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THE SELECTIVE LOCALIZATION OF *p*-RADIOIODOPHENYLHYDROXYLAMINE IN RED CELLS: ITS RELATION TO METHAEMOGLOBINAEMIA

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A recent communication reported the selective localization of radioactive material in the red cells and spleen of the rat after administration of *p*-iodophenylurethane and *p*-iodoaniline labelled with radioactive iodine (Crick and Jackson, 1952). From this work it was apparent that a common metabolic product was responsible for the localizing effect. Accordingly, *p*-iodophenylhydroxylamine (IC_6H_4NHOH) and *p*-iodonitrosobenzene (IC_6H_4NO) have been prepared in radioactive form and their distribution in rat tissues examined. The results presented in this paper show that these two substances possess a very high affinity for erythrocytes by virtue of their reaction with haemoglobin to form a stable complex, and either or both are likely metabolic products of iodophenylurethane and iodoaniline in the rat.

METHODS

Preparative.—Bamberger (1895) appears to have been the first to prepare *p*-iodophenylhydroxylamine although no details were given. Lapworth and Haworth (1921) prepared the same compound by sulphide reduction of *p*-iodonitrobenzene and recorded the same melting point as the previous author (104–105° C.). We have found reduction of iodonitrobenzene with an aluminium-mercury couple in moist ether to be a superior and convenient method; in numerous preparations of iodophenylhydroxylamine by this method the melting point of the pure compound has been consistently 95–96° C. (decomp.).

Preparation of p-*Iodonitrobenzene Labelled with Radio-iodine*

p-Nitroaniline (2 g.) was dissolved in hydrochloric acid (8 ml., sp. gr. 1.1) cooled in ice-water and diazotized with sodium nitrite (1.07 g.) in water (5 ml.). The solution was transferred to a beaker, and, with continued cooling, potassium iodide (2.4 g. in 5 ml. of water containing a suitable quantity of radioactive iodide) was added in the course of a few minutes. The dark brown mixture was allowed to stand for 1.5 hours at room temperature when the brown solid which had separated was filtered off and washed successively with water, potassium iodide (5%), sodium thiosulphate (5%), and water. After drying for 1 hour *in vacuo* at 100° C. the product was treated with charcoal in chloroform solution and crystallized by addition of alcohol. Yield, 2.0 g., m.p. 166–167° C.

Preparation of Radioactive p-*Iodophenylhydroxylamine.*—*p*-Iodonitrobenzene (1 g.) suspended in anaesthetic ether (50 ml.) was added to a freshly prepared aluminium-mercury couple (from 1 g. of aluminium foil) and the solution boiled for 40 minutes. The mixture was filtered into a round-bottomed flask (250 ml.), a little porous pot added, and the solvent rapidly removed *in vacuo* by direct connection to a water pump. The flask may be immersed in *warm* water to aid the process. The solid residue in the flask was left *in vacuo* for a further 15 minutes at room temperature and recrystallized from benzene or benzene-light petroleum (b.p. 40–60° C.), from which the iodophenylhydroxylamine separates in very light, colourless plates m.p. 95–96° C. (decomp.). Yield 0.4 g.

Preparation of Radioactive p-*Iodonitrosobenzene.*—*p*-Iodonitrosobenzene was originally prepared by Bamberger (1895) from *p*-iodophenylhydroxylamine by chromic oxidation and steam distillation; in our hands this method yielded poor results. The following procedure is simple and particularly suited to the preparation of the radioactive compound. *p*-Iodophenylhydroxylamine (0.25 g.) was dissolved in dry, anaesthetic ether (20 ml.), a mixture of dry silver oxide (prepared from hot silver nitrate solution with less than the equivalent requirement of barium hydroxide solution) and anhydrous magnesium sulphate (1 g. of each substance) added, and the mixture allowed to stand for 30 minutes with occasional shaking. An emerald green colour rapidly developed. After filtration the solvent was removed and the iodonitrosobenzene separated from a high-melting product (m.p. 195° C.) by crystallization from light petroleum. It forms green needles, m.p. 97–98° C. Yield, 0.15 g. The volatility of this compound necessitates especial care in handling the radioactive material.

In aqueous alcohol *p*-iodophenylhydroxylamine rapidly changes to 4:4′-di-iodoazoxybenzene m.p. 195° C., which is also produced in the preparation of the iodonitrosobenzene referred to above. Both iodophenylhydroxylamine and iodonitrosobenzene slowly undergo the same change at room temperature and should be kept at −20° C.

Animal Techniques and Measurement of Radioactivity.—For intravenous administration the iodophenylhydroxylamine was dissolved in a minimal quantity of ethanol, 2–3 volumes of water were added with shaking, and the solution used immediately. For intraperitoneal administration freshly prepared solutions in olive oil were used. The iodonitrosobenzene is insoluble in water and alcohol and appeared to decompose in olive oil. Its solubility in liquid paraffin was adequate for intraperitoneal use. The other techniques used have been previously described (Crick and Jackson, 1952).

RESULTS

p-Iodophenylhydroxylamine (IC_6H_4NHOH).—This substance, like phenylhydroxylamine, causes immediate and intense methaemoglobin formation when added to blood or washed red cells or injected intravenously into rats. The blood and tissue distributions of radioactivity produced by a single intravenous injection are shown in Fig. 1. There is a very rapid and specific localization of radioactivity (about 50% of the dose administered) in the erythrocytes and, with the exception of the spleen, no significant retention in other tissues. No appreciable fall in the level of activity in the blood occurs at this dose level during 24 hours after administration of the substance. A similar result is obtained after intraperitoneal injection (10 mg./kg. in olive oil), for in spite of the insolubility of the compound in water the effects appear very rapidly (Fig. 2); in a few minutes the animals pass into a state of collapse with severe methaemoglobinaemia. The duration of the latter is surprisingly short, visible methaemoglobinaemia having disappeared in about 2 hours, with complete recovery of the animals. In spite of this, the radioactive indicator shows that the drug is still present in some form within the red cells (Fig. 2).

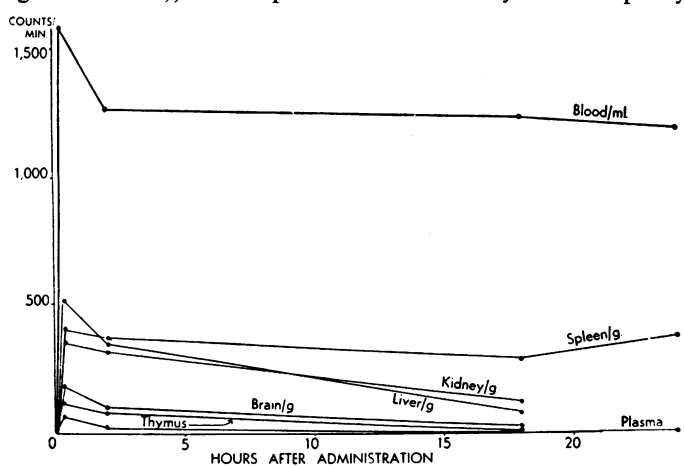


FIG. 1.—The distribution of radioactive material after intravenous administration of *p*-iodophenylhydroxylamine (5 mg./kg., specific activity 29 counts/μg./min.). Each point represents the mean value from a group of five rats.

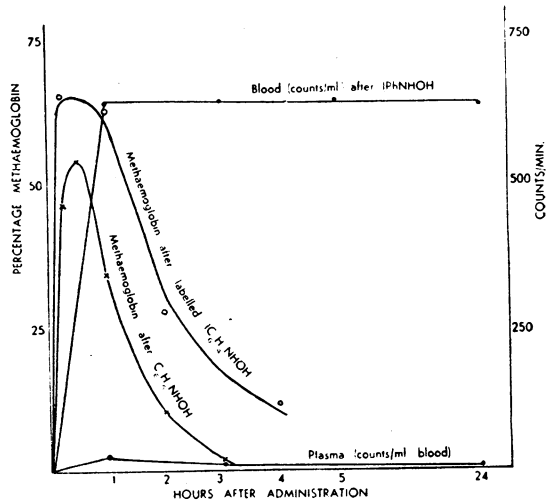


FIG. 2.—The intraperitoneal administration of iodophenylhydroxylamine and methaemoglobinaemia. Dose, 10 mg./kg., 10 counts/μg./min. Methaemoglobinaemia is rapidly induced by the drug but disappears quite quickly, whereas radioactive material is retained within the red cells. Iodonitrosobenzene produces essentially the same results as the iodohydroxylamine. Methaemoglobinaemia induced by an equivalent dose (5 mg./kg.) of phenylhydroxylamine is shown for comparison, and is less intense and persists for a shorter time.

The quantitative aspects of the reaction between iodophenylhydroxylamine and the cells has been studied in washed red cells in saline suspension by adding the iodo compound dissolved in a small quantity of aqueous alcohol and measuring the cell/saline distribution of radioactivity after varying periods of time (Table I). Maximal reaction occurs within a few minutes, the cells becoming intensely "methaemoglobinaemic," and the radioactivity in the cells cannot be dislodged by washing, nor does it emerge into the surrounding fluid on standing overnight at room temperature or 37° C. Over a wide range of concentration of iodophenylhydroxylamine the percentage radioactivity retained by the cells remained constant (Table I), but above 2,000 μg./ml blood haemolysis became significant

TABLE I

REACTION OF OXYGENATED ERYTHROCYTES WITH IODOPHENYLHYDROXYLAMINE AND IODONITROSOBENZENE

Drug	μg. Added/ml. "Blood" (5 ml. Suspension)	Percentage Uptake After (hr.)			
		0.5	2	5	24
IPhNHOH	50	92	92	—	—
	100	92	90	—	—
	250	92	90	92	85
	375	90	91	93	91
	900	92	93	93	92
	2,000	97	90	92	36
	3,150	89	82	75	—*
	4,000	82	75	23	—*
IPhNO ..	700	94	93	—	—
	1,500	94	93	—	—

5 ml. citrated blood added to 20 ml. saline, centrifuged, the cells washed three times with saline and made up to the initial volume. 5 ml. of the suspension is thus equivalent to 1 ml. blood.

* Complete haemo'ysis.

and the corpuscular retention could not be assessed with accuracy. Estimation of the associated methaemoglobin formed in these experiments, using the method described by Evelyn and Malloy (1938), gave the results shown in Fig. 3. Obviously, small quantities of the iodo-compound are able to initiate the conversion of much haemoglobin to the ferric state, although the increase is

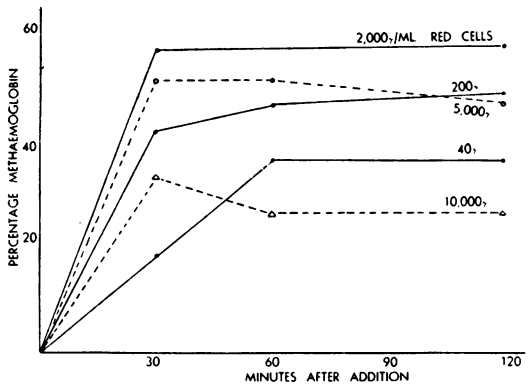


FIG. 3.—The production of methaemoglobin *in vitro* by addition of *p*-iodophenylhydroxylamine to washed rat erythrocytes in saline. A maximum of 60% methaemoglobin was produced by 2,000 μg./ml. red cells.

not maintained with increasing concentration of drug, a maximum of about 60% conversion being achieved. In spite of the rapid disappearance of the methaemoglobinaemia *in vivo* (Fig. 2), reversal was not achieved *in vitro* using radioactive cells washed repeatedly with saline and finally shaken with fresh plasma or saline, even with added glucose or cysteine, either at room temperature or at 37° C. This happened when the amount of radioactive compound corresponded to quantities of retained drug comparable to those achieved *in vivo* (e.g. 100 μg./ml. blood), where reduction of the methaemoglobin is complete in 2–3 hours. Reduction may easily be carried out with sodium hydrosulphite, however, and followed by shaking to oxygenate the pigment. Neither stage was associated with significant loss of radioactivity from the cells although haemolysis occurred during the reduction ; subsequent treatment with carbon monoxide also failed to dislodge the radioactive compound, the ratio of radioactivity to haemoglobin remaining constant (Table II).

TABLE II

Conversion of the methaemoglobin of labelled erythrocytes to haemoglobin and carboxyhaemoglobin does not remove radioactivity, although considerable haemo'ysis occurs. The ratio counts/haemog'obin remains steady.

Suspension of Cells	Counts/ml. Suspension	mg. Haemoglobin/ml. Suspension	Counts/ Haemoglobin
Original	18,950	77	250
After hydrosu'phite	6,560	29	230
After carbon monoxide	1,825	7	260

Experiments with cell suspensions in saline in which the haemoglobin was maintained in the reduced state by sodium hydrosulphite show that added iodophenylhydroxylamine can still be concentrated to some degree in the red cell (Table III), although this is now unaccompanied by any alteration in colour of the pigment. The concentration ratio (counts/ml. cells to counts/ml. saline) is less than one-tenth of that found for the oxygenated cells, so that the latter have a much greater affinity for the drug.

TABLE III

REACTION OF "REDUCED" ERYTHROCYTES WITH IODOPHENYLHYDROXYLAMINE AND IODONITROSOBENZENE

Drug	μg. Added/ml. "Blood" (5 ml. Suspension)	Percentage Uptake After (hr.)		
		0.5	1	2
IPhNHOH..	100	30	31	21
	500	40	36	40
	1,000	41	43	41
	2,000	44	45	45
IPhNO ..	500	47	47	47 (overnight)
	1,000	44	47	47 (overnight)

Conditions of reaction as described in Table I.

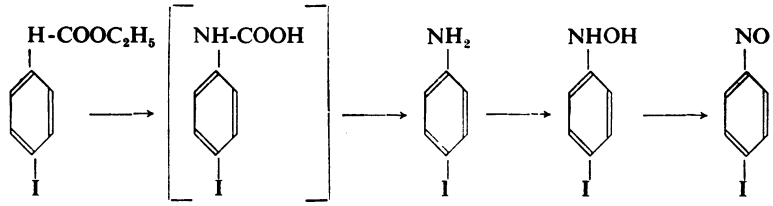
p-*Iodonitrosobenzene* (IC_6H_4NO).—Experiments have been carried out with this substance along similar lines to those described above, but were more restricted by the insolubility of the material. Administration of iodonitrosobenzene to rats by the intraperitoneal route in liquid paraffin solution (Fig. 2) was followed by the rapid onset of

collapse with intense methaemoglobinaemia. The distribution of radioactive material in the blood was very similar to that after iodophenylhydroxylamine. The *in vitro* reactions of iodonitrosobenzene with washed erythrocytes have been studied to a limited extent, and the concentration effects observed using both oxygenated and reduced cells were similar to those found after iodophenylhydroxylamine (Tables I and III).

It is indeed remarkable how rapidly these two insoluble compounds find their way into red cells from saline suspensions, even in relatively high concentrations.

Discussion

The mechanism by means of which various aromatic nitro- and amino-compounds cause methaemoglobinaemia has been investigated for many years on account of its importance in relation to the use of such compounds in industry and therapeutics. Acetanilide has been especially singled out for study, and available evidence points to either phenylhydroxylamine or nitrosobenzene being directly responsible for the methaemoglobinaemia (Brodie and Axelrod, 1948; Greenberg and Lester, 1947; Bodansky, 1951). Only these substances, of likely metabolites, are really potent agents in inducing methaemoglobin formation directly in animals or when added to washed suspensions of red cells; neither substance has been actually demonstrated to be a metabolite of acetanilide or aniline. This is not surprising in view of the instability of both phenylhydroxylamine and nitrosobenzene in aqueous media. The facility with which the *para*-iodo derivatives of phenylhydroxylamine and nitrosobenzene produce methaemoglobinaemia in spite of their relative insolubility in water, and the fact that they may carry a radioactive label, has enabled their metabolism and relation to methaemoglobin formation to be studied in more detail *in vivo* and *in vitro* with interesting results. We had already observed that the retention of radioactivity by red cells after exposure to *p*-iodophenylurethane or *p*-iodoaniline was associated with methaemoglobin formation *in vivo* only; even so the abnormal pigmentation was a transient phenomenon whereas the radioactivity persisted within the cell (Crick and Jackson, 1952). Both *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene are much more potent agents than the iodourethane or iodoaniline and are also rapidly effective *in vitro*. The likelihood is that one or both of them are formed in the rat during the metabolism of the iodourethane and iodoaniline and is responsible for the localization of radioactivity within the erythrocyte. Thus:



The red cell component involved in the reaction is haemoglobin or methaemoglobin and the resultant union is very firm and appears to be independent of the state of the pigment, whether subsequently reduced, oxygenated, or converted to carboxyhaemoglobin. These findings are surprisingly different from the results previously reported by Keilin and Hartree (1943) using phenylhydroxylamine (C_6H_5NHOH), where the combination of this substance with haemoglobin (as followed spectroscopically) was stated to be very unstable and readily broken down by exposure to oxygen or carbon monoxide. They found that combination between haemoglobin and phenylhydroxylamine occurred in the proportion of 1:2 based on the iron content of the pigment, and that the new unstable compound possessed a characteristic absorption spectrum. The reactions of labelled iodophenylhydroxylamine and iodonitrosobenzene with crystalline haemoglobin and some of its derivatives are being examined in more detail, and an account will be published at a later date. It may be stated here that combination occurs in stoichiometric proportions to form compounds of considerable stability; the globulin fragment of the haemoglobin is not involved in these reactions.

It is a curious observation that these two iodocompounds appear to be incapable of converting more than about 60% of the haemoglobin in erythrocytes to methaemoglobin *in vivo* or *in vitro* —an observation which has been previously made with phenylhydroxylamine (Lester, Greenberg, and Shukovsky, 1944). There are related compounds, on the other hand, like *p*-aminopropiophenone which are stated to cause practically complete conversion of the pigment *in vivo* in the dog to the ferric state (Vandenbelt, Pfeiffer, Kaiser, and Sibert, 1944; Bodansky and Gutman, 1947). The change to methaemoglobin appears to be a catalytic process in the lower concentration ranges of iodophenylhydroxylamine (Fig. 3); apparently the oxidation is complicated at higher concentrations

by the combination of either iodophenylhydroxylamine or iodonitrosobenzene with haemoglobin or methaemoglobin which may interfere with the spectroscopic estimation of the latter when the method of Evelyn and Malloy (1938) is used. The intermediate formation of a violet pigment may also be observed at higher concentrations before the final development of brown "methaemoglobin." This presumably is analogous to the unstable violet pigment referred to in the reaction between haemoglobin and phenylhydroxylamine (Keilin and Hartree, 1943).

The ability of phenylurethane, *p*-iodophenylurethane, and *p*-iodoaniline to induce methaemoglobinaemia in the rat depends upon the metabolic conversion of each of them into the hydroxylamine. Reference to Fig. 4 illustrates the results obtained after administration of these substances by the intraperitoneal route. Phenylurethane, although rapidly absorbed, does not apparently produce sufficient metabolite (aniline or phenylhydroxylamine) to induce methaemoglobinaemia, even at this high dose level. Both iodophenylurethane and iodoaniline, however, cause marked methaemoglobinaemia, from which one may infer that iodophenylhydroxylamine is more potent than phenylhydroxylamine in this respect, possibly by virtue of its greater stability, although both substances are unstable in solution. Moreover, the end-products are different; phenylhydroxylamine is converted into *p*-aminophenol and its *para*-iodo derivative into 4:4'-di-iodoazoxybenzene.

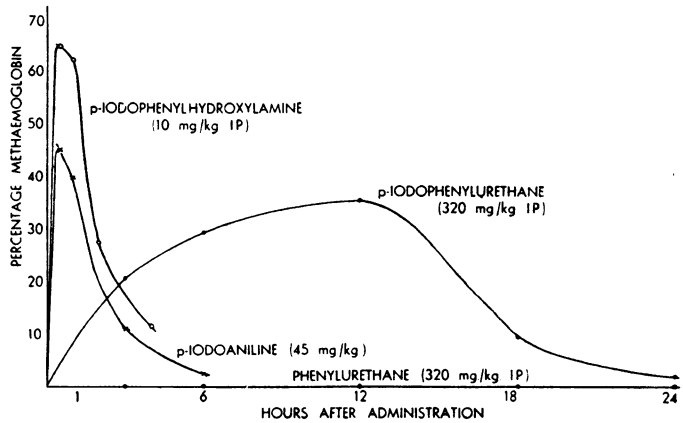


FIG. 4.—A comparative picture of the induction of methaemoglobinaemia in rats by *p*-iodophenylhydroxylamine (10 mg./kg.), *p*-iodoaniline (45 mg./kg.), *p*-iodophenylurethane (320 mg./kg.), and phenylurethane (320 mg./kg.), all by the intraperitoneal route. The sustained methaemoglobinaemia after the iodourethane is due to its more gradual metabolism involving the production of iodoaniline and then iodophenylhydroxylamine which is the active agent. Phenylurethane differs from its *para*-iodo derivative in not causing methaemoglobinaemia even at this high dose level. Presumably insufficient aniline and hence phenylhydroxylamine accumulates to cause any effect.

It is recognized that the methaemoglobin induced by chemical agents is easily reduced to haemoglobin by a reductase system present in the erythrocytes (for a review of the evidence see Bodansky, 1951). We have been unable so far to produce reversal in cells containing methaemoglobin induced by either iodophenylhydroxylamine or iodonitrosobenzene *in vitro* even when the amounts of these substances used produce corpuscular concentrations similar to those attainable *in vivo* where reversal readily occurs after a short time (Fig. 2). Nor was reduction promoted by addition of cysteine or glucose as has been the experience of other workers (Kiese, 1943, 1947; Bodansky, 1951). However, labelled methaemoglobinaemic cells are quickly reduced by sodium hydrosulphite without loss of radioactivity, although a fair amount of haemolysis occurs. Subsequent oxygenation of the cells or treatment with carbon monoxide produces the expected change in the cell pigment, also without loss of the radioactive label, which emphasizes the stability of the complex formed. Although phenylhydroxylamine produces the same sequence of events in the red cells as its *para*-iodo derivative, there appears to be no firm union between the former substance and haemoglobin. Thus preliminary treatment of washed red cells with as much as 6,000 μg./ml. of cells of phenylhydroxylamine failed to block subsequent reaction with iodophenylhydroxylamine. The intensely methaemoglobinaemic cells behave like normal cells in their ability to absorb and retain the radioactive material. The presence of the iodine atom in the aromatic ring is presumably responsible for the stability of the pigment complex.

SUMMARY

1. The preparation of *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene labelled with radioactive iodine is described.

2. These substances localize specifically in the erythrocytes of rats and are firmly bound to the haemoglobin.

3. The associated methaemoglobinaemia which develops *in vivo* soon disappears leaving the radioactive material still bound within the cell. Reduction of methaemoglobinaemic cells produced *in vitro* was only achieved with the aid of sodium hydrosulphite, again without loss of radioactivity.

4. The radioactivity retained in cells is not removed by conversion of the haemoglobin to oxyhaemoglobin or carboxyhaemoglobin. The stability of the complex formed appears to be due to the *p*-iodo-substituent, since phenylhydroxylamine forms an unstable complex with haemoglobin.

5. The localization of radioactive material in erythrocytes observed after administration of *p*-iodophenylurethane or *p*-iodoaniline to rats is presumably due to the metabolic conversion of these two substances to *p*-iodophenylhydroxylamine and/or *p*-iodonitrosobenzene.

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