Distribution of C\textsuperscript{14}-labeled Urethans in Tissues of the Mouse and Subcellular Localization in Lung and Liver*

ISAAC BERENBLUM, NECHAMA HARAN-GHERA, RUTH WINNICK, AND THEODORE WINNICK

(Department of Experimental Biology, The Weizmann Institute of Science, Rehovoth, Israel)

The striking sensitivity of the lung to the carcinogenic action of urethan (12) has long been considered a unique feature of the action of this compound. More recently, Salaman and Roe (13, 15) demonstrated that urethan, though noncarcinogenic for skin, could induce the initiating phase of carcinogenesis in this tissue when applied topically; and Harán and Berenblum (7) subsequently showed that this effect is also elicited when the compound is given orally. Added interest was aroused by the demonstration of tumor-inhibitory properties of urethan (6); and in view of the simplicity of the molecule, numerous investigations have been conducted on modifications of the structure, and on the metabolic behavior in the body, of urethan in relation to its carcinogenic and tumor-inhibitory properties.

In studies concerned with the metabolism and mechanism of action of urethan, Skipper and co-workers (5, 11, 16) found that this substance was distributed rather uniformly in various organs of the mouse, without marked localization in any single tissue. With either carbonyl- or ethyl-C\textsuperscript{14}-labeled urethan, more than 90 per cent of the radioactivity was eliminated (chiefly as carbon dioxide) in 24 hours. Boyland and Rhoden (4), using a chemical method of analysis, reached similar conclusions regarding the distribution and rate of elimination of urethan.

With the carbonyl-C\textsuperscript{14} urethan, 1.3 per cent of the isotope was retained in body tissues of mice at 24 hours (bicarbonate-C\textsuperscript{14} giving the same value), while the C\textsuperscript{14} of ethyl-labeled urethan was retained to the extent of 7.3 per cent (methylene-C\textsuperscript{14} labeled ethanol giving 6.2 per cent) (16). Accordingly, the more extensive incorporation of ethyl-C\textsuperscript{14} urethan suggested metabolism via ethanol to acetate.

Urethan is known to act on nuclei, causing pyknosis and producing chromosome breaks at higher concentrations (Boyland and Koller [5]). It does not reduce respiration or glycolysis of lung tissue (Boyland and Rhoden [4]). In recent studies in connection with the induction by urethan of pulmonary adenomas in mice, Rogers (14) concluded that the process was intimately related to nucleic acid synthesis, with deoxyribonucleic acid as the critical site of action.

The present study is concerned with the metabolism of urethan-C\textsuperscript{14} in several tissues of the mouse, and with the distribution of the radioactivity in subcellular fractions of lung and liver. Both in vivo and in vitro experiments have been employed in the search for the locus of the tumor-inducing action of urethan.

MATERIALS AND METHODS

Animals.—Young, adult, male Swiss mice were employed throughout the present work. These mice have very low incidence of spontaneous lung tumor but have proved very responsive to the action of urethan, not only in the initiation of skin carcinogenesis but also in the induction of lung tumors.

Isotopic compounds.—Ethyl-1-C\textsuperscript{14} urethan (1.3 mc/millimole) and carbonyl-C\textsuperscript{14} urethan (1.8 mc/millimole) were obtained from the New England Nuclear Corporation, Boston. Specified quantities of each substance were injected intraperitoneally into mice, together with sufficient nonlabeled urethan to make the total dosage correspond to 1000 mg/kg body weight. In in vitro experiments, the C\textsuperscript{14}-urethans were used without isotopic dilution.

Radioactivity determinations.—All samples were measured in the mica end-window Geiger counter as solid layers and were corrected for self-absorption. The counts/min of C\textsuperscript{14} were converted to moles, with the aid of the specific activities of urethan standards.

Isolation and analytical procedures.—Animals were bled through the eye socket, and citrated blood plasma was prepared. Lung, liver, and portions of skin were minced and then homogenized with \(\frac{1}{3}\) parts of water, with the use of the Potter homogenizer. For determination of total C\textsuperscript{14}, 0.05-ml. aliquots of the homogenates (or 0.06 ml. of plasma) were oxidized with 3 ml. of hot 50 per cent H\textsubscript{2}SO\textsubscript{4} saturated with K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}, with distillation of about 1 ml. of H\textsubscript{2}O from this system into 1 ml. of CO\textsubscript{2}-free \(\frac{1}{2}\) NaOH. Subsequently, excess 1 M BaCl\textsubscript{2} was added, and the resulting BaCO\textsubscript{3} was centrifuged, washed with H\textsubscript{2}O and alcohol, and plated for counting. In certain in vitro experiments with carbonyl-C\textsuperscript{14} urethan, "alkali-labile C\textsuperscript{14}" was determined by treatment of samples with 1 ml. of \(\frac{1}{2}\) NaOH.
at 100° C. for about 5 minutes, then cooling to 0° C., acidifying with excess 12 N H₂SO₄ (with care to avoid loss of CO₂), and then collecting the C₁⁴OS by distillation as described above.

The radioactivity due to urethan was also measured by a specific carrier method. Non-labeled urethan (100-mg. quantities) was added to 0.2-ml aliquots of homogenates (or plasma). After deproteinisation with an equal volume of 10 per cent trichloroacetic acid, 0.9 ml. of glacial acetic acid was added to 0.1 ml. of each filtrate. Next, 60 mg. of xanthydrol was added, with warming, to dissolve this reagent. On being cooled to 0° C., the crystallized dixanthyl urethan derivative was collected (1), recrystallized twice from 50 per cent n-propanol, dried, weighed, and counted for radioactivity. This value was corrected for loss of urethan-C₁⁴ in the isolation procedure, in accordance with the carrier principle. The C₁⁴ concentration in liver proteins was determined, with isolation according to customary methods (17).

Cellular particulate fractions of liver and lung were prepared essentially as described by Hogeboom (9). About twelve mice were used to provide adequate material for this purpose. The nuclear and mitochondrial preparations from lung were less pure than corresponding liver preparations but were considered adequate for present purposes. The yield of lung mitochondria was markedly lower per gram of tissue than with liver. In studying distribution in particulate fractions, "non-volatile C₁⁴" represented the residual radioactivity after samples were dried on planchets and the latter had stood overnight in air at room temperature. "Non-dialyzable C₁⁴" corresponded to material which did not pass through cellophane membranes when preparations were dialyzed overnight in the cold against 0.85 M sucrose. This residual activity was measured by the previously mentioned "alkali-labile" method. In all experiments, replicate samples were employed in C₁⁴ determinations.

The data in the charts and tables are considered generally accurate to within approximately 5 per cent, except for the residual C₁⁴ values after dialysis and after volatilization. In these cases, owing to the relatively low radioactivities, the probable errors were larger, about 10–20 per cent. Because of the small magnitudes of these residual activities and the thoroughness of the processes employed, it was not considered worth while to prolong the treatments beyond 24 hours or to vary the conditions further.

RESULTS

The data in Chart 1 confirm the findings of others that administered urethan appears in approximately equal concentrations in different organs of the mouse. It may be seen that, initially, nearly all the C₁⁴ in each of the four tissues represented unmetabolized carbonyl-C₁⁴-urethan. The 6-hour liver had the most radioactivity in substances other than urethan. At 24 hours only very low concentrations of C₁⁴ could be detected in the tissues. The results with ethyl-C₁⁴-urethan (Chart 2) differ somewhat from those in Chart 1 in that, with increasing time, a greater proportion of the C₁⁴ from catabolism appeared in nonurethan forms and considerable radioactivity was retained after 24 hours. These observations agree with the report by Skipper and associates (16) that ethyl-C₁⁴-urethan is more extensively incorporated into various tissue components than is the carbonyl-labeled carbamate.

Table 1 shows that differences between total C₁⁴ and urethan-C₁⁴ in liver are partly accounted for by incorporation into proteins.

While there was no selective accumulation of
urethan in whole lung, as compared with other organs, it was of interest to examine the subcellular distribution of isotope in this tissue, with liver as a control. Table 2 gives the results of such a fractionation, following the administration of carbonyl-C\(^{14}\)-urethan to a group of mice. Measurement of total C\(^{14}\) by the oxidation method was not feasible, because of the presence of large amounts of sucrose. However, the "alkali-labile" values represent essentially urethan. The "non-volatile" and "non-dialyzable" figures largely reflect derived radioactive substances.

Of the alkali-labile C\(^{14}\) in the original homogenates, 85 per cent was found in the supernatant mitochondria/lung nuclei is about 3.5 by the alkali-labile method.

It was also of interest to determine the distribution of C\(^{14}\) in subcellular particulates following the \textit{in vitro} addition in the cold of labeled urethan to whole homogenates of liver and lung. The uptake of urethan under these conditions obviously represents a physical, rather than a metabolic, process.

The left half of Chart 3 shows that small but definite quantities of urethan-C\(^{14}\) were retained by the particulate preparations after the washing with sucrose solution. The greatest uptake (per gram original tissue) occurred with lung nuclei, and the least with the small quantity of lung mitochondrial.

**TABLE 2**

**DISTRIBUTION FROM LABELED URETHAN IN LIVER AND LUNG TISSUE**

Ten mice in each group; dosage, 25 mg. (7\textmu c.) urethan-carbonyl-C\(^{14}\)/mouse; sacrificed after 5.5 hours. The livers and lungs of each group were pooled prior to analysis.

Figures represent \mu moles C\(^{14}\)/gm original tissue.

<table>
<thead>
<tr>
<th>SUBSTANCES ANALYZED FOR</th>
<th>LIVER</th>
<th>LUNG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole homogenate</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Alkali-labile</td>
<td>24.0</td>
<td>1.05</td>
</tr>
<tr>
<td>Nonvolatile</td>
<td>3.35</td>
<td>0.54</td>
</tr>
<tr>
<td>Nondialyzable</td>
<td>0.90</td>
<td>0.09</td>
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</tbody>
</table>

* Microsomes + nonsedimentable fraction.

**TABLE 3**

**CONCENTRATION OF LABELED URETHAN IN SUBCELLULAR FRACTIONS OF LIVER AND LUNG**

The data are expressed as \mu moles C\(^{14}\)/gm protein of each subcellular fraction.

<table>
<thead>
<tr>
<th>SUBSTANCES ANALYZED FOR</th>
<th>LIVER</th>
<th>LUNG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole homogenate</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Alkali-labile</td>
<td>188</td>
<td>31</td>
</tr>
<tr>
<td>Nonvolatile</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>Nondialyzable</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

* Microsomes + nonsedimentable fraction.

fraction with liver and about 95 per cent with lung. While much of the nonvolatile and nondialyzable C\(^{14}\) was likewise in the nonsedimented phase, appreciable radioactivity was retained in nuclear and mitochondrial fractions.

To compare the results in terms of concentration of C\(^{14}\) in the fractions, the data of Table 2 were recalculated and expressed in relation to protein content, the latter being taken as an arbitrary criterion of the quantity of organic material in each fraction (Table 3). On this basis, the C\(^{14}\) concentration remained highest in the supernatant phase, but the levels of isotope in nuclei and mitochondria were relatively elevated. It is noteworthy that, while nuclei exceeded mitochondria in C\(^{14}\) concentration in the alkali-labile, nonvolatile, and nondialyzable analyses with liver, in the case of lung the reverse was true. Thus, the ratio for lung mitochondria/lung nuclei is about 3.5 by the alkali-labile method.
sorbed or had penetrated into nuclei and mitochondria. An extension of the in vivo technic to isolated particulate preparations made possible the measurement of urethan retention by comparable quantities of nuclear and mitochondrial material, following exposure to the same concentration of urethan. One additional procedure was employed, in which the particulate preparations were separated from the urethan solution, without subsequent washing, in order to estimate the capacity for uptake under conditions of relatively higher urethan concentration. Table 4 gives the results of two such independent experiments. It is seen that, of the preparations tested, only lung mitochondria were able to concentrate urethan. The elevated ratios in this case are actually minimal values, since considerable urethan solution of lower C14 concentration must have adhered to the mitochondria.

### DISCUSSION

Microsomes were not isolated or studied in the present work, and hence discussion must be restricted to the results with nuclei and mitochondria. In all experiments with these particulates from lung and liver, the mitochondria of lung were most active in taking up or retaining urethan. The resemblance of the in vitro results to those in vivo suggests, but does not prove, that the process is physical or physico-chemical in the intact animal. It is possible that urethan, like urea, combines with protein through hydrogen bond formation. However, mitochondria are also rich in other substances, notably ribonucleic acid, which may be able to bind urethan.

Neither the loose complexes of unchanged urethan with tissue products, on the one hand, nor the incorporation of CO2 derived from the total breakdown of urethan, on the other, can be compared with the protein binding behavior of carcinogenic hydrocarbons (8) or azo dyes (10). Whether there is in addition an intermediate metabolite of urethan, which binds specifically with proteins, cannot be decided from the results so far available.

While the present experiments with lung mitochondria might offer a clue to the mechanism of...
the carcinogenic action of urethan in mice, considerable further work is necessary before such assumptions can be made. Urethan is also taken up in vivo by lung nuclei, and the present research does not disprove the suggestion of Rogers (14) that deoxyribonucleic acid is the target of urethan action in the induction of pulmonary adenomas. However, to concentrate attention on this aspect, with the exclusion of the mechanisms involving the participation of mitochondria, would not seem justifiable according to our present results. Last, the possibility still remains that metabolites derived from urethan are the carcinogenic agents. This possibility is under further study in this laboratory.

SUMMARY

C14-labeled urethan, injected into mice, appeared in approximately equal concentrations in liver, lung, skin, and plasma. The C14 of the carbonyl-labeled compound was almost completely absent from these tissues after 24 hours, while, in the case of ethyl-C14-urethan, the isotopic carbon of the catabolized urethan was more extensively incorporated into tissue constituents.

Only a minor part of the urethan-C14 of liver and lung was found in nuclei and mitochondria. Of these particulates, lung mitochondria had the highest C14 concentration. The same result was obtained with in vitro experiments in the cold, involving the addition of labeled urethan to whole homogenates and to isolated particulate preparations.

The present results suggest the possibility that the induction of pulmonary tumors by urethan is related to the uptake of the carbamate by the lung mitochondria.

REFERENCES

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