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METABOLISM OF ADRENOCHROME SEMICARBAZONE IN THE RAT

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Abstract—Adrenochrome monosemicarbazone was administered to rats. The bulk of the compound was found to be excreted unchanged in a comparatively short period of time. At a dose level of 10 mg/kg, approximately half the administered dose could be accounted for by urinary excretion within 6 hr after administration.

Radioactive tracer studies indicated that 85 to 90 per cent of the urinary product is unchanged adrenochrome semicarbazone. The remaining 10 to 15 per cent represents three minor metabolites. Two of these have been characterized as sulfate conjugates; the third product was not characterized because of difficulties in isolation. One of the metabolites appears to be a sulfate conjugate of 6-hydroxy-N-methylindole semicarbazone and the other a sulfate conjugate of 5-amino-6-hydroxy-N-methylindole.

FOR A number of years adrenochrome semicarbazone has been used in the form of a salicylate complex under the trade name of Adrenosem as a hemostatic agent. Little, however, is known of its mode of action or metabolic fate.

The most extensive piece of work on the metabolism of adrenochrome semicarbazone appears to be that of Fischer and Lecomte,[1] who studied the metabolism of adrenochrome semicarbazone in man. They gave the compound by mouth and found that about 20–30 per cent was excreted unchanged, while 20 per cent was excreted as an indole that had lost the semicarbazide moiety. The authors believed the latter conversion was due to bacterial action. They found some evidence for conjugated products but were not able to prove their presence conclusively. Since this publication, relatively little work has been done on the metabolism of the compound. We felt, therefore, that it would be of value to reinvestigate the metabolism of this compound in an experimental animal such as the rat in order to determine the nature of metabolites formed.

The present work is concerned with the preparation of labeled and unlabeled adrenochrome semicarbazone, its metabolism in the rat, and the identification of urinary metabolites derived from it.

EXPERIMENTAL

Preparation of adrenochrome semicarbazone

Adrenochrome semicarbazone was prepared from adrenochrome, which had been made from epinephrine by silver oxide oxidation, according to the procedure of Heacock *et al.*[2] To 250 mg adrenochrome, 180 mg semicarbazide hydrochloride and 250 mg sodium acetate were added; the reaction mixture was shaken in an ice bath until crystallization occurred. After standing in the cold for 2 hr the crystals were

filtered, washed with a little ice water, and dried. After recrystallization from ethanol the compound melted at 236° (uncorr.). Elemental analysis: $C_{10}H_{12}N_4O_3$. Calculated: C, 50·84; H, 5·12; N, 23·72. Found: C, 50·56; H, 4·83; N, 23·71.

Preparation of ^{14}C-*adrenochrome semicarbazone and adrenochrome-*^{14}C-*semicarbazone*

Adrenochrome labeled with carbon-14 was prepared from β-^{14}C *dl*-epinephrine bitartrate, sp. act. 1 mc/m-mole (Chem. Trac. Baird Atomic) as described by Noval *et al.*,[3] except that the action was carried out in an aqueous medium instead of in methanol. The product, after removal of residual traces of silver by passage through a Reeve Angel SB-1 ion-exchange paper, was converted subsequently to the semicarbazone by the addition of 67 mg semicarbazide hydrochloride and 100 mg sodium acetate. The mixture was shaken in an ice bath for 15–20 min to allow the ^{14}C-adrenochrome semicarbazone to crystallize; the product was then washed with a small amount of cold water. Adrenochrome-^{14}C-semicarbazone was prepared in the same manner, except that 63 mg unlabeled semicarbazide and 5 mg of ^{14}C-semicarbazide (sp. act. 6·1 mc/m-mole) were added.

*Preparation of 6-hydroxy-5-semicarbazido-*N*-methylindole*

One gram adrenochrome semicarbazone was suspended in 150 ml methanol, 100 mg of 5% palladium on carbon as a catalyst was added. Catalytic hydrogenation was carried out at 60 lb pressure for 18 hr. The catalyst was removed by filtration and the filtrate concentrated *in vacuo* until the product crystallized out; after drying in an inert atmosphere, and it was re-crystallized from methanol. On heating, the compound turned red at 196° and melted, with decomposition, at 204°. Elemental analysis: $C_{10}H_{12}N_4O_2$. Calculated: C, 54·53; H, 5·49; N, 25·44. Found: C, 54·50; H, 5·62; N, 24·05.

*Sulfation of adrenochrome semicarbazone and 6-hydroxy-5-semicarbazido-*N*-methylindole*

Ten milligrams of either adrenochrome semicarbazone or 6-hydroxy-5-semicarbazido-N-methylindole was dissolved in 1 ml pyridine at 0° and added to a solution of 0·1 ml chlorosulfonic acid in 1 ml pyridine, also at 0°. The mixture was agitated at 45° for 30 min. The sulfate conjugates were isolated by chromatography on Whatman 3MM paper. The reaction mixture was first chromatographed with water as the solvent. The R_f 0·65 material was eluted with water and subsequently rechromatographed on Whatman 3MM paper, in the butanol:acetic acid:water system (see below).

Colorimetric determination of the excretion of adrenochrome semicarbazone. Pairs of Sprague–Dawley rats weighing approximately 200 g were given adrenochrome semicarbazone at the following dose levels: 10, 20, 40, 80, and 100 mg/kg respectively. Urine was collected at the stated intervals, and the amount of adrenochrome semicarbazone excreted was determined colorimetrically, essentially as reported by Fischer and Lecomte,[1] by the addition of 5 ml of a 5% solution of lead acetate to 5 ml of an appropriate urine dilution. The solution was allowed to stand 5 min, filtered, and the filtrate read in the Beckman DB spectrophotometer at 540 mμ.

Chromatographic procedures. Urinary metabolites were identified and purified by means of paper chromatography. Butanol:acetic acid:water (8:2:2) and water were the two principal solvent systems employed. The chromatography was carried out

ascending on Whatman 3MM paper for 18 hr with the butanol:acetic acid:water system, and for 4 hr with water. These two systems also were employed for two-dimensional chromatography. On occasion isopropanol:ammonia:water (20:1:2) and ethyl acetate:acetic acid:water (12:4:1) were used as solvent systems.

The urinary metabolites were isolated by chromatography on a cellulose powder column, 40 × 4·5 cm. Rat urine (20–30 ml) was placed on this column. The column was developed and eluted with water as described in the discussion of each of the metabolites.

Isotope detection and counting procedures. Labeled metabolites on two-dimensional chromatograms were detected by radioautography. Kodak daylight brand X-ray film was exposed to paper chromatograms.

Urine samples and one-dimensional chromatograms cut into sections and the radioactivity were counted in a Packard Tri-Carb scintillation counter, with appropriate internal standardization.

Radioactivity on paper strips also was counted with a Nuclear Chicago Actigraph system in a windowless gas-flow detector.

Color reactions used in the detection and identification of urinary metabolites. Ehrlich's reagent (1%-dimethyl aminobenzaldehyde in 10% hydrochloric acid in methanol) was used to detect indoles and, on subsequent exposure to ammonia fumes, for the detection of indoxyl compounds (fluorindal reaction).

The ninhydrin, acidic diazo, and Ekman reactions, as well as the persulfate oxidation reaction, were carried out as described by Jepson.[4]

N-Chloro derivatives were prepared by spraying the paper with an aqueous solution of sodium hypochlorite having about 2% available chlorine. After drying and the removal of excess chlorine by placing the paper in a hood for 1 hr, the paper was sprayed with a 2% starch solution containing 1% potassium iodide.

One volume of 20% perchloric acid diluted with four volumes of ethanol was used for hydrolysis of phenolic conjugates. The chromatograms were sprayed with this reagent and placed in an oven at 100° until the paper began to char. The paper was then dipped into acidic diazo reagent to detect free 6-hydroxy compounds. Sulfate was detected with the sodium rhodizonate reaction of Burma.[5]

RESULTS AND DISCUSSION

Excretion of adrenochrome semicarbazone and metabolites

When adrenochrome semicarbazone was administered to rats, a considerable portion was excreted fairly rapidly as unchanged adrenochrome semicarbazone. Table 1 gives the results of two experiments on the excretion of a dose of adrenochrome semicarbazone (10 mg/kg i.p.). In one experiment the labeled compound was employed; in the other, excretion was followed by means of spectrophotometric assays. The results of the two experiments are comparable.

Results with intraperitoneal and intravenous (i.v.) administration are very similar, although less variability was encountered with i.v. injections. Table 2 shows that, dependent on the dose level, one third to two thirds of the administered adrenochrome semicarbazone could be found in the urine. The lesser recovery at higher dose levels is undoubtedly due to a greater conversion to other metabolites.

Toxic effects, noted only at dose levels above 100 mg/kg, were exhibited by the susceptibility of the rats to seizures which are especially triggered by audiogenic

TABLE 1. EXCRETION OF ADRENOCHROME SEMICARBAZONE

Collection interval (hr)	Experiment A		Experiment B	
	Total dis/min	Per cent of total activity	Adrenochrome semicarbazone excreted (μg)	Per cent of injected dose
0–6	903,600	38	1,608	45
6–24	374,772	16	466	13
24–48	803,823	3	70	2
48–72	13,665	0·59	37	1
72–96	2,550	0·11		
96–120	6,801	0·03		
Total	1,375,621	59		61

Comparison of two experiments in which adrenochrome semicarbazone was administered (10 mg/kg) to Sprague–Dawley rats. In each experiment the average excretion from three rats weighing approximately 100 g is measured. In one experiment the labeled compound, specific activity 0·35 μc/mg was used; in the other, nonlabeled compound, with the excretion being followed by spectrophotometric assay.

TABLE 2. ADRENOCHROME SEMICARBAZONE EXCRETION AS A FUNCTION OF DOSE LEVEL

Dose (mg/kg)	Time (hr)	Recovered (%)
100	6	7
	24	29
	48	33
	72	35
80	6	8
	24	34
	48	37
	72	39
40	6	68
	24	78
	48	82
	72	82
20	6	58
	24	71
	48	73
	72	74
10	6	45
	24	58
	48	60
	72	61

Results based on duplicate experiments of average excretion from two rats at each dose level (after the animals had received unlabeled adrenochrome semicarbazone and the excretion was measured spectrophotometrically).

stimuli. This phenomenon has been reported by a number of investigators[6, 7] to be general upon administration of hydrazides and related compounds. The effect has been also noted with hydrazones and semicarbazones such as acetone semicarbazone.

Identity of the urinary metabolites

Radioautography of a two-dimensional chromatogram of a urine concentrate from rats that had received ^{14}C-adrenochrome semicarbazone indicated that, in addition to a large amount of unchanged adrenochrome semicarbazone, there were traces of three or four other metabolites.

Further paper chromatographic studies, in which the activity of each metabolite was counted by means of the Actigraph paper-strip counter, indicated that the radioactivity was distributed among the metabolites as shown in Table 3, which also

TABLE 3. RADIOACTIVITY AND R_f OF THE METABOLITES

Metabolite	Per cent of total radioactivity	Semicarbazide moiety attached	Butanol: acetic acid: water 8:2:2 (R_f)	H_2O (R_f)
A	6	Yes	0·00	0·65
B	5	No	0·18	0·64
C	69	Yes	0·41	0·36
D	17	Yes	0·53	0·45
E	3	No	0·66	0·48

gives the R_f of the various metabolites in the butanol:acetic acid:water system and in the water system.

Metabolite A. Metabolite A accounted for about 6 per cent of the radioactivity in the urine. It was partially purified by passing the rat urine through the cellulose powder column. The initial fraction was collected until the yellow adrenochrome semicarbazone band was about to be eluted from the column. The eluate was concentrated *in vacuo* to about 1 ml, spotted as a streak on Whatman 3MM paper, and chromatographed in the butanol:acetic acid:water system. The material remaining at the origin was eluted and referred to as metabolite A.

After administration of either ^{14}C-adrenochrome semicarbazone or adrenochrome-^{14}C-semicarbazone, radioactivity was found in areas on chromatograms corresponding to metabolite A, indicating that the semicarbazone moiety was intact. This was confirmed by the absorption spectrum of this compound, which is very similar to adrenochrome semicarbazone, and by a positive reaction with hypochlorite reagent, indicating the presence of an NH or NH_2. The compound also reacted in a manner similar to that of adrenochrome semicarbazone, since it was both Ehrlich- and ninhydrin-negative. Color reactions for various metabolites and standards are given in Table 4. The Ehrlich reaction, on standing for 24 hr, was only weakly positive. The persulfate oxidation and fluorindal reactions for indoxyl were negative. The diazotization and coupling with Ekman's reagent were negative, indicating the absence of an aromatic amine. The chromatographic behavior of this metabolite indicated that in all probability it was a conjugate. A negative naphthoresorcinol test and the absence of change on treatment with glucuronidase ruled out the

possibility that the metabolite was a glucuronide. A positive test with the rhodizonate reaction indicated that metabolite A was a sulfate conjugate. This was confirmed by sulfation of some 6-hydroxy-5-semicarbazido-N-methylindole: products behaving in a manner identical with that of metabolites A and B were obtained.

TABLE 4. COLOR REACTIONS AND STANDARDS FOR METABOLITES

Metabolites	Structural formula in Fig. 1	Ehrlich fluorindal	Diazotization for aromatic amine	Hypochorite starch KI	Perchloric acid followed by acidic diazo reagent	$BaCl_2$ rhodizonic acid
A	VI	–	–	+	+	+
B	VII	Yellow	+	+	+	+
C	I	–	–	+	–	–
D	I	–	–	+	–	–
Standards						
Adrenochrome semicarbazone	I	–	–	+	–	–
6-Hydroxy-5-semicarbazido-N-methylindole	III	Blue	–	+	+*	–
Sulfation mixture						
R_f 0·00 product	VI	–	–	+	+	+
R_f 0·18 product	VII	–	+	+	+	+

* Positive acidic diazo without perchloric acid treatment.

The occurrence of reduction and internal rearrangement to give 5,6-dihydroxyindoles is a quite common phenomenon with aminochromes. The analogous reaction appears to occur with adrenochrome semicarbazone upon catalytic hydrogenation, resulting in the formation of 6-hydroxy-5-semicarbazido-N-methylindole. The same reaction occurs *in vitro* when adrenochrome semicarbazone is incubated with rat liver minces. The exact nature of this reaction is currently under investigation.

The infrared spectrum of material prepared by the reduction of adrenochrome semicarbazone is compatible with the structure of 6-hydroxy-5-semicarbazido-N-methylindole (Fig. 1, III). The main features of this spectrum, compared with those of adrenochrome semicarbazone, are the appearance of a peak at 3497 cm^{-1}, which is due to an intramolecular bonded OH, and a shift of the 1690 cm^{-1} peak of adrenochrome semicarbazone to 1653 cm^{-1} due to loss of the semicarbazone-conjugating system. This loss is also evident in the visible spectrum by the loss of the 354-mμ peak and 440-mμ inflection. 6-Hydroxy-5-semicarbazido-N-methylindole is easily reoxidized on shaking a solution in the presence of air. The reoxidized compound has a spectrum identical with metabolite A and differs from adrenochrome semicarbazone in that the 440-mμ inflection has shifted to 425–475 mμ.

If 6-hydroxy-5-semicarbazido-N-methylindole was an intermediate in the formation of metabolite A, position 3 would be unsubstituted. The negative indoxyl reaction indicates that the conjugation does not occur in position 3.

Conjugation occurring at position 6 is likely, since the acidic diazo reaction of Jepson[4] became positive after perchloric acid hydrolysis, indicating the presence of a free 6-hydroxy group after hydrolysis.

On the basis of the evidence presented, the structure labeled VI in Fig. 1 is postulated for metabolite A.

Metabolite B. This compound was purified in the same manner as metabolite A. Material corresponding to an R_f 0·18 was eluted from the paper chromatogram run in the butanol:acetic acid:water system. Tracer studies indicated that it accounts for about 5 per cent of the activity in the urine and that in this metabolite the semicarbazone moiety was absent, since it could not be detected when the animal received adrenochrome-^{14}C-semicarbazone.

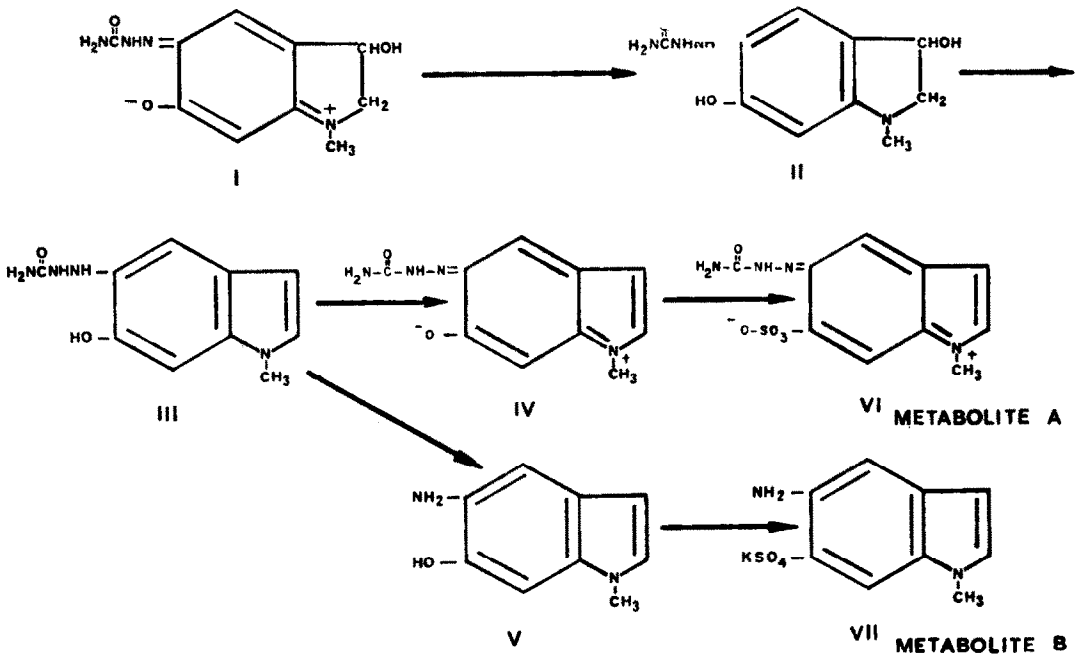

FIG. 1. Pathway of adrenochrome semicarbazone metabolism in the rat, giving rise to urinary metabolites A and B.

The metabolite gave a yellow color with Ehrlich's reagent and a positive diazo test for an aromatic amine, indicating that a part of the semicarbazone moiety had been split off, leaving an aryl amine. The compound gave a negative ninhydrin reaction.

The test for sulfate conjugation also was positive for this compound, as was the acidic diazo test for conjugation occurring in position 6. On the basis of this evidence, structure VII in Fig. 1 is postulated for metabolite B.

Metabolite C. This compound is undoubtedly unchanged adrenochrome semicarbazone. The evidence for this is the chromatographic similarity between it and the injected material, as well as identical absorption spectrum in the visible, u.v., and i.r. ranges. The metabolite was isolated by chromatography on cellulose powder. The fraction corresponding to the adrenochrome semicarbazone band was eluted and concentrated *in vacuo*; the adrenochrome semicarbazone that precipitated was recrystallized from ethanol.

Metabolite D. This metabolite is adrenochrome semicarbazone, the chromatographic behavior of which has been slightly modified by protonation of the molecule, resulting in a slower rate of migration, for example, in the butanol:acetic acid:water system (R_f 0·41, in contrast to adrenochrome semicarbazone standard R_f 0·53); on treatment with 1 N hydrochloric acid, adrenochrome semicarbazone exhibited this slow migration rate. If metabolite D and adrenochrome semicarbazone, which had been treated with 1 N hydrochloric acid, were put into a neutral or slightly basic medium prior to chromatography, both exhibited a migration rate similar to that of the adrenochrome semicarbazone standard. This form of adrenochrome semicarbazone accounts for about 17 per cent of the activity in the urine as estimated by chromatographic studies. Some of this is undoubtedly an artifact due to isolation and chromatographic procedures, as it is doubtful that protonation to the extent of 17 per cent would occur at the pH of rat urine.

Metabolite E. On the basis of tracer experiments, metabolite E accounts for about 3 per cent of the radioactivity. These studies also indicate that the semicarbazide moiety has been lost, as this compound cannot be detected radioautographically on chromatograms of urine from rats which had received the semicarbazide-labeled adrenochrome semicarbazone. The compound could be detected consistently in the urine when the adrenochrome-labeled adrenochrome semicarbazone was administered. Because of difficulties in its purification it has not been characterized further.

From these results, the metabolic sequence illustrated in Fig. 1 is thought to account for urinary metabolites A and B. The initial sequence of reactions resulting in the reduction and rearrangement of adrenochrome semicarbazone to 6-hydroxy-5-semicarbazido-N-methylindole, III, occurs *in vitro* when a rat liver mince was incubated under anaerobic conditions with adrenochrome semicarbazone. The product obtained in this reaction is identical with the compound obtained by catalytic hydrogenation of adrenochrome semicarbazone.

The 6-hydroxy-5-semicarbazido-N-methylindole, III, is subsequently either oxidized or further reduced, resulting in either 6-hydroxy-N-methylindole semicarbazone or 5-amino-6-hydroxy-N-methylindole. Both of these compounds are excreted as the sulfate conjugate with the conjugation occurring in position 6.

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REFERENCES

1. P. FISCHER and J. LECOMTE, *C.r. Séanc. Soc. Biol.*, **142**, 1446 (1948).
2. R. A. HEACOCK, C. NERENBERG and A. N. PAYSA, *Can. J. Chem.* **36**, 853 (1958).
3. J. NOVAL, A. SOHLER, S. P. STACKHOUSE and A. C. BRYAN, *Biochem. Pharmac.* **11**, 467 (1962).
4. J. B. JEPSON, *Chromatographic and Electrophoretic Techniques*, (Ed. I. SMITH), vol. 1, p. 183. Interscience, New York (1960).
5. D. P. BURMA, *Analyt. Chem. Acta* **9**, 513 (1953).
6. E. H. JENNY and C. C. PFEIFFER, *J. Pharmac. exp. Ther.* **122**, 110 (1958).
7. H. DIEKE, *Proc. Soc. exp. Biol. Med.* **70**, 688 (1949).