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CHRONIC TOXICITY OF CARBOETHOXYPHTHALAZINO-HYDRAZINE AND ITS INFLUENCE ON THE EXCRETION OF 5-HYDROXYINDOLEACETIC ACID AND THE ACTIVITY OF 5-HTP-DECARBOXYLASE IN WHITE RATS

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Morrow, Schroeder & Perry (1953) and Dustan, Taylor, Corcoran & Page (1954) observed multiple side-effects and noted many complaints in patients treated for several months with hydralazine (1-hydrazinophthalazine, Apresoline) in doses of 400–800 mg daily. These symptoms closely resembled those of acute disseminated lupus erythematosus. They included: fatigue, loss of appetite, fever, arthralgia, pains in the abdomen and thorax, erythematoid rashes on the arms and forearms, albuminuria, haematuria and many blood abnormalities. Cells similar to those found in lupus erythematosus (LE), anaemia, increased sedimentation rate, decrease in haemoglobin content, changes in the polysaccharide level, leukopenia, decrease in albumin content and hyperglobulinaemia were found in the peripheral blood. Non-specific pneumonia, pericardial and pleural effusions were frequently observed. Several of these signs have also been observed in dogs (Comens, 1956, 1960; Dubois, Katz, Freeman & Garbak, 1957) and guinea-pigs (Siguier, Bétourne & Bonnet de la Tour, 1958; Braverman & Lerner, 1962) during chronic treatment with hydralazine.

Recently a new derivative of hydralazine (N_1-carboethoxy-N_2-phthalazino-hydrazine (CEPH)) was synthesized by Biniecki, Haase, Izdebski, Kesler & Rylski (1958). Its pharmacological properties were analysed by Kubikowski, Majcherczyk & Szymańska (1959), Chruściel, Janiec, Herman, Brus, Kleinrok & Pojda (1963) and Kleinrok (1964), and it has been used in the treatment of hypertension with good results (Rozwadowska, Dowženko, Olejniczak, Przybył & Raszewski, 1957).

Haverback, Sjoerdsma & Terry (1956) and Chruściel & Górka (1965) have observed decreased excretion of 5-hydroxyindoleacetic acid (5-HIAA) in lupus erythematosus. The similarities between the symptoms of lupus erythematosus and the changes observed during prolonged treatment with hydralazine prompted a study of the metabolism of tryptophan during the chronic treatment of experimental animals with hydrazinophthalazines.

The excretion of 5-HIAA, a metabolite of tryptophan, was investigated and compared with the activity of 5-hydroxy-L-tryptophan carboxy-liase (5-HTP-decarboxylase), in

various tissues. Histological analyses of these tissues were also carried out. CEPH was chosen for these experiments because of its low acute toxicity (the chronic toxicity of CEPH in white rats is reported later). Other hydrazinophthalazines have also been used with results which will be reported elsewhere.

METHODS

Twenty female white rats of the Wistar strain were fed for 8 months with a diet of granules containing CEPH. The diet contained wheat 45%, barley 25%, corn 12%, maize 5%, meat and bone flour 5%, ground dried fruit 4%, salt mixture (calcium carbonate 40%, bone flour 35%, sodium chloride 19.48%, magnesium sulphate 4%, ferrous sulphate 0.32%, manganese sulphate 0.06%, cupric sulphate 0.1%, cobalt sulphate 0.02%, potassium iodide 0.02% and herbs 1%) 1%, cod liver oil 1%, and margarine 1%. The amount of food eaten averaged 50 g/kg. The content of CEPH was gradually increased from 20 mg/kg body weight to 1 g/kg (see Fig. 1). In the ninth month, after an interval of 2 weeks in which no CEPH was administered, the animals were fed with a diet containing CEPH 500 mg/kg body weight for 2 weeks. A control group of 10 female rats of the same strain was fed with the same diet without the addition of CEPH.

During the 9 months animals died or were killed (see Fig. 1). Kidney, liver, spleen and lung sections were made and stained with haematoxylin and eosin. The activity of 5-HTP-decarboxylase in homogenates of kidney and liver was determined manometrically (Blaschko & Chruściel, 1960).

The animals were kept in large cages in groups of five. They had free access to fresh milk and water renewed daily. The weight of the animals was recorded weekly. At intervals during the 9 months the animals were placed in metabolism cages and fasted for 24 hr while urine was collected. Because the volumes of urine over 24 hr were very small, from the fourth month of the experiment measured volumes of water were available to the animals to prevent dehydration. Urine was collected in tubes containing glacial acetic acid. The excretion of 5-HIAA was measured by Pierce's (1959) method. Routine laboratory examination of the urine and its sediment was performed. In the eighth month blood was collected by tail puncture and a test for LE-cells was carried out as described by Snapper & Nathan (1955); the cholesterol level (Sperry & Webb, 1950) was assayed in the serum.

Additional experiments were carried out to investigate the influence of CEPH and other hydrazinophthalazines on DOPA-decarboxylase activity *in vitro* and *in vivo*. The activities of DOPA- and 5-HTP-decarboxylase in fresh homogenates of rat liver tissue with the addition of CEPH, hydralazine or 1:4-dihydrazinophthalazine (Nepresol) were compared with control activities. In *in vivo* experiments the decarboxylase activities were analysed in liver and kidney homogenates of 100 white rats treated for 3 weeks with either CEPH (50 or 100 mg/kg daily, given subcutaneously with an additional 10 or 20 mg/kg respectively in drinking water) or hydralazine (10 or 20 mg/kg subcutaneously with 2 or 4 mg/kg respectively in drinking water). Freshly collected livers from individual animals and kidneys from numerous animals were frozen and ground in a mortar cooled in the deep freeze. The homogenates were diluted 1:1 or 1:2 with 0.067 M sodium phosphate buffer, pH 6.5 and the activities of 5-HTP- and DOPA-decarboxylases were assayed manometrically as described by Blaschko & Chruściel (1960).

RESULTS

The acute toxicity of CEPH is shown in Table 1.

CEPH added to the diet did not cause any behavioural changes or toxic effects in white rats when given at a low dosage (20–50 mg/kg) for 3 months. Within this period three rats were killed and no pathological changes in the internal organs were found. Doses of CEPH 20 and 50 mg/kg did not affect the body weight of the animals but at

a dose level of 50 mg/kg the rats grew at a slower rate. A slight decrease in the mean body weight of the rats given 200 mg/kg was noted. Treatment with 1,000 mg/kg of CEPH caused proteinuria and slight glycosuria, as well as a sharp decrease in the body weight of the animals. After several days six rats died; a further group of four was killed to obtain samples for biochemical and histological analysis. The treatment with CEPH was interrupted for 2 weeks. Then the surviving rats were given CEPH 500 mg/kg in the diet and because three more died the remaining animals were killed. No differences in the weight of heart, liver, spleen, kidneys, brain and suprarenals were found between experimental and control rats.

TABLE 1
ACUTE TOXICITY OF CEPH
Doses expressed in mg CEPH/kg body weight

Species	Treatment	LD_{min}	LD50	LD100
Rat	Intravenous	80	110	170
	Intraperitoneal	340	337	400
	Intramuscular	250	333	400
Mouse	Intravenous	60	60	75
	Intraperitoneal	350	382	450
	Intramuscular	300	417	500
	Oral	500	834	1200
Frog	Oral	500	650	1100
	Lymph sac	400	636	900

The histological examinations showed that the following changes had taken place:

Kidneys. A thickening of the glomerular basement membrane was found (Fig. 2). In some glomeruli symptoms of degeneration—for example, hyaline changes and capillary obstruction—were seen.

Spleen. Changes within the spleen were : oedema of the connective tissue, the sporadic development of new collagen fibres, and reduction of lymphopoietic tissue with a concomitant increase of the red pulp. Phagocytic cells containing yellowish-brown pigment, most probably a haemoglobin derivative, were more numerous than in the controls.

Liver. Changes within the liver were slight. Some hydropic changes within the cells were occasionally observed and a small focus of atrophying cells filled with a mass of homogenous protein was found in one rat.

Lung. There were no characteristic changes in the pulmonary tissue. Such changes as were observed were limited to a medium degree of thickening of interalveolar septa and the development of small infiltrations consisting of lymphocyte-like cells. In some animals the development of collagen fibres was limited and small hyaline changes were seen. In one rat a formation similar to Gross bodies was found.

The extent of these structural changes differed in different animals, but when the changes in one tissue were marked the same held for other tissues from the same animal. The greatest changes were found in rat No. 1/9. Slightly smaller changes were seen in animals 1/10 and 1/3; in the remaining rats of Table 2 they were much smaller.

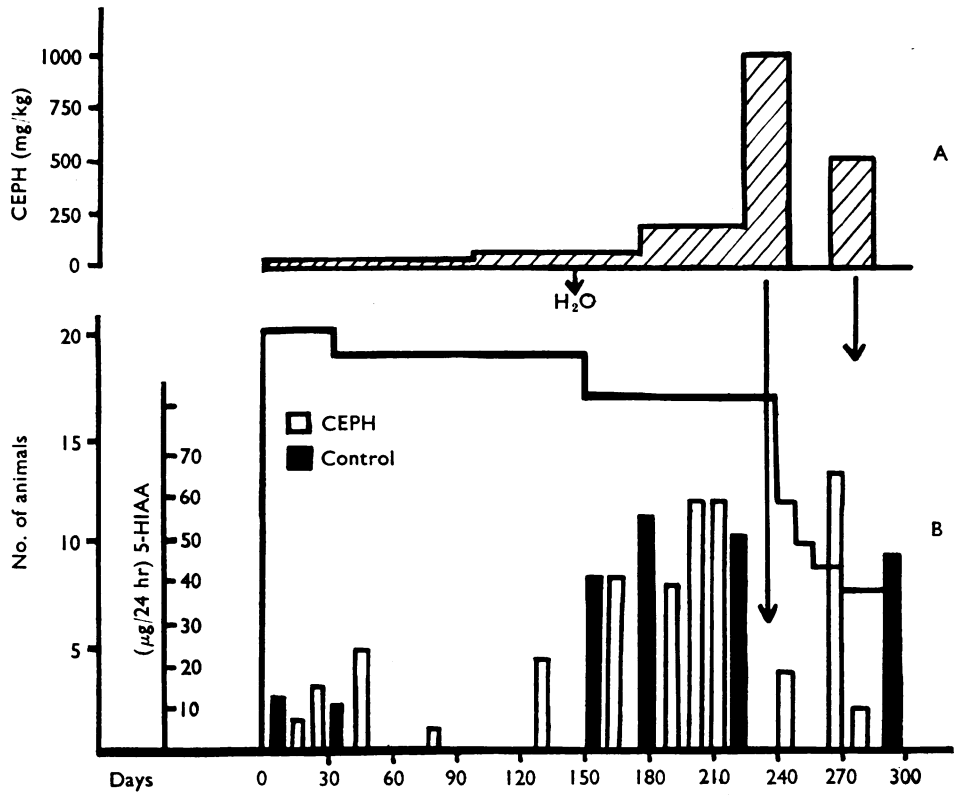

Fig. 1. Influence of prolonged treatment with carboethoxyphthalazinohydrazine (CEPH) on 5-hydroxyindoleacetic acid (5-HIAA) excretion in white rats. The dose of CEPH (mg/kg body weight) is shown in A. B shows the decrease in the number of animals during the 9 months and the excretion of 5-HIAA during 24 hr periods. Initially no water was given during the collection of urine but at 4 months (indicated by arrow) measured amounts of water were given. Additional arrows indicate significant reductions in the excretion of 5-HIAA.

TABLE 2

INFLUENCE OF CEPH ON THE DEGREE OF CHANGES RESEMBLING LUPUS ERYTHEMATOSUS AND THE 5-HTP-DECARBOXYLASE ACTIVITY IN LIVER AND KIDNEY HOMOGENATES OF WHITE RATS

* Killed after 2 week interval in the treatment with CEPH. † Combined homogenates.

	Treatment with CEPH			5-HTP-decarboxylase activity	
Rat No.	Time (months)	Total amount (approx.) (g)	Histopathological changes (degree of severity)	(μml. CO_2/g fresh tissue/hr) Kidney	Liver
1/3	8	8·0	+++	89	0
1/9	8	8·0	+++++	0	0
1/10	8	8·0	++++	0	0
1/5	8*	8·5	++	126	0
2/6	8½*	10·5	+	}68†	}148†
2/8	8½*	8·0	+		

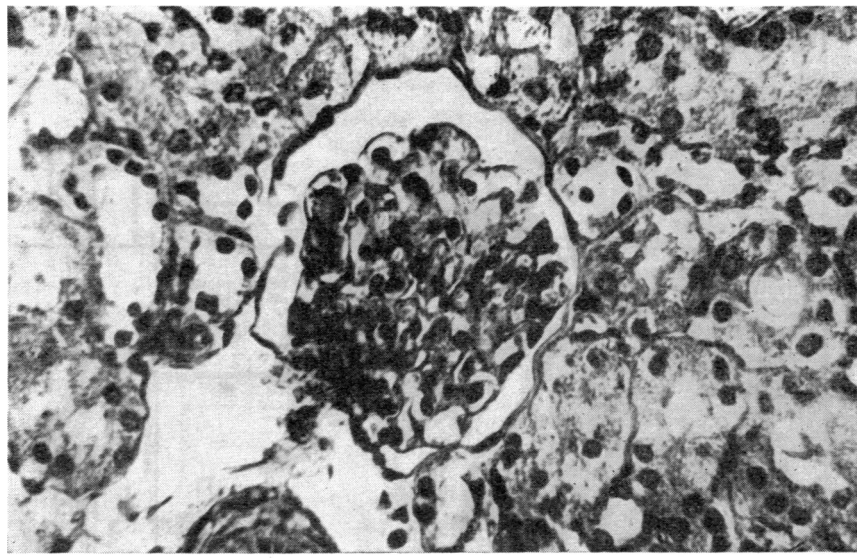

Fig. 2. Kidney of a white rat (No. 1/9) treated for 8 months with CEPH showing thickening of the basement membrane in the glomeruli. Haematoxylin and eosin, ×400.

There is a striking parallelism between the inhibition of 5-HTP-decarboxylase activity in liver and kidney homogenates of these animals and the intensity of the histopathological changes (Table 2). In rat No. 1/9 which had the greatest pathological changes, the inhibition of 5-HTP-decarboxylase from both liver and kidney was complete.

The excretion of 5-HIAA in urine fluctuated throughout the treatment with a lower dose of CEPH. The decrease in 5-HIAA excretion was evident, however, (Fig. 1) when larger doses (500 and 1,000 mg/kg) were used. Variations in urine volume were statistically non-significant. Serum cholesterol was measured from the eighth month onwards and by the end of the experiment the mean total cholesterol level (150 mg%) had increased as compared with control rats (80 mg%). The test for LE-cells was positive in some of the CEPH-treated rats. However, no true LE cells were found. Single pseudo-LE-cells were observed and rosettes, erythro- and leuco-phagocytosis were encountered. The inhibitory effect *in vitro* of both hydralazine and 1:4-dihydrazinophthalazine on the activity of kidney and liver decarboxylases was confirmed (Table 3). Furthermore, CEPH was shown to be a much weaker inhibitor of both 5-HTP- and DOPA-decarboxylase.

In a preliminary experiment, doses of hydrazine of 10, 50 and 100 mg/kg added to the diet completely abolished the 5-HTP-decarboxylase activity in the kidney and reduced it in the liver.

Treatment for 3 weeks with hydrazinophthalazine injections depressed the tissue decarboxylase activity. No 5-HTP-decarboxylase activity was found in kidneys of rats treated with CEPH in the doses employed or hydralazine in a dose of 10 mg/kg. A slight activity of this enzyme remained in kidneys of rats treated with hydralazine 20

mg/kg (11 as compared with 71 μml. CO_2 g^{-1} hr^{-1} in the control). In the liver the activity of both decarboxylases was depressed (Fig. 3). The decrease in enzymatic activity does not seem to be dose-dependent. The effect of CEPH on 5-HTP-decarboxylase was slightly greater than on DOPA-decarboxylase. A dose of hydralazine 10 mg/kg depressed the liver 5-HTP-decarboxylase completely. A higher dose of hydralazine (20 mg/kg) did not produce such a severe inhibitory effect.

TABLE 3

INFLUENCE OF HYDRAZINOPHTHALAZINES *IN VITRO* ON THE DECARBOXYLASE ACTIVITY OF LIVER HOMOGENATES OF WHITE RATS

*As compared with control activity (100%).

Substance	Conc. (M)	Substrate	Activity* (%)
Hydralazine	0·0007	DOPA	72
Hydralazine	0·007	DOPA	0
Dihydralazine	0·01	DOPA	20
Dihydralazine	0·05	DOPA	0
CEPH	0·01	DOPA	90
CEPH	0·05	DOPA	76
CEPH	0·1	DOPA	50
CEPH	0·1	DOPA	0
CEPH	0·001	5-HTP	85
CEPH	0·01	5-HTP	69
CEPH	0·1	5-HTP	0

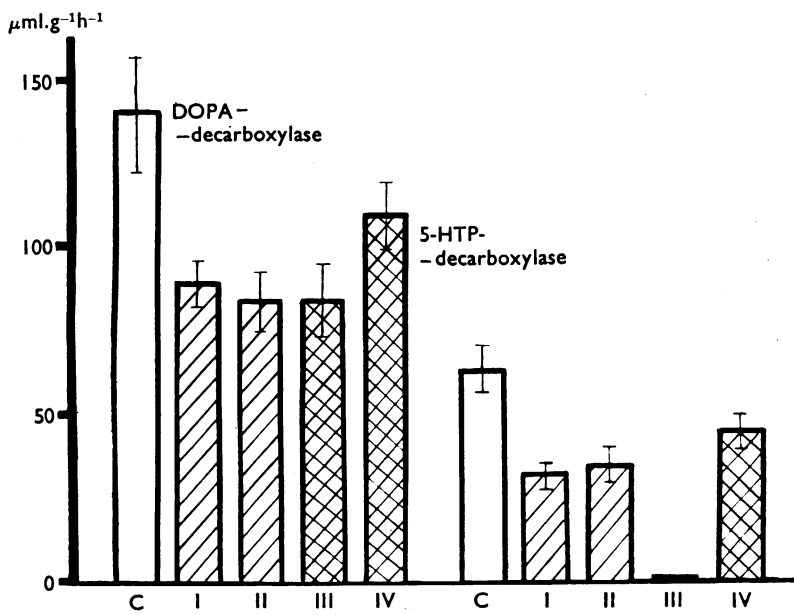

Fig. 3. DOPA- and 5-HTP-decarboxylase activity in homogenates of liver tissue from white rats treated for 3 weeks with carboethoxyphthalazino-hydrazine (CEPH) or hydralazine. Activity was expressed in μml. CO_2 formed by 1 g of fresh tissue in 1 hr. C: control animals. Treated animals: I: CEPH 50 mg/kg subcutaneously with 10 mg/kg in drinking water; II: CEPH 100 mg/kg subcutaneously with 20 mg/kg in drinking water; III: hydralazine 10 mg/kg subcutaneously with 2 mg/kg in drinking water; IV: hydralazine 20 mg/kg subcutaneously with 4 mg/kg in drinking water.

DISCUSSION

Chronic treatment with low doses of CEPH (20–50 mg/kg) did not produce any toxic symptoms in rats. A dose of 200 mg/kg inhibited the gain in weight but doses of 500 mg/kg or more in the diet were toxic and resulted in the death of many animals. CEPH is, however, less toxic than hydralazine (Kubikowski *et al.*, 1959).

Möllerberg (1958) did not observe any changes similar to lupus erythematosus in rats treated with hydralazine. He used only four rats in the experiment, however, and the treatment with hydralazine (50 mg/kg) lasted for only 17 weeks. After a more prolonged period of treatment using much larger doses of the less toxic CEPH we have noted histopathological changes which are remarkably similar to the changes characteristic of subacute systemic lupus erythematosus. These changes varied in intensity in different animals. In some animals, for unknown reasons, they were practically absent.

There is a striking correspondence between the extent of the histopathological changes and the intensity of inhibition of the liver and kidney 5-HTP-decarboxylase activity. The disturbance of 5-hydroxytryptophan metabolism occurred only in those animals which showed changes in collagen formation and metabolism which suggests that there is a link between these metabolic pathways. The decrease in 5-HIAA excretion in the urine observed after large dose of CEPH is a significant pointer to interference with 5-HTP-metabolism. It is interesting that kidney 5-HTP-decarboxylase was inhibited *in vivo* to a greater extent by both CEPH and hydralazine than the liver enzyme. This may be caused either by greater susceptibility of the kidney enzyme or by greater accumulation of the hydralazinophthalazines in the kidney tissue during excretory processes.

Recently in our laboratory Chruściel (1967) and Chruściel, Chruściel, Brus, Pojda, Trzeciak, Mai, Plech, Lenartowicz & Kleinrok (1967) have obtained hydralazine syndrome in rats treated for several months with hydralazine. The symptoms resembled those observed in susceptible patients treated with hydralazine: leucopenia, increase of sedimentation rate, hypoalbuminaemia and α- and β-hyperglobulinaemia were found. Histologically, in rats treated with hydralazine in a dose of 50 mg/kg for 3 months (Chruściel *et al.*, 1967), thickening of glomerular basement membranes and wire-loops were found in the kidney. In some of these animals accumulation of 5-hydroxytryptophan in liver tissue and increase in kynurenine excretion after treatment with tryptophan (100–500 mg/kg) were found, confirming the disturbance of tryptophan metabolism (Trzeciak, Chruściel & Chruściel, 1967). Because similar changes were seen in rats after a prolonged treatment with a potent decarboxylase inhibitor, trihydroxyphenyl seryl-hydrazine, Ro-4-4602 (Chruściel, 1967), it is tempting to assume that inhibition of 5-HTP-decarboxylase may be an important factor in the development in collagenosis. Further experiments are in progress.

SUMMARY

1. The action of carboethoxyphthalazinohydrazine (CEPH) on 5-HTP- and DOPA-decarboxylases activities and on morphological changes in rats has been investigated in reference to (*a*) the development of changes similar to lupus erythematosus disseminatus and (*b*) a model for studying the connexions between disturbances of 5-HTP metabolism and collagenosis.

2. Although CEPH is much less toxic than other hydrazinophthalazines, after prolonged treatment with very high doses it causes a variety of toxic effects. In kidney and spleen some changes characteristic of experimental collagenosis were found (for example, the thickening of basement membranes and wire-loops in the glomeruli, the decrease in white pulp in the spleen). In some rats no changes were seen.

3. A parallelism between the extent of characteristic pathological changes and the degree of inhibition of 5-HTP-decarboxylase activity was found.

4. The significance of these findings is discussed.

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