Brit. J. Pharmacol. (1964), **23**, 285–294.

DISTRIBUTION OF (2-BROMOETHYL)ETHYL(NAPHTH-1-YLMETHYL-[^{14}C])AMINE HYDROBROMIDE IN THE RAT

BY

J. D. P. GRAHAM

From the Department of Pharmacology, Welsh National School of Medicine, Cardiff

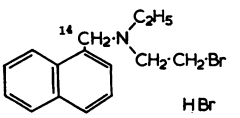
(*Received January* 13, 1964)

(2-Bromoethyl)ethyl(naphth-1-ylmethyl)amine hydrobromide (SY28) is a halogenoalkylamine related to dibenamine. A dose of 10 mg/kg injected intravenously into rats antagonizes the pressor response to adrenaline for 36 hr. If this amount of SY28 labelled with ^{14}C in the 1-methyl position is administered, specific radioactivity is present in blood and tissues many days after the antagonism of adrenaline is relieved. The ^{14}C is excreted in bile and in urine, but not in expired air. It is present in fat but not to a greater extent than it is in other tissues. It does not cross the placental barrier. There is no evidence that slow release from a lipid depot accounts for the long duration of action.

(2-Bromoethyl)ethyl(naphth-1-ylmethyl)amine hydrobromide, familiarly known as SY28, is a potent member of the 2-halogenoalkylamine series of drugs related to dibenamine and phenoxybenzamine. On intravenous injection it antagonizes the pressor response to adrenaline (Graham & Lewis, 1953) and to noradrenaline. Once fully established, the antagonism cannot be overcome by increasing the dose of adrenaline given. The duration of this effect varies with the dose administered: 1 mg/kg exerts it for approximately 6 hr and 10 mg/kg for approximately 48 hr in cats (Graham & Lewis, 1954). A fully effective dose of dibenamine in cats (20 mg/kg) lasts for 4 to 6 days. An equi-effective dose of dibenamine in the mouse is 40 mg/kg (Kuschinsky & Vorherr, 1958). The reason for the long duration of action of these compounds, as compared with substances such as phentolamine, has been a subject of controversy.

After an effective dose of dibenamine has been injected into the blood of a donor, it is not possible to block the tissues of a recipient cat by circulating donor blood through them at times when the donor is still blocked (Graham & Lewis, 1954; Nickerson & Goodman, 1948). Sodium thiosulphate inactivates ethyleneiminium ion, the active chemical species formed by these compounds (Graham, 1957) and, if injected intravenously before administration, it will prevent an otherwise active compound from blocking. If, however, blockade is established, thiosulphate will not reverse it or reduce its duration. It might be concluded from these and similar observations that the hypothesis originally advanced by Nickerson & Goodman (1948) to the effect that dibenamine acts by prolonged or irreversible interaction with a tissue constituent is probably correct. Nevertheless, once Axelrod, Aronow & Brodie (1952) and Brodie, Aronow & Axelrod (1954) had shown that

dibenamine and phenoxybenzamine are to be found in the fat of dogs after injection into the blood, and that substantial quantities are still there 24 hr later, it was logical to suggest that the long duration of action might be due to continuing release of active compound from this depot, despite the failure to modify the block, once established, with thiosulphate, or to demonstrate the presence of active compound in cross-circulating blood. Intravenous injection of 20 mg/kg of [^{14}C]-phenoxybenzamine into the dog demonstrated that total clearance from the blood took 4 to 5 days and that the pressor response to injected adrenaline was gradually restored as the detectable plasma levels of ^{14}C fell. Horowitz & Nickerson (1954) injected mice with 5 mg/kg of [^{14}C]-phenoxybenzamine intravenously and found radioactivity in kidney and liver 24 hr later, but failed to demonstrate accumulation in body fat. None was detected in expired carbon dioxide sampled at 24 hr. The report is brief and lacking in detail.



[^{14}C]-SY28

The present work was undertaken in order to gain a more complete picture of the distribution and clearance of SY28, an active dibenamine-like compound labelled with ^{14}C in the 1-methyl position, in an attempt to throw light on the part played by storage in fat depots on the duration of action of this compound.

METHODS

The [^{14}C]-SY28 provided had a specific activity of 4 μC/mg (for preparative details see appendix), and it was used to examine its distribution with time in the tissues of rats injected intravenously with a standard dose of 10 mg/kg in 1 ml./kg of solvent (acid ethanol, 0.1 ml., to which was added Tyrode solution, 0.9 ml.).

Unlabelled SY28: onset and duration of effect. Groups of five atropinized spinal rats injected with 5 mg/kg of hexamethonium bromide were used to determine the delay in onset and the duration of the effective antagonism to the pressor action of 0.5 μg/kg of noradrenaline produced by 1 and 10 mg/kg of unlabelled SY28. A dose of 10 mg/kg in a volume of 0.5 ml. was not injected in less than 1 min because of acute toxic effects. For a dose of 1 mg/kg male rats were prepared, the response to 0.5 μg/kg of noradrenaline was recorded, SY28 was injected intravenously and the administration of noradrenaline was repeated every 15 min until an unequivocal pressor response was obtained once more. For the 10 mg/kg dose, male rats were atropinized, rendered docile by inhalation of ether, and the SY28 injected into the vein on the dorsum penis. They were again atropinized and prepared as spinal rats after 24, 30, 36 and 48 hr, and 10 days (twenty-five rats), and the effect of the pressor amines was examined every 15 min for 4 hr. At 1 min after the finish of the injection blockade had not been effected. At 5 to 10 min it had, after 15 min it was fully established in the rat, cat or dog. The duration of blockade was approximately 6 hr for the former dose, and 36 hr for the latter in the rat.

^{14}C-labelled SY28. As a result of the tests described above, male rats were taken in groups of three, rendered docile by inhalation of ether, and freshly dissolved [^{14}C]-SY28 in a dose of 10 mg/kg was injected into the vein on the dorsum penis. Various procedures were adopted to collect specimens. Usually rats were kept in individual metabolism cages. At appropriate times they were narcotized with ether, blood was collected and then they were decapitated and dissected, and the organs were placed at $-12°$ C in individual plastic bags duly labelled. Three

pregnant (14 day) rats were anaesthetized, an external jugular vein was cannulated, [^{14}C]-SY28 (5 mg/kg) was injected and the rats were used for collection of expired air. After killing, foetuses were extracted as free as possible from contamination with maternal fluids.

Further procedures were as follows.

Blood. In three unanaesthetized rats 20 μl. of blood were collected from a free-flowing incision at the tip of the tail, at intervals of 1 and 5 min, 1, 8 and 24 hr, 2, 3, 4, 6 and 10 days after injection of 1 μC of SY28. The blood was transferred to 1.0 ml. of distilled water, well mixed and three 0.1 ml. aliquots were each added to 5 ml. of phosphor for counting. Blood from rats injected with saline was used as a control. When the rats were killed, 0.5 ml. of blood was collected by cardiac puncture into a syringe wetted with heparin solution, and was added to 9.5 ml. of distilled water, mixed, frozen at $-12°$ C, and after thawing three 0.1 ml. aliquots were used for counting. To determine the distribution of 1 μC of activity in blood, four rats were injected and two were bled after 1 min, and two after 10 days; one anaesthetized rat was injected with 5 mg/kg of [^{14}C]-SY28 and 2 ml. of blood was collected from an intra-arterial cannula after 1 and 15 min and 4 hr. Diluted, lysed whole blood, washed red cells, subsequently lysed by dilution and freezing, and plasma were separately examined after the haematocrit and haemoglobin values had been taken. Plasma proteins were precipitated with 0.08 ml. of 50% trichloroacetic acid/ml. and centrifuged, and the aqueous supernatant fluid was examined.

Urine. This was collected from the bladder of each rat at death by syringe and needle, and stored in vials at $-12°$ C. In rats which survived for longer periods after injection of [^{14}C]-SY28, urine was collected from the metabolic cages into ice-packed vessels, filtered, and stored in plastic bottles at $-12°$ C. Water was allowed *ad libitum* but feeding was restricted to periods of 8 hr at night. After the total volume had been measured, three aliquots of 0.1 ml. were used for counting, and compared with the urine of uninjected rats.

Bile. In two male rats (250 g. anaesthetized with pentobarbitone sodium, 20 mg/kg injected intravenously) the common bile duct was cannulated, the tip of the cannula being tied near the liver. Bile was collected for 1 hr as a control, and 1.0 mg/kg of [^{14}C]-SY28 was injected intravenously. After an interval of 30 min bile was collected for 2 hr, the volume was measured and samples of 0.1 ml. were added to 5 ml. of phosphor for counting.

Anterior chamber fluid (eye). When injected rats had been killed, the fluid from the anterior chambers of both eyes was collected by needle and syringe, and the whole was washed into 5 ml. of phosphor. The eye was then enucleated, opened and immersed temporarily in the phosphor which was stirred before counting.

Expired air (carbon dioxide). Air was sucked through a 40% solution of sodium hydroxide, a water trap, and then an airtight chamber into which an injected rat could be placed. From here it passed through sintered tubes through three traps each containing 25 ml. of a freshly-prepared molar solution of methylbenzethonium (Hyamine 10X) hydroxide in methanol. Collection was continued for 3 hr. Aliquots of 0.2 ml. were added to 5 ml. of phosphor for counting. An uninjected rat was used as control.

Tissues. After an animal was killed it was rapidly dissected, the organs were rinsed in saline, dried on blotting paper and stored at $-12°$ C. Each organ was subsequently weighed and portions totalling 1 g were taken. These were frozen by immersion in liquid nitrogen, and pulverized, then transferred as a cake to a glass Potter's homogenizer, thawed and emulsified with water. The fresh homogenate (0.1 ml.) was then transferred to 5 ml. of phosphor for counting. This procedure gave satisfactory homogenates of liver, kidney, spleen, skeletal and heart muscle, and aorta. Emission rates took 15 min to become stable. Specimens were collected from the panniculus, perirenal and omental fat in a stereotyped manner. From each rat 1 g of fat was made up of approximately equal amounts from these samples and dissolved in 10 ml. of a 10% (v/v) methanol-chloroform mixture, and 0.1 ml. of the solution was added to 5 ml. of toluene phosphor for counting. SY28 and the fat containing it are readily soluble in methanol-chloroform, which mixes homogeneously with dioxan or toluene. A phosphor of the latter was preferred, as the extinction coefficient is much less than with dioxan when a

solution of this fat is added. Brain was treated in the same way. After removal of the intestine, it was divided into three sections—upper and lower small intestine, and large intestine. Each section of small gut was washed through with 2 ml. of water, the washings were centrifuged, and aliquots of 0.1 were counted. Formed faeces was discarded, and the semi-solid content of the caecum was homogenized with 5 ml. of water and aliquots were used for counting.

Radioactivity determinations. Scintillation counting by using liquid phosphors was performed at 10° C, as described previously (Graham & Nicholls, 1959). Two samples were pipetted from each preparation and were counted repeatedly until consecutive figures agreed closely. Fluids such as urine became stable within 5 min, homogenates of tissue required 15 min. Specific activity is reported as a percentage of the initial values in counts/min/ml. or in counts/min/mg wet weight; occasional reference is made to a percentage of the initial total activity injected, derived from the specific activity resulting from addition of known amounts of labelled compound to preparations of tissues or fluids.

RESULTS

Distribution

Blood. After intravenous injection of [^{14}C]-SY28 (10 mg/kg) the specific activity of whole blood declines at a rate such that the half-clearance time is about 36 hr. The curve of clearance (taken from groups of rats) is shown in Fig. 1, and the

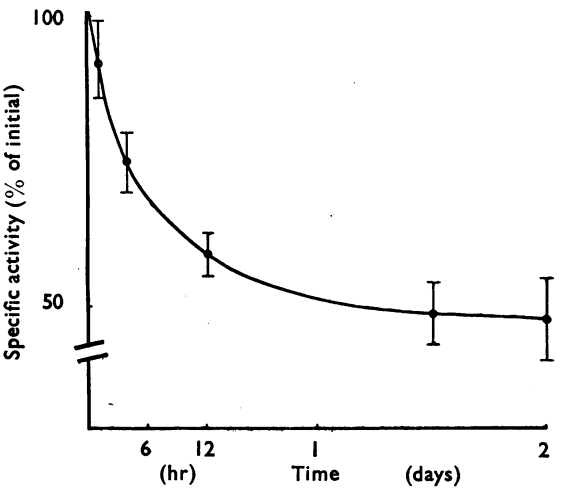


Fig. 1. The specific activity in whole blood of groups of three rats measured at intervals after injection of 10 mg/kg (40 μC/kg) of [^{14}C]-SY28, expressed as a percentage of the initial (1 min) level. The scale is linear, the abscissa truncated for convenience.

clearance in three rats studied for 10 days is displayed in Table 1. The rate of clearance declines as if exponentially at first and then more slowly, as if different mechanisms were involved. The adrenaline antagonism at this dosage fails at 36 to 48 hr. There is therefore about half the original radioactivity present in whole blood after blockade has ceased. The plasma—cell distribution of specific activity in the blood of rats has been studied, and varies as shown in Fig. 2. Initially the ^{14}C-label is mainly located in the plasma (80 to 90% of it) and not in

TABLE 1

CLEARANCE OF ^{14}C-LABEL FROM THE BLOOD OF UNANAESTHETIZED RATS

The specific activity was measured in blood samples taken from the tip of the tail at the intervals of time stated. Values are means of three

Time	Approximate % of initial level
5 min	100
1 hr	75
8 hr	62
1 day	52
2 days	49
3 days	42
4 days	38
6 days	38
10 days	25

the cells. It is largely attached to the protein which is precipitable with trichloracetic acid (84% of the total activity in plasma). The albumin fraction has been shown by electrophoresis to be the binding site preferred (Graham, 1960). Apart from passing out of the blood so that the total amount is reduced to approximately 70% of the initial level in 4 hr this distribution is altered. The percentage of the activity in the whole blood which is found in washed red cells rises from 16% at 1 min to approximately 77% of the total amount present during the period of total

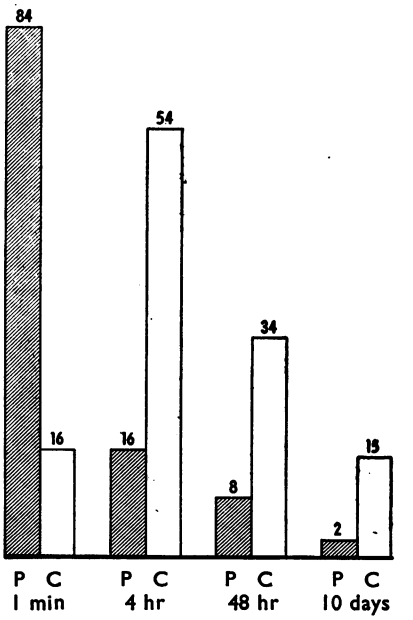


Fig. 2. Levels of radioactivity in plasma, and washed red cells of rat blood, expressed as percentages of the initial whole-blood count. The percentage values are given above, the times of sampling below. P = plasma ; C = red blood cells.

blockade (54% of the initial amount) and declines slowly over a period of 10 days, after adrenaline antagonism is relieved ; the percentage of the total which is found initially in plasma (84%) declines rapidly (23% of the total present after 4 hr, 16% of the initial amount) and levels out (19% of the total present, 8% of the initial amount, at 2 days ; 12% of the total present, 2% of the initial amount, at 10 days) ; some remains for many days after pharmacological activity is lost. The fraction of injected drug represented by this activity may not be involved in the initial active blocking process in tissue cells. It follows that the ready supply of large protein molecules provided by the plasma albumin initially and rapidly binds most of the activity. Binding to red blood cells is a slower but longer lasting process. Subsequently specific activity leaves the blood, more rapidly from plasma than from red cells so that the proportion in the latter becomes relatively high. There is therefore a firmer binding site in red cells than in plasma albumin. At the time when blockade is lifted (48 hr), the red cells contain approximately 60% of the activity which they contained when it was newly established (4 hr). The proportion of the total plasma content in the watery fraction rises from 16% at 1 min, to 30% at 15 min and at 4 hr, which indicates a passage from protein to water.

Anterior chamber fluid. This was found to contain specific activity within 1 min of intravenous injection in the relatively high concentration of 4 μg of compound per ml. of fluid. Within 15 min this value had fallen to half, and persisted at 6% of its initial level for 48 hr.

Other tissues. The distribution with time in fat, brain, liver, kidney, skeletal muscles, spleen, and heart is shown in Table 2. Peak concentrations were achieved immediately in spleen and brain, both of which contain much blood, and 15 min

TABLE 2

SPECIFIC ACTIVITY OF RATS AS A PERCENTAGE OF THE MAXIMUM FOUND AFTER INTRAVENOUS INJECTION OF 10 MG/KG OF [^{14}C]-SY28

Activities per g of wet tissue except for anterior chamber fluid which is total activity

	Time after injection					
Tissue	1 min	15 min	4 hr	36 hr	48 hr	10 days
Fat	11	100	82	28	19	25
Brain	100	57	54	43	47	22
Liver	79	100	44	15·6	7	4
Kidney	85	100	59	40	40	34
Muscle	19	42	100	28	27	8
Spleen	100	80	10	—	11	1·3
Heart	94	100	52	42	48	43
Anterior chamber fluid	100	45	40	7	6	0·2

after injection with most other tissues. Activity was retained to a varying extent. Heart muscle showed firm binding, skeletal muscle did not. Spleen did not retain activity, kidney did. The renal activity was not related closely to urinary or blood concentration and must be a function of renal tissue. The 14-day foetus does not

contain any ^{14}C-activity at 3 hr after injection into the maternal blood. The values obtained from aorta freed from blood were consistently low and of doubtful significance after the initial reading.

The concentrations of specific activity in counts/min/mg wet weight show the relative distribution in different organs at different times (Table 3).

TABLE 3

VALUES OF ^{14}C-ACTIVITY PRESENT AT 4 HR AND 2 AND 10 DAYS.

Each value is the mean of two counts on two samples from three rats, expressed in counts/min/mg of wet tissue, except for blood which is in counts/min/μl.

	Tissue							
Time	Blood	Fat	Heart	Muscle	Kidney	Liver	Spleen	Aorta
4 hr	40	20	34	60	35	43	27	5
2 days	24	5	32	16	24	7	30	3
10 days	15	6	28	5	20	4	4	0

Excretion

Urine. ^{14}C-Activity appears in the urine within a few minutes of intravenous injection but only in traces (much less than 1 μg/kg after 15 min with an original injection of 10 mg/kg of compound). The rate of excretion continues low for 4 to 8 hr and may be related to the oliguria secondary to hypotension, which lasts for some hours after a large dose. The concentration of ^{14}C-activity is low and the total output in 4 to 8 hr is still less than 1 μg/kg. The rat recovers some of its vigour after this and the rate of excretion rises. After 36 to 48 hr at which time the adrenaline antagonism is lifting or is gone and the rat appears to be normal, some 25 to 33% of the initial dose has been excreted, the rate of clearance is high and the concentration in the urine has risen fourteenfold, in spite of the diuresis. Urinary loss thereafter drops to a low level at which it stays, so that at the end of 10 days only 2 to 3% more has been excreted.

Bile. The mean volume of bile secreted during the 2 hr subsequent to injection of [^{14}C]-SY28 was 3.7 ml. A small dose of compound was administered in order to avoid undue hypotension. Bile sampled 30 min later contained radioactivity and approximately 5% of the total injected appeared in the bile during these 2 hr. The chemical nature of the excreted material is not known.

The washed content of the upper small intestine contains this specific activity in apparently higher concentration (counts/min/ml. of wash) than does bile. The upper small gut contains more than the ileum, which contains much more than the caecum. The activity may be excreted in pancreatic juice as well as in bile, and must be largely reabsorbed in the jejunum.

Expired air. This, when passed through methylbenzethonium hydroxide, gives up 95% of its carbon dioxide under favourable circumstances. No evidence of radioactivity was found in the samples taken after 3 hr of collection, and it is concluded that none is excreted by this route.

DISCUSSION

These distribution studies with the ^{14}C-labelled compound are of limited value, but certain points emerge. There are several stages in clearance from blood into which has been injected a freshly prepared solution (consisting largely of unreacted parent compound and some ion): (1) rapid binding to plasma protein in competition with passage into cells; (2) after renal function is resumed an exponential pattern of clearance, largely into the urine and bile; (3) slow and insignificant loss which may come from plasma protein and from cells. Specific activity accumulates quickly in fat (see Table 2) but the concentrations found after 4 hr in fat are less than those in blood, whereas the concentrations in skeletal muscle are higher (Table 3). In view of its bulk and the high concentration in it, the skeletal muscle must contain the major portion of the activity at the time of stable adrenaline antagonism (4 hr) in the rat. This muscle does not hold its specific activity content as does cardiac muscle (27% of the peak initial concentration as against 48% at 48 hr), and it may be transference from muscle rather than from fat which maintains the blood level. At 36 to 48 hr, which is the time of pharmacological recovery with the dose given, the concentration in blood remains higher than that in fat. Radioactivity or pharmacological activity could not pass from fat to tissues via blood at this ime, as postulated by Brodie *et al.* (1954), unless there is a transport mechanism. The findings do not support the contention (Axelrod *et al.*, 1952; Brodie *et al.*, 1954), based largely on experiments with dibenamine, that storage in fat plays a significant part in prolonging the activity of typical halogenoalkylamine antiadrenaline compounds, but it must be realized that the rate of reactivity of dibenamine in various solvents is much slower and less complete than that of SY28. There can be no doubt that a high percentage of SY28 specific activity is firmly bound to various tissues, probably to protein, at a time greater than that needed for recovery from pharmacological blockade. The prolonged presence of specific activity in a variety of muscular tissues supports the contention (Nickerson & Goodman, 1948; Nickerson, 1962) that this type of compound exerts its action by a firm but reversible union with the site of pharmacological action (presumably the α-receptor for catechol amines). It also indicates that much of the binding is to nonspecific sites and more prolonged than this. Some of the radioactivity detected in watery tissues is doubtless due to the presence of transformation products, the alcohol among others. In 2:1 (v/v) acetone—water mixture SY28 is converted very rapidly to the ethyleneiminium ion. After 30 min, the level of this ion as measured by thiol consumption declines to 25% of the amount found at 1 min. The effect of protein and other body constituents on the reactivity and the amount and proportion of products formed is not known. The proportion of the compound injected which exerts the pharmacological action must be very small. The liver rapidly acquires a high concentration and actively excretes it in bile, from which it is largely reabsorbed in the small intestine. None of the carbon label appears in expired air; total degradation cannot, therefore, have taken place and ultimate clearance is probably in the urine and faeces. The aorta is a very different structure from the arteriole but it is unexpected that so little activity can be extracted from it, and it may be that only autoradiographic studies will reveal

whether or not significant amounts are present in arterioles after pharmacological blockade is raised.

CHEMICAL APPENDIX

BY

K. CLARKE

From the Department of Chemistry, University of Hull

Synthesis of (2-bromoethyl)ethyl(naphth-1-ylmethyl)amine hydrobromide, labelled with ^{14}C in the 1-methyl position

Naphth-1-ylmethanol, $C_{10}H_7.^{14}CH_2OH$. Acetyl chloride (2 to 3 drops) was added to a solution of naphth-1-oic acid (carboxyl-[^{14}C], 100 mg) in absolute methanol (10 ml.). After 48 hr the solution was evaporated to dryness under reduced pressure to give the methyl ester as a white crystalline residue, melting point 106° C. The ester in dry ether (10 ml.) was added to a solution of lithium aluminium hydride (75 mg) in dry ether. The mixture was boiled for 60 min, then treated with water and the product was filtered through Celite, and shaken with ether. The ethereal solution was dried with sodium sulphate and evaporated to give a colourless oil. Yield, about 95%.

Naphth-1-ylmethyl chloride, $C_{10}H_7.^{14}CH_2Cl$. Dry pyridine (0.025 ml.) and freshly distilled thionyl chloride (0.075 ml.) were added to a solution of the above oil in anhydrous ether (10 ml.). The mixture was boiled under reflux for 60 min, decomposed with water and shaken with ether. The ethereal solution was washed with sodium bicarbonate solution, then with water, and dried with sodium sulphate. Evaporation gave a colourless oil which slowly solidified. Yield, about 85%.

(2-Hydroxyethyl)ethyl(naphth-1-ylmethyl)amine, $C_{10}H_7.^{14}CH_2.NEt.CH_2.CH_2OH$. The naphth-1-ylmethyl chloride, *N*-ethylethanolamine (0.07 ml.) and anhydrous potassium carbonate (0.2 g) in butan-2-one (40 ml.) were boiled together for 4 hr. Water was added and the product was shaken with ether. The ethereal solution was dried (with potassium carbonate) and was evaporated under reduced pressure and the resulting oil was carried forward to the next stage. Yield, about 90%.

(2-Bromoethyl)ethyl(naphth-1-ylmethyl)amine hydrobromide, $C_{10}H_7.^{14}CH_2NEt.CH_2.CH_2Br$, HBr. The aminoalcohol, redistilled phosphorus tribromide (0.09 ml.) and dry chloroform (8 ml.) were boiled under reflux for 7 hr. The solvent and any volatile phosphorus compounds were removed by distillation under reduced pressure. The residue was treated with boiling absolute ethanol (4 ml. twice) and the combined extracts were filtered through Celite. Anhydrous ether was slowly added until a fine flocculent precipitate formed. This was filtered off (through Celite) and more ether was added until the solution became cloudy. The solution was kept in a refrigerator overnight when it deposited white crystals (85 mg) of the required halogenoamine, melting point 161 to 164° C. Yield, about 72%. Overall yield, about 54%.

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