SOME EFFECTS OF HYDRALLAZINE ON BLOOD IRON AND AN IRON-CONTAINING ENZYME SYSTEM

BY S. M. KIRPEKAR* AND J. J. LEWIS

From the Department of Materia Medica and Therapeutics, University of Glasgow

Received April 20, 1959

A hydrallazine-iron chelation complex has been prepared and shown to have a m.p. of 210°, and no ultra-violet absorption peak at 265 mμ. It has no hypotensive properties and does not antagonise the pressor response to adrenaline in the rat. At high dose levels hydrallazine inhibits catalase activity *in vitro* but not *in vivo*. The iron catalysed oxidation of cysteine to cystine is also inhibited by hydrallazine. Hydrallazine at high dose levels has been shown to cause haemolysis *in vivo* and *in vitro*. A consistent effect upon iron-excretion could not be shown.

SCHROEDER and Perry[1] found that compounds capable of forming complexes with metals could sometimes lower the blood pressure of renal-hypertensive rats but not of the normotensive animal. They suggested that these compounds and the weak metal complexes formed, combined *in vivo* with metal ions and so caused a fall in blood pressure. Fallab and Erlenmeyer[2] have studied complex formation between hydrallazine, Fe^{2+}, Zn^{2+} and Co^{2+} but did not prepare an hydrallazine-iron chelate. We have therefore investigated the ability of hydrallazine to combine with iron; to inhibit an iron-containing enzyme system and the iron-catalysed oxidation of cysteine to cystine.

Amongst the side effects of prolonged use of hydrallazine is anaemia. Some hydrazine derivatives, for example phenylhydrazine, are known to cause haemolysis and since hydrallazine has a reactive hydrazine group we have investigated this possibility.

METHODS

Preparation of Hydrallazine Chelate

A 2 per cent w/v solution of hydrallazine hydrochloride was added to a 10 per cent w/v solution of ferric chloride hexahydrate. A grey precipitate sparingly soluble in water was formed and was recrystallised from absolute methanol until the melting point was constant at 210°. A 0·02 mg./100 ml. solution of hydrallazine in ethanol has one absorption peak below 210 mμ and a second peak between 255 and 268 mμ, while the hydrallazine-iron complex, 0·072 mg./100 ml. in ethanol, did not display the second peak. The chelate was micro-analysed for nitrogen.

Pharmacological Properties of the Hydrallazine-iron Chelate

A 5 mg./ml. solution of the hydrallazine-iron chelate was prepared in propylene glycol, and the effects of this solution were compared with those of a 5 mg./ml. aqueous solution of hydrallazine hydrochloride and propylene glycol itself. Rats of either sex weighing between 300 and

* Squibb Fellow.

400 g. were anaesthetised by intraperitoneal injection of 0·7 ml./100 g. body weight of a 25 per cent w/v solution of urethane. Drug solutions were injected into the cannulated jugular vein and blood pressure recorded from the common carotid artery.

Effects upon Catalase Activity

Crystalline catalase prepared by the Sigma Chemical Co., was used. Catalase activity *in vitro* and *in vivo* was estimated by the method of Euler and Josephson[3]. In *in vivo* experiments rats of either sex weighing between 150 and 200 g. were injected intraperitoneally with 5 mg./100 g. body weight of hydrallazine hydrochloride and killed after 3 hours. The livers were rapidly removed, weighed and homogenised in ice-cold glass distilled water using a Potter-Elvehjem homogeniser. The homogenate was made up to a volume corresponding to 10 ml./g. of liver and a 1 ml. aliquot of this diluted 10 times with ice-cold distilled water. For estimation of catalyse activity 0·2 to 0·4 ml. of this dilute suspension was added to 50 ml. of 0·01N H_2O_2. Controls were done with normal saline. In both *in vitro* and *in vivo* experiments velocity constants were calculated and plotted against time.

Effects upon Oxidation of Cysteine

Oxidation of cysteine to cystine is catalysed by trace metals and iron and copper are especially effective. The iron-catalysed reaction was studied by the Warburg "direct" method. The main chamber contained 1·9 ml. borate buffer at pH 7 together with 0·4 ml. of a 15 mg./ml. cysteine solution. The side-arm contained 0·1 ml. of $3{\cdot}3 \times 10^{-3}$M ferric chloride solution and 0·6 ml. of water. In experiments with hydrallazine the water was replaced by 0·6 ml. of hydrallazine solution to give final flask concentrations after tipping of 0·01, 0·05, 0·10, 0·50, 1·00 or 2·00 mg./ml. The temperature was 24 $\pm$ 0·1°. After an equilibration period of 10 minutes the contents of the side-arm were tipped into the main chamber and readings taken at 5 minute intervals for 30 minutes.

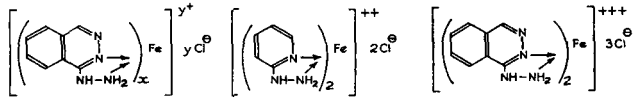
Effects upon Red Blood Corpuscles

For *in vivo* studies rabbits weighing 2·5 to 3·0 kg. were injected intravenously with a solution of neutral $^{59}FeCl_3$ containing 2 μc. of radioactivity. Radioactive iron was obtained as a solution of $^{59}FeCl_3$ containing 20 μc. per 10 ml. Blood samples were collected into heparinised bottles after 24 hours and then on subsequent days for about 30 days. Blood was not collected from the injected ear to avoid contamination from adherent ^{59}Fe. One ml. samples of blood were made up to 10 ml. with water and counted on an Ekco scintillation counter. No attempt was made to separate plasma from corpuscles since almost all of the activity goes into the cells. When the activity had become stable (7 to 9 days) hydrallazine (40 mg./kg.) was injected subcutaneously and blood samples collected 24 hours after the injection and counted. The same procedure was repeated on three subsequent days making a total dose of 160 mg./kg. over a 4 day period. The controls received saline. In some experiments

haemoglobin concentrations and haematocrit values were estimated. Blood samples were collected at intervals for about 21 days after the last injection of hydrallazine. Radioactivity was plotted against time after correcting for decay.

Effects upon Iron Excretion

Rats of either sex weighing about 150 g. were injected intraperitoneally with 2 μc. of ^{59}Fe in the form of neutral $^{59}FeCl_3$ solution. The urine and faeces were collected after 24 hours, combined, partly dried at 100° and



a b c

FIG. 1.

then transferred to a muffle furnace and ashed at 700° for 18 hours. The ash was taken up in dilute hydrochloric acid and adjusted to 5 ml. It was not completely soluble so that the samples were counted in a well-type scintillation counter. Two groups of 4 animals were used. Two in each group served as controls and 2 as tests. Excretion of ^{59}Fe was maximum on the third or fourth day; it then fell and reached a plateau. Hydrallazine

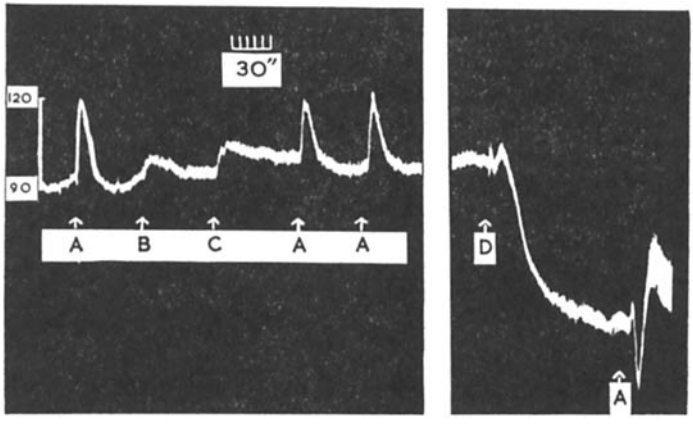


FIG. 2. Rat. Urethane anaesthesia. Blood pressure recorded from common carotid artery and drugs injected *via* the jugular vein.

At A, adrenaline, 0·5 μg./kg.
B, propylene glycol, 0·1 ml./kg.
C, hydrallazine-iron complex, 0·50 mg./kg.
D, hydrallazine, 0·25 mg./kg.

hydrochloride (2·5 mg./100 g.) was injected intraperitoneally on 4 successive days. The urine and faeces were collected, ashed and counted as above.

RESULTS

Hydrallazine Chelates

Certain diamines, for example ethylenediamine, are capable of forming metal chelates. In these the bonds are formed by electron donation and the number of groups associated with the metal is known as the co-ordination number. Iron is known to form chelates and it seems probable that it is forming one with hydrallazine. The five membered ring shown in the general formula (Fig. 1*a*) is theoretically the most stable structure and a similar structure is known in the case of the iron-β-pyridylhydrazine complex (Fig. 1*b*). The analyses obtained for N gave figures of from 22·1 to 23·0 per cent. On the basis of these figures the more probable structure is the one in which the co-ordination number of iron is 4 (Fig. 1*c*). It is probable, however, that we obtained a mixture.

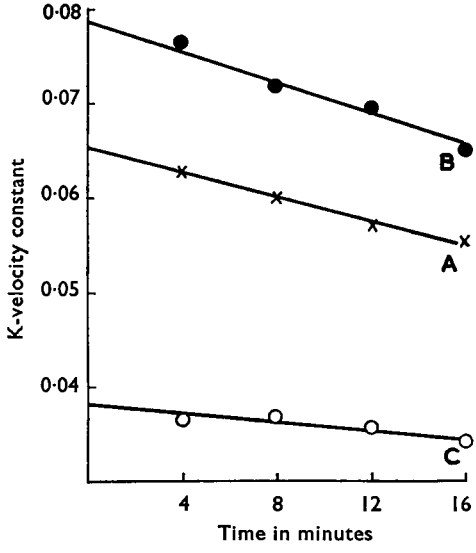


FIG. 3. Effect of hydrallazine on *in vitro* catalase activity.

A, control.
B, hydrallazine, 0·10 mg./ml.
C, „ 10·0 „

Pharmacological Properties of the Hydrallazine-iron Chelate

Hydrallazine causes a marked fall in the blood pressure of the anaesthetised rat and antagonises the pressor effects of adrenaline. The chelate caused no fall in blood pressure but induced a small rise, probably due to the propylene glycol solvent. It did not antagonise the pressor response to adrenaline. Ferric chloride was found to be inert in these respects (Fig. 2).

TABLE I

EFFECT OF HYDRALLAZINE ON IRON-CATALYSED CYSTEINE OXIDATION
Oxygen uptake in μl. ± S.E.

	Concentration μg./ml.	Number of observations	Incubation time (minutes)		
			10	20	30
Hydrallazine ..	10	6	136 (± 6·08)	156 (± 6·24)	152 (± 5·29)
„ ..	50	6	128 (± 10·44)	154 (± 8·06)	153 (± 7·21)
„ ..	100	6	128 (± 8·18)	159 (± 6·16)	154 (± 5·65)
„ ..	500	6	10 (± 0·85)	9 (± 0·87)	7 (± 1·1)
„ ..	1,000	6	12 (± 1·73)	12 (± 2·44)	14 (± 3·31)
„ ..	2,000	3	9·6 (± 1·30)	8·5 (± 2·5)	8·3 (± 7·2)
Control	—	29	132 (± 2·6)	152 (± 2·75)	152 (± 2·91)

Effects upon Catalase Activity

Hydrallazine (0·1 and 1·0 mg./ml.) had little or no effect upon catalase activity *in vitro* but 10 mg./ml. caused about 50 per cent inhibition (Fig. 3). A single dose of 5 mg./100 g. of hydrallazine hydrochloride in rats did not reduce the catalase activity of the liver 3 hours after injection.

Effects upon Oxidation of Cysteine

The results are shown in Table I. Cysteine itself was slightly oxidised at pH 7. Hydrallazine caused a slight oxidation of cysteine. The iron-catalysed oxidation was significantly inhibited ($P < 0.001$) by hydrallazine hydrochloride (0·5, 1·0 or 2·0 mg./ml.), lower concentrations (e.g. 0·1 mg./ml.) had no effect.

Effects upon Red Blood Corpuscles

For these experiments a total of 7 animals of both sexes were used, two of these were controls. The results of a typical experiment are shown in Figure 4. Iron is slowly incorporated into the red corpuscles; the maximum blood radioactivity is reached in 7 to 9 days and this level, once reached, remains constant over the period of the observations. The radioactivity of the blood remained unchanged for 24 to 48 hours after the first or second injection of hydrallazine, but from the third day onwards the activity began to fall. This fall continued on the fourth and subsequent days even though hydrallazine injections were stopped. Activity returned to normal in about 15 days. Four out of five test animals showed these effects but one animal showed no changes in blood radioactivity—treatment being stopped because it had convulsions. In one animal in which haemoglobin and haematocrit estimations were made both showed a fall after hydrallazine, haemoglobin falling from 13·16 to 10·86 per 100 ml. and the haematocrit from 40 to 33. About 16 days after stopping hydrallazine treatment these values returned to normal.

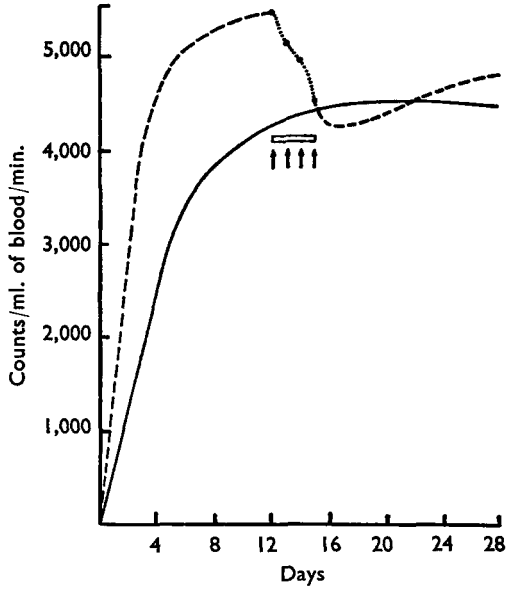


FIG. 4. Effect of hydrallazine on red blood corpuscles. Thick and dotted lines indicate control and test animals respectively. Arrows indicate administration of hydrallazine (40 mg./kg.) to the test animal.

Effects upon Iron Excretion

The counts excreted were plotted against time in days. The number of counts excreted in the urine and faeces increased in 2 animals, 2 others showing no change.

Discussion

The iron-hydrallazine chelate has no hypotensive action in normal rats and does not antagonise the pressor response to injected adrenaline. It has thus lost two of hydrallazine's more important pharmacological properties. It has been reported[4,5] that when hydrallazine is incubated with sera, proteins or polypeptides it loses much of its hypotensive effect. When incubated with an ultrafiltrate of serum there is less loss of activity indicating that the presence of the serum colloids is important. It has also been observed that serum which has first been heated to 56° still causes loss of activity so that an enzyme-catalysed reaction is not likely[3,4]. Hydrallazine is also inactivated by incubation with ground arteries. When it combines with iron, hydrallazine loses its hypotensive activity and the ability to antagonise the pressor response to adrenaline, probably owing to the formation of a stable ring compound. There is no detailed evidence that hydrallazine acts in this way with protein but information on the extent to which it is bound and inactivated by plasma and serum proteins would be valuable.

Hydrallazine inhibits catalase *in vitro* only at very high concentrations; lower concentrations are inactive. Liver catalase activity *in vivo* was not reduced by even the large doses used but the iron-catalysed oxidation of cysteine to cystine was inhibited. This effect may be due to chelation of iron by hydrallazine.

Hydrallazine appears to cause some haemolysis *in vivo* and when a blood sample was incubated at 37° with hydrallazine haemolysis was seen. In rabbits high doses cause a sharp decline in blood radioactivity which rises to normal levels within a few days. This indicates that the iron liberated from the haemolysed corpuscles is apparently used again in the formation of new cells and there is no evidence of inhibition of haemoglobin-synthesis. The results obtained from iron excretion studies do not enable us to draw any conclusions.

Our results do not support the view that hydrallazine acts as an anti-hypertensive by virtue of forming metal chelates. It must be borne in mind that hydrallazine lowers the blood pressure of normotensive animals. The effect of hydrallazine on the red blood corpuscles may explain the anaemias associated with the prolonged clinical use of this drug, especially at high dose levels. Some of the other side effects of hydrallazine may be due to inhibition of cellular oxidation mechanisms.

Acknowledgements. We thank Dr. C. Dale Falconer of Ciba Laboratories Ltd.. for supplies of hydrallazine hydrochloride, Dr. A. H, Beckett, Dr. J. A. Blain, Dr. M. Martin-Smith, Mr. S. Reid and Dr. M. M. Bluhm for help and advice, Miss Sheena MacPhee and Mr. Adam Ritchie for technical assistance and Mr. R. Callander for the drawings.

REFERENCES

1. Schroeder and Perry, *J. Lab. clin. Med.*, 1955, **46**, 416.
2. Fallab and Erlenmeyer, *Helv. chim. Acta*, 1957, **40**, 369.
3. Euler and Josephson, *Ann. Chem.*, 1927, **452**, 158.
4. Tripod and Meier, *Helv. Physiol. Acta*, 1954, **12**, C.33.
5. Tripod, *Hypotensive Drugs*, Pergamon Press, London, 1956.

After Mr. Lewis presented the paper there was a DISCUSSION.