correspondingly limit the reutilization of activity from dying cells. Evidence in support of this is presented in the following paper. Reutilization has proven a complication in studies with thymidine ( ). Double tracer measurement of urinary activity indicates (Fig. ) that there is probably no turnover of DNA in the first 10 hours after labeling. The spleen and bone marrow then show the earliest loss of activity and largely account for the decline of the total body activity between 12 and 36 hours. The sudden loss observed between 13 and 17 hours coincides with the appearance of labeled phagocytized nuclei following $H^3$ thymidine administration ( ) suggesting that it is due to the destruction of red cell nuclei following their extrusion.

Cell turnover has been most dramatically observed in the intestine beginning abruptly some 36 hours after labeling (Fig. 1). This agrees well with the known maturation, migration and death of the mucosal cells as they travel from the crypts along the villi to desquamate into the lumen. Following this loss a portion of the original activity remains which continues to disappear, but at a much slower rate (dashed line), suggesting another labeled compartment. This compartment may represent lymphatic tissue since the thymus shows a similar slow decline.

The skin probably has a very slow turnover of radioactivity, although the problem of contaminating iodide makes precise determination difficult. Slow turnover has been observed in a tumor transplanted in the tail to facilitate precise measurement. (This technic should prove useful as a biochemical assay <u>in vivo</u> of carcinolytic procedures and toxicity as measured by death of body cells could be simultaneously determined.)

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2. Utility - rates of synthesis.

IDU as an analogue of thymidine enters into a crucial step in DNA synthesis and therefore can act as a very sensitive indicator of changes in DNA metabolism. However, care must be exercised in the interpretation of results since several factors in addition to the rate of DNA synthesis may alter utilization. Thus, as an example, FDU which is known to inhibit DNA synthesis via a block in thymidylate synthesis (  ) can potentiate the utilization of IDU (Fig.  ). The mechanism presumably involves a decrease in the pool size of competing thymidine resulting in an increased efficiency of utilization of IDU.

The simplest model to explain IDU (and thymidine) utilization would seem to involve (Fig.  ) first a rapid distribution (within seconds) into an extracellular pool, then its uptake within minutes into proliferating cells or its catabolism by liver cells. As little as 0.05 (  ) μmoles of thymidine injected simultaneously with IDU can depress the 3 minute incorporation into intestinal DNA by 50%, suggesting either competition at the cell membrane or a flooding of the intracellular pools.

Since competition is maximal when the enzymatic site, or transport site, is saturated with substrate, this data indicates that the effective pool size of DNA precursors in the thymidine pathway cannot be larger than 0.05 μmoles, but of course might be much smaller, and since the time course of incorporation closely approximates that for disappearance from the blood, this pool must exist in extra-cellular space as free thymidine. This is not to say that local concentrations within synthesizing cells may not be higher, and probably are higher (footnote) but that these pools must be small in total content.

Footnote: Feinendegen has shown autoradiographically a distribution of soluble precursors within bone marrow cells which is similar to the distribution of labeled DNA, suggesting that cells in DNA synthesis selectively concentrate thymidine.

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The major fate of IDU is catabolic and this presumably is carried out in the liver by thymidine phosphorylan. This enzyme has been shown to convert IDU to iodouracil and deoxyribose phosphate more rapidly than it attacks thymidine. This presumably explains why total uptake of IDU (integrated over its availability time) is also depressed by a thymidine load. However, in this case 0.1 μmoles of thymidine is required for a 50% depression in incorporation and 10 μmoles completely blocks uptake.

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This study is intended - as previously stated - to be carried out in patients with advancing, incurable malignancies, and with their full understanding and cooperation. The investigative maneuvers will be timed individually so as not to interfere with the normal clinical care of the patients.

Plan to administer I.V. 100 μc of $I^{131}$ deoxyuridine which has been premixed with 100 μc of $I^{125}$ iodide both in sterile isotonic solution. Serial blood samples and urine samples will be taken to study the disappearance of breakdown products and the beginning of cell turnover. Whole body counting will be undertaken to check retention in DNA.

After approximately 8 to 10 days the injections will be repeated, but immediately following an injection of fluorodeoxyuridine to determine whether this agent potentiates the utilization of IDU as it does in mice. For this purpose 50 mg of FDU in sterile isotonic solution will be injected intravenously. This is less than one percent of the dose used for therapeusis (30 mμ/kg/day for 5 days - Burchenal and Ellison Clin. Pharm. and Terap. 2, 523 (1961) ).

Radiation doses:

Whole Body

$I^{131}$ DU - 16 mr

$I^{125}$ - energy = 35 kev γ + 30 kev x-ray

= 65 kev

since it is all absorbed can use β formula

$$(D_\beta = 73.8\ \bar{E}_\beta\ C_o\ T\ eff)$$

$$= 73.8 \times 0.065 \times 2 \times \frac{10}{24}$$

$$= 4 \text{ mr}$$

Doses to labeled organs

$I^{125}$ - no additional

$I^{131}$ as previously calculated

assuming 2% uptake into intestine = 2 μc

localized doze = 150 mr

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# $I^{131}$ RADIATION DOSES

Using formula (17) p. 828, reference (1):

$$D_\beta(\infty) = 73.8\ \bar{E}_\beta\ C_o\ T_{Eff} \qquad \text{rad}$$

and formula (88) (modified) p. 861, reference (1):

$$D_\gamma(\infty) = 0.0346\ (IA)\ g\ C_o\ T_{Eff} \qquad \text{rad}$$

where $\bar{E}_\beta$ = 0.187 MeV (p. 899, ref. (1))

IA = 2.18 $cm^2$-r/mc-hr (p. 899, ref. (1))

$T_{Eff}$ = Effective half-time in days

$C_o$ = 1 μc/g (initial concentration)

g = geometry factor, assuming $\mu = 0.03\ cm^{-1}$

For choice of g, see p. 858, ref. (1)

| | $D_\gamma$ rads for $C_o$ = 1 μc/g | | | | | | | | | | | $D_\beta$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | g | | | | | | | | | | | |
| $T_{Eff}$ | 10 | 20 | 30 | 40 | 50 | 60 | 80 | 100 | 125 | 150 | 200 | |
| 0.5 | 0.4 | 0.8 | 1.1 | 1.5 | 1.9 | 2.2 | 3.0 | 3.8 | 4.7 | 5.6 | 7.5 | 6.9 |
| 1 | 0.8 | 1.5 | 2.3 | 3.0 | 3.8 | 4.5 | 6.0 | 7.6 | 9.4 | 11.3 | 15.1 | 13.8 |
| 2 | 1.5 | 3.0 | 4.5 | 6.0 | 7.5 | 9.0 | 12.1 | 15.1 | 18.8 | 22.6 | 30.2 | 27.6 |
| 3 | 2.3 | 4.5 | 6.8 | 9.0 | 10.6 | 13.6 | 18.1 | 22.6 | 28.3 | 33.9 | 45.2 | 41.4 |
| 4 | 3.0 | 6.0 | 9.0 | 12.1 | 15.1 | 18.1 | 24.1 | 30.2 | 37.7 | 45.2 | 60.3 | 55.2 |
| 5 | 3.8 | 7.5 | 10.6 | 15.1 | 18.8 | 22.6 | 30.2 | 37.7 | 47.2 | 56.5 | 75.5 | 68.0 |
| 6 | 4.5 | 9.0 | 13.6 | 18.1 | 22.6 | 27.2 | 36.2 | 45.2 | 56.5 | 67.8 | 90.4 | 81.8 |
| 7 | 5.3 | 10.5 | 15.8 | 21.1 | 26.4 | 31.6 | 42.2 | 52.7 | 65.9 | 79.2 | 105.5 | 96.6 |
| 8 | 6.0 | 12.1 | 18.1 | 24.1 | 30.2 | 36.2 | 48.2 | 60.3 | 75.5 | 90.5 | 120.5 | 110.4 |

Ref. (1): Hine and Brownell, Radiation Dosimetry, 1956.

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