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A TRACER STUDY OF THE METABOLISM OF *p*-IODOPHENYL URETHANE; THE SELECTIVE LOCALIZATION OF RADIOACTIVE MATERIAL

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The ability of certain urethanes (carbamates) to cause nuclear changes in dividing cells and inhibitory effects on the growth of some experimental tumours is well known. There is little knowledge of the mechanism whereby these effects are brought about. In view of the possibility that the active agent may be some breakdown product, studies of the metabolism and distribution of ethyl carbamate have been made in man, rat, and mouse ; in man and the rat by direct estimation in the body fluids and tissues (Boyland and Rhoden, 1949 ; Archer, Chapman, Rhoden, and Warren, 1948) and in the mouse by means of ethyl carbamate labelled in the carbonyl and methylene groups with radioactive carbon (Bryan, Skipper, and White, 1949 ; Skipper, Bennett, Bryan, White, Newton, and Simpson, 1951). The simple conclusions have emerged that ethyl carbamate is rapidly absorbed from the alimentary tract and distributes itself uniformly throughout the body fluids and tissues. It is rapidly broken down into carbon dioxide and ammonia (over 90 per cent in 24 hr.) without any evidence of selective localization in normal or tumour tissue.

No metabolic studies of aryl urethanes appear to have been carried out previously ; in the present work *p*-iodophenyl urethane was chosen as starting material because of the opportunity of introducing radio-iodine. The results obtained so far are interesting in that they provide a remarkable example of selective localization of organic material.

EXPERIMENTAL

(*a*) *Preparative.*—In earlier experiments labelled N-*p*-iodophenyl ethyl carbamate (*p*-iodophenyl urethane) was prepared from iodoaniline containing radioactive iodine by a process similar to that previously described by Basterfield, Woods, and Wright (1926). A method has been developed for the direct iodination of phenyl urethane based on the procedure described by Jurd (1949) for the iodination of acetanilide.

Preparation of iodine containing radioactive iodine.—A solution containing I^{131} (20–30 mc.) as iodide was added to a solution of KI (3.3 g.) in water (10 ml.). Dilute H_2SO_4 (10 ml. of a 10 per cent solution) was added slowly, followed by H_2O_2 (35 ml. of a 10 per cent solution). The flask was cooled in ice and after standing for at least 0.5 hr. the precipitated active iodine was filtered off at the pump, washed with water, and dried *in vacuo* over P_2O_5 for 5 hr. at room temperature. The subsequent iodination process was found to proceed much more slowly if the iodine was not thoroughly dried.

N-phenyl ethyl carbamate.—Aniline (45 ml.) in dry ether (100 ml.) was treated with ethyl chloroformate (26 ml.) in dry ether (50 ml.), added dropwise, with shaking and

cooling in ice. The precipitate of aniline hydrochloride was removed by filtration. Evaporation of the filtrate furnished an oil which solidified on cooling and was recrystallized from ethanol-water. Yield 29 g. (73%); m.p. 51° C.

Radio-iodination of N-phenyl ethyl carbamate.—N-phenyl ethyl carbamate (1.65 g.) was dissolved in dry ethanol (20 ml.) and HgO (1.6 g.) added. The mixture was contained in a round-bottomed flask provided with a stirrer. Iodine (2.5 g.) containing the radioactive element (see above) was added during the course of 10 min. after which vigorous stirring was continued for 1 hr. with cooling in ice-water. The mixture was left overnight at room temperature and then filtered. The residual mercuric iodide and oxide was washed with a little boiling ethanol and the combined filtrate and washings poured into water (100 ml.) containing a few drops of 10 per cent $Na_2S_2O_3$ solution. The precipitated iodo-compound was filtered at the pump and washed with thiosulphate solution and water. Two recrystallizations from ethanol-water gave the pure product as fine colourless needles, m.p. 114–115° C. Yield 2.4 g. (80%).

Preparation of radioactive p-*iodoaniline.*—The following method is based on that described by Brewster (1931) and gives superior results for a small-scale preparation. Dry, active iodine (approx. 2.5 g.) was dissolved in dry ethanol (10 ml.) and aniline (0.9 ml. A.R.) in ethanol (5 ml.) added. After standing for 0.5 hr. or more the mixture was poured into $NaHCO_3$ solution (100 ml. of 1.5 per cent), stirred well, and allowed to stand a further 0.5 hr. The dark precipitate containing iodoaniline was separated at the pump, washed with thiosulphate solution and water, and air-dried overnight. This solid was then extracted repeatedly with light petroleum (b.p. 40–60°) from which the product crystallized in colourless needles, m.p. 62–63° C. Yield 1.2 g. (55%).

(*b*) *Animal techniques.*—The distribution of radioactivity after administration of labelled N-*p*-iodophenyl ethyl carbamate and *p*-iodoaniline was studied in rats. Both substances were administered, either intraperitoneally or orally, to animals lightly anaesthetized with ether. The drugs were dissolved in olive oil, the dose volume being usually 4 ml. per kg. body weight, although alterations of this volume to 2 or 6 ml. did not appear to affect the results. Rats of a Wistar strain were used, in groups of five of the same age (100–150 g. weight). In each experiment zero hour was taken as the time of administration of the drug and all subsequent estimations of radioactivity were corrected for decay to this time. The specific activity of the drug at zero time was also estimated, so that, assuming the number of counts in a tissue to be proportional to the number of molecules of the drug present, its concentration could be determined. Animals were killed with ether, the thorax opened immediately, and the animals exsanguinated via the right auricle. Various organ specimens were then removed for assessment of radioactivity. After intraperitoneal injections the viscera were contaminated by the drug so that only blood, brain, and muscle were examined in these experiments. Care was taken to avoid cross contamination while dissecting the tissues; instruments were washed after removal of each piece of tissue and again after each carcass had been discarded.

(*c*) *Preparation of samples for estimation of radioactivity.*—*Blood.*—After collection the blood was citrated, 0.5 ml. diluted to 10 ml., and counted directly. The remainder of the blood was centrifuged. Aliquots of plasma were taken from each rat in the group, mixed, and 1 ml. of the combined plasma diluted to 10 ml. for counting. By centrifuging each blood sample separately haemolysis was considerably reduced.

Tissues.—Weighed samples were digested by the technique recommended by Howarth (1949), 2–3 ml. of LiOH suspension (10 per cent in 20 per cent ethanol) being used. We also added KI to 1 per cent; this iodide prevented losses of radioactivity which otherwise unexpectedly occurred. A similar loss has been mentioned by Free, Page, and Woollett (1951). It was convenient to leave the tissues overnight in this solution, by

which time digestion was advanced and completed with a few minutes' boiling. The contents of the tubes were again made up to 10 ml. for counting.

Urine.—The radioactivity in a suitable sample of combined urine and washings was first estimated . Ethanol (1 ml.) was then added to 10 ml. of this sample together with silver phosphate (ca. 100 mg.) and shaken for 2–3 min.; this procedure effectively removed any inorganic iodide. After filtration the radioactivity remaining was assessed and represented organically bound iodine.

Results and Discussion

p-Iodophenyl urethane, like phenyl urethane, possesses hypnotic or anaesthetic properties according to dose. Both substances are readily and almost completely absorbed from the alimentary tract or peritoneal cavity from olive oil solution. Their toxicities, however, are very different, which may be related in part to solubility, for the iodo-compound is much less soluble as the following figures show :

Iodophenyl urethane at 20° C.	Plasma, 1.7 mg./ml.
	Water, 0.085 mg./ml.
Phenyl urethane at 20° C.	Plasma, 2.19 mg./ml.
	Water, 0.62 mg./ml.

The onset of depression of the central nervous system is more rapid with phenyl urethane and less prolonged. In addition, it is associated with marked vasodilatation of the skin vessels ; the ears and tails become very pink. The iodophenyl urethane (in doses of 500 mg./kg. in oil, by the intraperitoneal route) produces light anaesthesia without capillary dilatation, associated with marked methaemoglobinaemia—an effect which is not observed with phenyl urethane. The appearance of these animals is similar to that observed after iodoaniline (see below). It will be seen from Table I that phenyl urethane is much more lethal than the substituted compound ; deaths with the former substance all occur within 24 hr., whereas the iodo-compound is lethal only after this time. As judged by the distribution of radioactivity

TABLE I

Drug	Dose (mg./kg.)	Percentage mortality 24 hr.	72 hr.
Phenyl urethane	250	0	0
	350	0	0
	500	60	60
	600	100	100
	750	100	100
p-Iodophenyl urethane	500	0	0
	750	0	20
	1,000	0	60
	1,250	0	80
p-Iodoaniline	250	0	0
	375	0	0
	500	0	60
	600	40	100

after oral administration, iodophenyl urethane (Fig. 1) undergoes a rapid metabolic transformation during the first 24 hr.; it is thus similar to ethyl carbamate except that 60 per cent of the radioactive material is excreted by the kidney during this time, whereas only a small percentage of ethyl carbamate escapes in this way (Boyland and Rhoden, 1949). Unlike ethyl carbamate, which is said to be uniformly distributed among tissues, iodophenyl urethane shows interesting tissue affinities. This is apparent

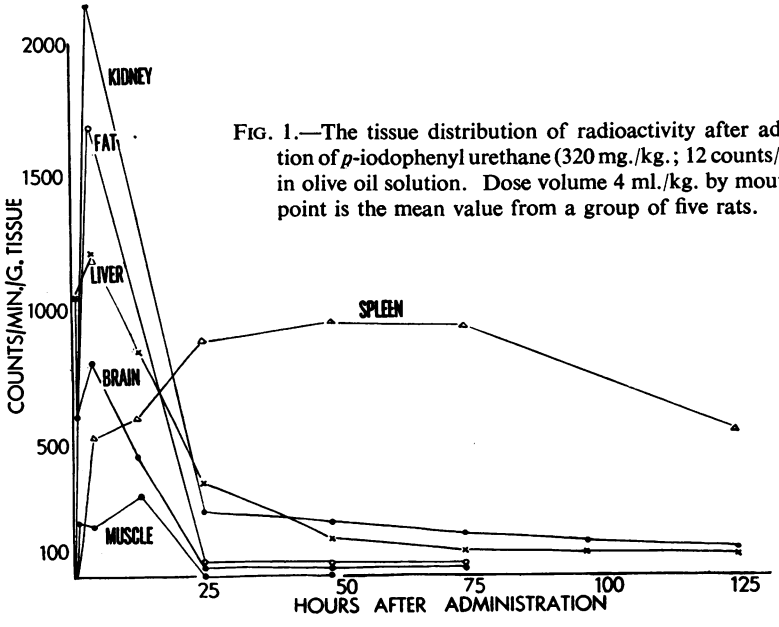

FIG. 1.—The tissue distribution of radioactivity after administration of *p*-iodophenyl urethane (320 mg./kg.; 12 counts/μg./min.) in olive oil solution. Dose volume 4 ml./kg. by mouth. Each point is the mean value from a group of five rats.

during the rapid metabolic phase referred to above and in its subsequent fate (Figs. 1–5 inclusive). It seems likely that the selective concentration exhibited during the 24 hr. phase is due to iodophenyl urethane itself. Of the major tissues kidney shows the highest transient radioactivity. Most of the radioactivity found in the urine is in organic combination, although some dehalogenation occurs (10 per cent of the ingested dose). In the main body tissues, the concentration ratios compared with plasma at the experimental peak of 4 hr. (oral dose, 320 mg./kg., Figs. 1 and 2) are as follows: Kidney 18, liver 10, spleen 4.2, fat 14, brain 6.4, muscle 1.4. The other tissues examined (lung, thymus, prostate, testis, and ovary) showed little uptake of radioactivity. The high concentration in fat (intra-abdominal or subcutaneous) was anticipated in view of the lipoid solubility of the compound and its probable absorption with fat; its rapid loss from the fat depots was rather unexpected. The brain concentration is associated with the period of drowsiness, although this effect is not so marked as when the drug is given by the intraperitoneal route.

It is in the blood and spleen, however, that the most interesting features occur. Whole blood shows a progressive uptake which does not follow the pattern of other tissues (Fig. 2), but reaches a maximum figure intermediate between that of kidney and fat, by which time, however, the activity of the other tissues referred to (spleen excepted) has declined to very low levels. Furthermore, the radioactivity

is almost entirely located in the cellular elements of the blood and persists for days, long after all significant radioactivity is cleared from the plasma and other tissues, so that the relative concentration effect is of a very high order. The level of radioactivity may be raised to any chosen level by suitably increasing the proportion of radioactive iodine in the drug administered. The plasma activity, even at its

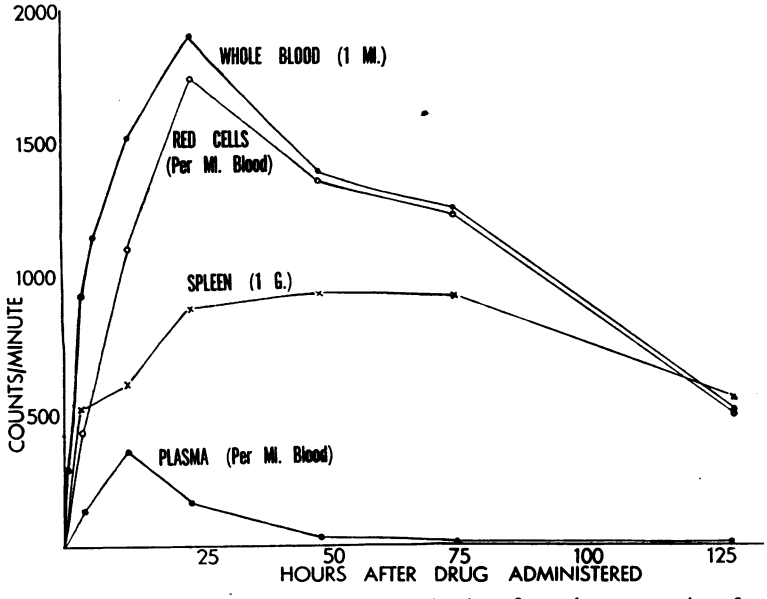

FIG. 2.—The localization of radioactivity in blood and spleen from the same series of experiments as in Fig. 1. In the blood almost all the radioactivity is in the cellular elements. Both blood cells and spleen show prolonged retention of activity.

maximum, is small, even though no correction is applied for haemolysis. The splenic activity parallels that of the blood but does not reach such high values ; it is probably related to the functions of the spleen in storing and destroying red cells. It is interesting that the liver does not share to the same degree in this activity distribution in spite of its participation in reticulo-endothelial function.

A similar experiment, although followed for a shorter time, was carried out using the intraperitoneal technique of administration (Fig. 3). By this route narcosis is much more marked, and its onset and duration is related to the gross brain uptake, which, at its peak, is five times higher than the level attained by oral administration of the same dose of drug. The animals used in this experiment carried an implanted tumour (the Walker carcinoma 256), and the relative concentrations compared to plasma at the time of maximum brain level were : brain 8.4, blood (cells) 3.4, and tumour 3.0. This emphasizes the affinity shown by brain tissue for the drug at this stage.

Once again the precipitous fall in tissue levels occurs, accompanied by the high corpuscular retention ; the red cell/plasma ratio after 24 hr. is 6 compared with 0.5 for the tumour/plasma and brain/plasma ratios. The amount of haemolysis in

the blood after centrifuging was greater after intraperitoneal injection of the drug and accounts for more of the plasma activity.

The next step was to ascertain whether the selective uptake occurred at a much lower dose level (60 mg./kg.). The specific activity of the starting material was necessarily raised for this purpose. The tissue picture was essentially the same (Figs. 4 and 5) except that the rapid metabolic phase was over in about one half the time required for the previous oral dose (Figs. 1 and 2) where the dose was five times greater. The plasma level was lower, and the activity concentration (plasma

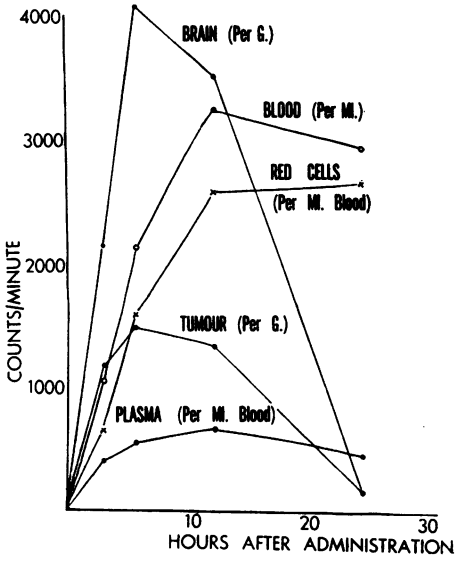

FIG. 3.—The distribution of radioactivity after administration of labelled *p*-iodophenyl urethane (320 mg./kg. in olive oil) by the intraperitoneal route, for comparison with Figs. 1 and 2.

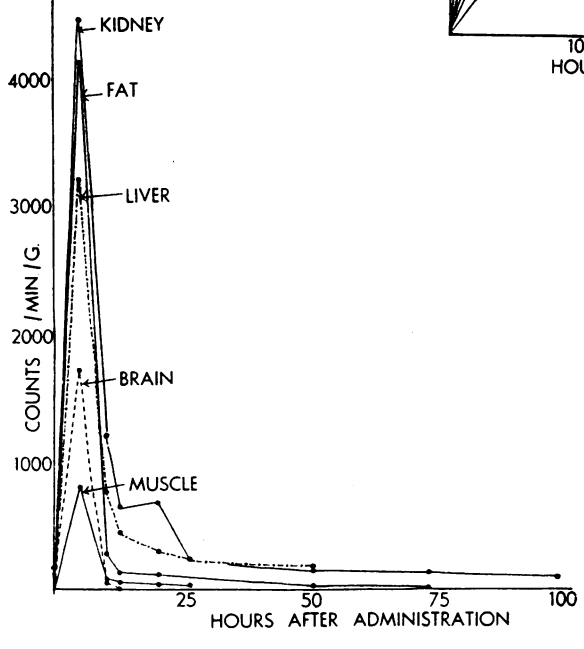

FIG. 4.—The mean tissue distribution of radioactivity after oral administration of a smaller dose of *p*-iodophenyl urethane (60 mg./kg. in olive oil). The more rapid turnover in tissues is apparent, although the activity levels are proportionately higher, because of the increased specific activity of the starting material (84 counts/μg./min.).

= 1) in corpuscles and spleen after 24 hr. was thus much higher (30 and 12.5 respectively). A curious feature of this series was the second peak of activity observed in the blood cells and spleen after 24 hr. (Fig. 5). The reality of this effect was confirmed by a subsequent experiment with freshly prepared material, but its significance is not known. After five days, in spite of almost complete clearance of plasma, the corpuscles still retained 1,400 counts per min., representing a content of

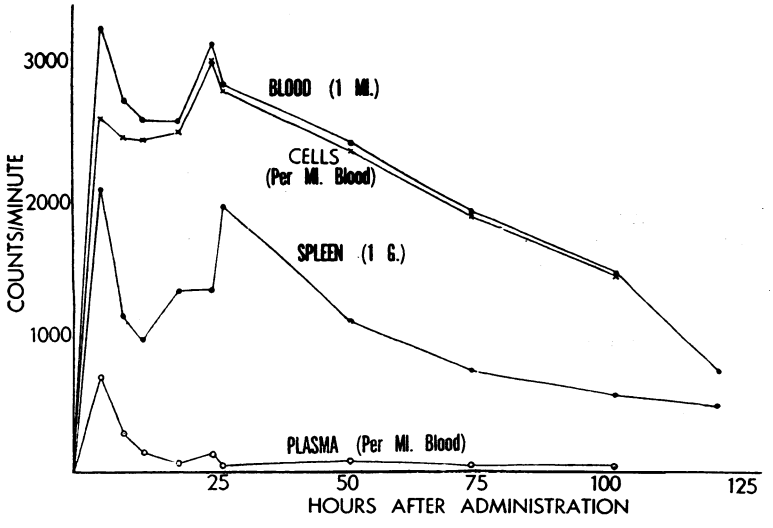

FIG. 5.—The same experiment as in Fig. 4 showing the blood and spleen pictures. Compared with Fig. 1, a proportionately greater quantity of material is localized in the red cells, although the dose is reduced to 1/5th. The amount of radioactivity present is a function of the specific activity of the starting material and may be increased at will.

about 18 μg. of original material per ml. of blood. This may be compared with a retention of 40 μg. after a dose of 320 mg./kg. by mouth after the same time interval (Figs. 1 and 2); the mean spleen content was 9 μg./g. tissue after the smaller dose and 40 μg. at the higher level.

It became clear from experiments carried out *in vitro* during the above studies that neither iodophenyl urethane nor phenyl urethane is selectively taken up from plasma solution by the red cells of man or the rat—only equilibration to approximately equal concentrations occurs. This suggested some metabolic product is the reactive substance with red cells. Hydrolysis such as occurs with ethyl carbamate seemed the most likely primary change. This suggested iodoaniline or aniline as possible metabolites, especially as aniline is well known to cause methaemoglobinaemia. However, we have not observed visible methaemoglobinaemia after phenyl urethane even in high dosage, whereas the iodinated compound produces methaemoglobinaemia in doses as low as 10 mg./kg. and this effect is marked with 50 mg./kg. This suggested that phenyl urethane, which has a rapid and intense, though short, action on the nervous system, is likewise rapidly excreted and insufficient aniline is produced by hydrolysis to cause methaemoglobinaemia. On the other hand, iodophenyl

urethane has a more prolonged effect and the iodoaniline formed is apparently a more effective agent for inducing methaemoglobinaemia.

The metabolic picture after oral administration of iodoaniline labelled with radio-iodine is shown in Fig. 6, which is on a similar activity scale to Figs. 4 and 5, and the dose chosen represents the iodoaniline equivalent of 60 mg./kg. of iodophenyl urethane. The overall appearance resembles that produced by the iodourethane ; especially is this so with the blood picture. In the tissues there is a rapid metabolic phase in the first 12 hr., but the relative concentrations of radioactivity

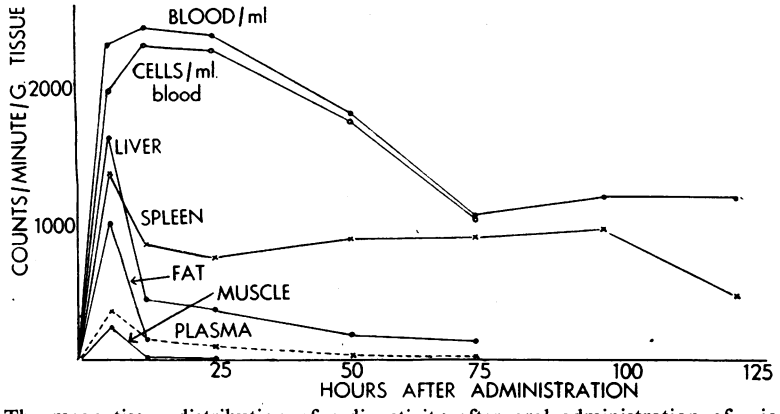

FIG. 6.—The mean tissue distribution of radioactivity after oral administration of *p*-iodoaniline (45 mg./kg. in olive oil; specific activity 89 counts/μg./min.). For comparison with Figs. 1, 2, 4, and 5. Note the general similarity of the curves in this figure to those from *p*-iodophenyl urethane, especially in regard to the blood. The fat uptake is considerably lower from iodoaniline than from the urethane.

are less intense than with the iodo-ester. Brain concentrates iodoaniline less than it does iodophenyl urethane ; both substances, however, may produce a sustained hypnosis. It is not possible to say at this stage whether iodoaniline is partly responsible for the central effects of the urethane. After intraperitoneal injection of iodoaniline the tissue distribution picture (Fig. 7) is again similar to that of the iodophenyl urethane (Fig. 3) except that the urethane produces a proportionately greater retention of material in the blood cells.

In previous work on the metabolism of acetanilide and related compounds in man and dog (Brodie and Axelrod, 1948) chemical estimations were made of the blood and tissue distribution of these substances. The authors suggest that aniline undergoes the following changes in the intact animal :

$$\text{Ph.NH}_2 \rightarrow \underset{\text{phenyl hydroxylamine}}{\text{Ph.NHOH}} \rightarrow \text{p-HO.C}_6\text{H}_4\text{NH}_2 \rightarrow \text{p-RO.C}_6\text{H}_4\text{NH.CO.CH}_3$$

It is suggested that phenyl hydroxylamine is the potent agent inducing methaemoglobin formation. A small dose of this substance (1 mg./kg.) intravenously in the dog resulted in the conversion of 45 per cent of its haemoglobin to the ferric pigment. We have found a dose of 5 mg./kg. intravenously in the rat produces immediate and intense methaemoglobinaemia (40 per cent after 10 min.) lasting for about an hour. After administration of radio-iodoaniline some 50 per cent of radioactive

material appears in the urine in 24 hr. of which most is in organic combination, which is neither unchanged material nor acetylated iodoaniline (compare the aniline picture illustrated above).

Various problems of interest have arisen out of this work on which we are endeavouring to throw light. Like aniline, iodoaniline is not taken up by the blood cells from plasma, thus confirming previous statements that some other site is the source of methaemoglobin inducing agents. The nature of the combination within

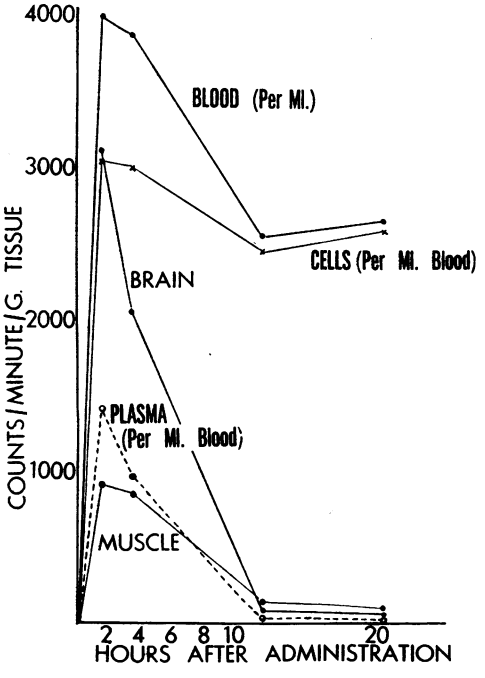

FIG. 7.—The mean distribution of radioactivity after intraperitoneal administration of *p*-iodoaniline (45 mg./kg. in olive oil; specific activity 89 counts/μg./min.). For comparison with Figs. 3 and 6.

the blood cell and its stability is particularly interesting since the methaemoglobinaemia is a transient phenomenon whereas the radioactivity persists long afterwards. Possible concentrations of radioactivity within bone marrow and leucocytes are being investigated. The effects of high radiation dosage administered in this way are being examined since it is possible to introduce literally any intensity of radioactivity desired into the blood cells and spleen with a very high degree of selectivity.

SUMMARY

1. The preparation of N-*p*-iodophenyl ethyl carbamate labelled with radio-iodine is described.

2. A preliminary examination has been made in rats of the tissue distribution of radioactivity after oral and intraperitoneal administration of this substance; a similar study has been carried out with labelled iodoaniline.

3. Both substances show an initial rapid metabolic phase during the first 24 hr. with transient concentration of varying extent in the major tissues—kidneys, brain,

and liver. No such effects were observed in other tissues, including muscle, lung, thymus, and gonads.

4. A remarkable concentration of radioactivity develops in the cellular elements of the blood—probably the red cells only—which is associated in its earlier stages with methaemoglobinaemia. However, this localization of radioactivity persists long after visible methaemoglobinaemia has disappeared ; the spleen shares in this activity, but on a somewhat smaller scale. Other tissues are then virtually free from radioactivity. This localization of activity occurs with both the substances referred to above.

5. It is likely that N-*p*-iodophenyl ethyl carbamate undergoes hydrolysis *in vivo* to produce iodoaniline ; this in turn is converted to another agent which presumably enters the erythrocyte and is fixed by some cellular component.

6. The metabolism of phenyl urethane is not accompanied by any visible methaemoglobinaemia even in sublethal doses.

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