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URINARY EXCRETION OF HYDROXYSTEROIDS*, 17-KETOSTEROIDS AND ALDOSTERONE IN RATS DURING A CYCLE OF TREATMENT WITH MORPHINE

E. PAROLI and P. MELCHIORRI

Institute of Pharmacology, University of Rome, Rome

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Abstract—The effects of morphine and nalorphine on excretion of hydroxysteroids (3 : 21-hydroxysteroids), 17-ketosteroids and aldosterone have been examined in rats. A short cycle of treatment (5–10 days) with morphine (15–75 mg/g body weight daily) has determined an increase of urinary levels of hydroxysteroids. A longer term treatment markedly decreased all steroids in urine as long the injected doses of morphine has shown a "stabilizing" effect. The depressing effect of morphine on adrenal steroid excretion appeared to be only slightly reduced by ACTH injections. Withdrawal of morphine as well as nalorphine injection induced a striking and sustained increase of both aldosterone and hydroxysteroids, whereas 17-ketosteroids attained normal levels. Nalorphine alone reduced the urinary steroids very slightly. A combination of morphine and nalorphine did not produce the same behaviour of urinary steroids previously observed in morphinized rats.

SINCE administration of certain steroids has been found to modify the analgesic effect of morphine and the development of tolerance for the drug,[1,2,3,5] the possibility cannot be rejected that during a repetitive morphine administration a new pattern in production and metabolism of endogenous steroids occurs and may account for the development of a modified behaviour towards the drug.

Previously we have reported[4] that rats briefly treated with a single daily dose of morphine (15 mg/kg body weight) excreted more hydroxysteroids and less 17-ketosteroids and conjugated forms, whereas animals which had long been fully tolerant to the analgesic effect of morphine excreted 17-ketosteroids at approximately normal levels and hydroxysteroids much above control values.

The present paper, which is a comprehensive account of our experiments on this subject from 1958, deals with urinary excretion of steroids in rats to different dosage levels of morphine, to the stage of treatment, and to the withdrawal of the drug or abstinence from nalorphine. For this purpose various rat strains were tested and estimation of steroids in urine was performed by properly sensitive methods. In some experiments the response of adrenal cortex of morphinized rats to injected ACTH or to cold was also investigated.

* According to Weinman and Jayle method in rat urine we found mainly 3 : 21-hydroxysteroids. This agrees with the observation that rat adrenals produce almost entirely corticosterone (Bush; Morris and Williams[12]).

METHODS

Seventy adult rats of the Sprague–Dawley strain and ten male rats of the following strains: Wistar, Long Evans, Swiss albino rats (weighing 150 ± 20 g) were used.

Influence of strain, diet, ambient temperature and relative humidity on basal excretion of steroids in urine was preliminarily examined to select proper experimental conditions (Tables 1 and 2). Fifty-five normal Sprague-Dawley rats, grouped as for experiments with morphine, were kept in separate metabolism cages on standard diet (ambient temperatures 20 ± 2 °C, relative humidity 70 ± 10 per cent) and studied for urinary excretion of steroids during 40 days. Each group of rats was then given morphine.

Two groups of twelve rats received, respectively, 25 mg and 75 mg/kg body weight of morphine daily, for 40 days; one group of seven rats received 10 mg/kg of morphine daily for 140 days; two groups of six rats received, respectively, 15 mg and 30 mg/kg of morphine daily for 40 days; three groups of four rats received, respectively, 5 mg and 20 mg/kg of morphine or 20 mg/kg of morphine plus 20 mg/kg of N-allilnormorphine daily for 40 days.

All rats (excepting 140-day treated animals) were autocontrols. Four normal rats received 50 mg/kg of N-allilnormorphine daily for 40 days. Morphine was injected i.p. as sulphate; N-allilnormorphine was injected i.p. as bromidrate.

Effects of cold and ACTH on adrenocortical steroid excretion were studied, respectively, by abruptly lowering the ambient temperature to 5 °C for 5 hr, and by injecting ACTH intraperitoneally at 15 I.U./kg body weight.

Hydroxysteroids and 17-ketosteroids were analysed on 72-hr collections of urine from each rat; in attempting to estimate urinary aldosterone, the detection of this steroid was performed on reunited fractions from 8-day collections of urine of each rat. Moreover when striking variations of urinary steroids were to be expected, as after ACTH injection, cold and on withdrawal, reunited 24-hr collections of urine from three rats or eight rats were analysed for hydroxysteroids, 17-ketosteroids and aldosterone, respectively, provided in the same manner for controls.

The samples of urine were hydrolysed and extracted for chromatography. A combination of acid and enzymatic hydrolysis was performed according to Romanoff *et al.*[6] After hydrolysis, 0·5 mg of purified NaCl was added to urinary specimens and extraction was made using a mixture of benzene–chloroform–ether (1:1:1) to avoid troublesome emulsions according.[7] The extracts were taken up in 50% ethanol, and passed through a kieselguhr reversed-phase partitional column pretreated with vapour phase of dialkyldichlorosilane, as described by Howard and Martin,[8] using benzene–ether for the stationary phase and 50% ethanol for the mobile phase. This column extracted very efficiently the interfering pigments.

Column chromatography

The first 6 ml of column effluent were collected, evaporated to dryness *in vacuo*, dissolved in 0·1 ml of equilibrated 25% ethanol and transferred to the analytical column for partitional chromatography. The column chromatography was carried out with a kieselguhr column packed according to Butt *et al.*[9] using 25% equilibrated ethanol as stationary phase and toluene as mobile phase. Only the most polar steroids were fractionated by this column,* whereas the less polar steroids (i.e. 17-ketosteroids),

* Aldosterone and hydroxysteroids.

TABLE 1. URINARY STEROIDS IN RATS RESPECTING TO SOME BASAL CONDITIONS

Basal conditions	Urinary steroids (μg/24 hr $\pm$ s.d.)					
	Hydroxysteroids		17-Ketosteroids		Aldosterone	
	mean	range	mean	range	mean	range
(a) Strain*						
Sprague–Dawley	29·3 $\pm$ 2·5	(23·5 – 34·5)	6·9 $\pm$ 1·0	(5·5 – 8·3)	0·23 $\pm$ 0·02	(0·15 – 0·30)
Wistar	36·5 $\pm$ 2·4	(32·5 – 40·8)	6·2 $\pm$ 1·8	(3·7 – 9·8)	0·28 $\pm$ 0·06	(0·20 – 0·35)
Long Evans	51·7 $\pm$ 2·0	(47·5 – 56·3)	2·4 $\pm$ 1·1	(0·8 – 3·9)†	0·28 $\pm$ 0·07	(0·25 – 0·38)
Commercial albino rats	15·2 $\pm$ 4·0	(10·3 – 22·2)	14·4 $\pm$ 1·7	(8·8 – 15·7(25))‡	0·12 $\pm$ 0·04	(0·10 – 0·20)
(b) Diet (Sprague–Dawley)						
Diet *ad libitum*	31·1 $\pm$ 2·0	(28·7 – 34·5)	7·3 $\pm$ 1·2	(5·5 – 10·4)		
Standard diet	29·3 $\pm$ 2·5	(23·5 – 34·5)	6·9 $\pm$ 1·0	(5·5 – 8·3)	0·23 $\pm$ 0·02	(0·15 – 0·30)
(c) Ambient temperature (Sprague–Dawley)						
20 $\pm$ 2 °C	29·3 $\pm$ 2·5	(23·5 – 34·5)	6·9 $\pm$ 1·0	(5·5 – 8·3)	0·23 $\pm$ 0·02	(0·15 – 0·30)
25 $\pm$ 2 °C	24·2 $\pm$ 2·2	(22·5 – 26·4)	5·6 $\pm$ 0·8	(7·5 – 3·4)		
(d) Relative humidity (Sprague–Dawley)						
45 per cent	31·0 $\pm$ 2·0	(28·7 – 32·7)	7·2 $\pm$ 0·7	(6·5 – 10·3)		
70 per cent	29·3 $\pm$ 2·5	(23·5 – 35·5)	6·9 $\pm$ 1·0	(5·5 – 8·3)	0·23 $\pm$ 0·02	(0·15 – 0·30)

* At 20 $\pm$ 2 °C, standard diet and relative humidity 70 per cent (ten rats for each strain).
† Low conjugation activity of steroids was found in Long Evans strain.
‡ Some rats excrete an unidentified 11–17-dioxysteroid.

running practically with toluene front, were collected in the first 3–4 ml of column effluent. Aldosterone and hydroxysteroids were separated and collected respectively in the fifth to eighth and tenth to fifteenth millilitre of column effluent.

Estimation of steroids in single fractions

An aliquot of each fraction was analysed by the following methods: (i) Weinmann and Jayle[10] method (a modification of the original Hill method) for hydroxysteroids (sensibility = 15 μg $\pm$ 3 per cent); (ii) Masson and Corcoran method[11] for α-ketolic steroids; (iii) polarography of Girard hydrazones for Δ^4-3-keto steroids and 17-ketosteroids, according to Morris and Williams[12]. The last two methods have been used by the authors to detect steroids in rat plasma or urine.

Paper chromatography

An aliquot of the various collected fractions from the chromatograph column were put on Whatman no. 4 filter paper for chromatography, cut according to Rubin *et al.*[13]

TABLE 2. COMPOSITION OF THE DIET

Main constituents (g/kg diet)		Vitamins (mg/kg diet)		Vitamins (mg/kg diet)		Vitamins (mg/kg diet)	
Vitamin free		Vit. A,	50	Pantothenic ac,	100	Vit. B_{12},	0·02
Casein,	220	Vit. D,	20	Niacinamide,	200	Vit. C,	1000·00
Starch,	630	Vit. E,	500	Choline,	2000		
Vegetable,		Vit. K,	10	Inositol,	1000		
Oils,	100	Vit. B_1,	50	PABA,	300		
Salt mixture,	10	Vit. B_2,	20	Biotin,	1		

Descending chromatograms of the most polar steroids (i.e. hydroxysteroids and aldosterone) were run with toluene, as mobile phase, previously equilibrated with 75% methanol. The 17-ketosteroids ones were run by the ligroin-propylene glycol equilibrated system at a temperature maintained at 23 ° $\pm$ 2 °C, according to Savard.[14] Parallel chromatograms of authentic samples of each steroid were run in the same manner.

Spot detection in chromatograms

In each chromatogram steroid spots were detected by means of the following reactions: (i) tetrazolium method for α-ketol grouping in C_{17} (using nitro bleu tetrazolium, NBT, in place of bleu tetrazolium to make it suitable for quantities down about 0·1 μg/cm^2) according to Chen and Twell;[15] (ii) Zimmerman reaction for 17-ketosteroids according to Savard[16] (sensibility = 2·5 μg/cm^2); (iii) Pincus method (antimony trichloride) for hydroxysteroids (sensibility 2·5 μg/cm^2);[18] (iv) NaOH fluorescence according to Simpson and Tait[17] for Δ^4-3-ketosteroids (0·25 –1 μg/cm^2 sensibility).

Alternatively, undeveloped spots were eluted and analysed for ketosteroids by polarography of Girard's hydrazones and by infra-red spectroscopy.

Estimation of aldosterone

Particular attention has been given to the intriguing problem of aldosterone detection in rat urine. Parallel chromatograms of aldosterone column fraction and of

authentic sample of aldosterone were run in the same manner and aldosterone zones were determined by comparison of standard strip on which the spots were developed using the NBT method. Undeveloped spots were eluted with methanol and after solvent evaporation the residue was partitioned between 10 ml of dichloromethane and 2 ml of water, and the water phase discarded.

A first sample (half) of dichloromethane phase was analysed by polarography of Girard hydrazones (sensibility = 0·2 μg)[12].

A second sample was put on chromatographic paper, acetylated and oxidized, and run into two different systems according to Mattox *et al.*[19] Parallel chromatograms of authentic sample of acetylated and oxidized aldosterone were run in the same manner for accurate detection of spots. Alternatively for the purpose of an exclusively qualitative identification, infra-red spectrum of aldosterone eluted spots was recorded and compared with an infra-red curve of standard aldosterone.

Apparatus, and chemicals

For polarographic procedure Sergent's polarograph was used with the micro-polarographic cell described by Morris and Williams.[12] For fluorescence analysis Beckman's DU spectrophotometer was employed. Infra-red spectra of steroids were registered in Beckman's infra-red spectrophotometer.

All the chemicals used, with the exception of standard steroids, were products of Merck, Darmstadt. Highly purified standard steroids were a gift from N. V. Organon, which we gratefully acknowledge.

NaCl for polarographic procedure was recrystalized three times from ethanol to free it from interfering impurities giving a polarographic wave approximately at −1·1 V *tert*-Butanol, used as solvent for the Girard reagent in the polarographic procedure, was purified by ten successive extractions with half-saturated KCl solution, dried and distillated according to Morris and Williams.[12]

A glass development chamber was used for descending development of chromatograms, previously saturated with the vapours of the mobile phase and maintained at a controlled temperature. Paper was saturated with the stationary phase and brought to equilibrium with the atmosphere in the chamber for 24 hr prior to irrigation with solvent. All the chromatograms were run in the machine direction of the paper.

RESULTS

(a) *Urinary steroids in normal rats with respect to some basal conditions*

As shown in Table 1 excretion of steroids was found to vary according to strain, diet, ambient temperature and relative humidity. In the present work an inbred strain, Sprague–Dawley, appeared to be preferable for its homogeneity. On standard diet, atmospheric pressure 760 ± 10 mm Hg, ambient temperature 20 ± 2 °C, relative humidity 70 ± 10%, the urinary levels of steroids were found in this strain to range, for hydroxysteroids from 23·5–34·5 μg/24 hr, for 17-ketosteroids from 5·5 to 8·3 μg/24 hr, and for aldosterone from 0·15 to 0·30 μg/24 hr.

Paper chromatography of hydroxysteroids in toluene–75%methanol has given three spots (X_1, X_2, X_3) on the strip of which the middle one (X_2) was found to run at the same rate of pregnane-3α:11β:21-triol-20-one (Fig. 1). The other spots are still unknown (X_1 and X_3).

Two of the three spots (X_1 and X_2) developed a blue colour with the NBT method and all of them a brown colour with the $SbCl_3$ method showing, respectively, a α-ketolic chain in C_{17} and 3-hydroxy grouping. No reaction occurred with the Zimmerman method nor with NaOH fluorescence, indicating that 17-keto and Δ^4-3 keto groupings are absent. Polarography of Girard hydrazones of eluted X_2 spot have shown the typical polarographic wave of the 20-keto grouping, in agreement with the NBT method, but no 3-keto wave. The infra-red spectrum of this spot has given a curve identical to that recorded from an authentic sample of pregnane-3α:11β:21-triol-20-one. Polarography of eluted X_3 spot has given no polarographic wave showing no keto grouping. This finding was confirmed by infra-red spectrum of eluted spot. The method of Weinmann and Jayle[10] for hydroxysteroids has given 1 μEquiv. of acetylable hydroxy radicals per 180 μg of steroid showing that two acetylable OH radicals probably occur in steroid molecules.

From these findings it may be assumed that the probable structure of this steroid is that of pregnane-3α:20-diol with or without a non-reactive βOH radical in C_{11}.

The X_1 spot has given such a small amount of substance that no investigation was performed on its steroid content.

A two-step paper chromatography of 17-ketosteroids was performed. The paper strips were first chromatographed for 96 hr (Fig. 2). The portion of steroid mixture which has run off the lower end of the chromatograms was rechromatographed on fresh sheets of paper for 24 hr (Fig. 3). In 96-hr chromatograms, two spots were detected (X_4–X_5) with *Rt* closely similar to etiocholanone (X_4) and androsterone (X_5). These spots have given only the Zimmerman reaction. In 24-hr chromatograms another two spots were found by the Zimmerman method (X_6–X_7). The steroids apparently run with the same rate of etiocholan-3α:11β-diol-20-one (X_6) and androstene 3α:11β-diol-20-one (X_7).

(b) *Urinary excretion of steroids in rats chronically treated with morphine at different dosage levels*

According to our findings the schematization of a cycle of treatment with morphine in rats respecting to urinary pattern of steroids and animal behaviour may be suggested. For this purpose we have divided the entire cycle of treatment into three stages: (i) first stage from 0 to 10 days; (ii) second stage from 10 to 40 days; (iii) third stage from 40 to 140 (or more) days. If the dose of morphine is not increased during the last stage of treatment, the third stage apparently resembles the abstinence stage since the injected dose of morphine is no more stabilizing.

First stage (Tables 3–7; Figs. 4–7). At dosage levels of morphine ranging from 15 mg to 75 mg/kg body weight daily, an increase of hydroxysteroids and a decrease of 17-ketosteroids and possibly of aldosterone were observed, whereas at lower dose levels of morphine a slight decrease of the aforementioned steroids has taken place after the onset of treatment. During this stage morphine has induced a full analgesic effect. Moreover the higher injected doses have produced typical "excitatory" phenomena in rats.

Second stage (Tables 3–7; Figs. 4–7). In this stage all the doses injected decreased the urinary excretion of the investigated steroids. At the end of this stage analgesic response to morphine appeared to be lessened although the reaction time to painful stimulus amounted to from one and a half times to twice the normal reaction time.

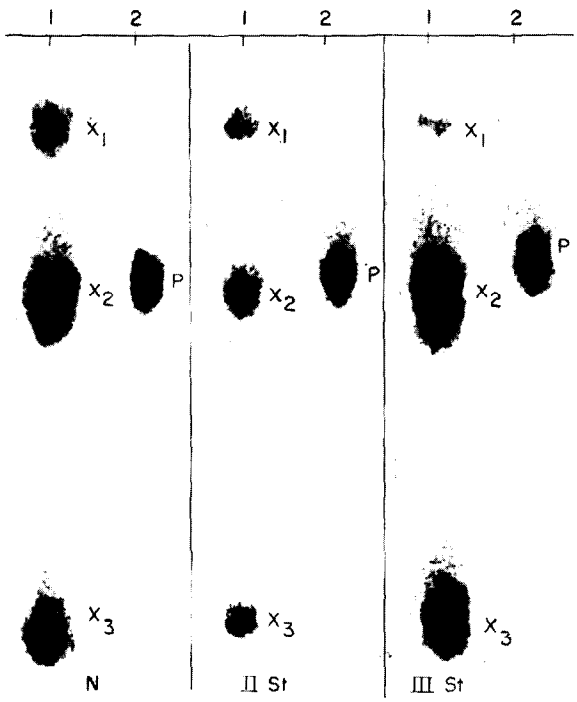

FIG. 1. Fractionation of urinary hydroxysteroids from: N, normal rats; II St, rats under 30 days treatment with morphine; III St, rats under 140 days treatment with morphine. X_1, X_2, X_3, spots of urinary hydroxysteroids; P, spot of standard steroid (pregnane-3α:11-β:21-triol-20-one).

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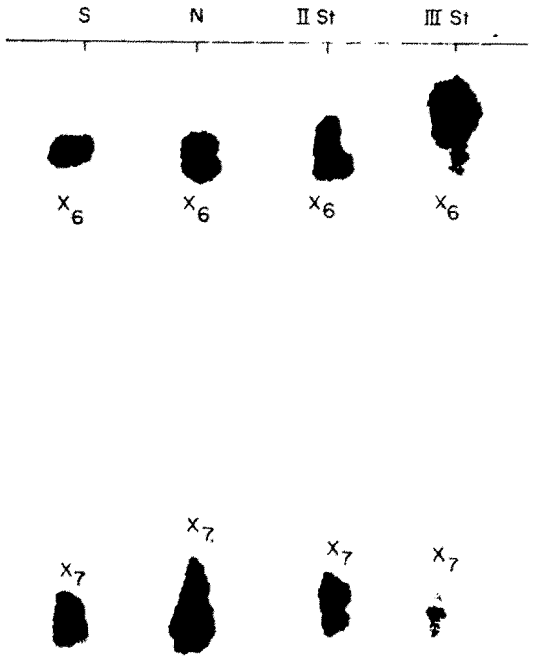

FIG. 3. Fractionation of urinary 17-ketosteroids in 24-hr chromatogram from: N, normal rats; II St, rats under 30 days treatment with morphine; III St rats under 140 days treatment with morphine S, standard steroids; E, ethiocolan-3α : 11β-diol-20-one; A, androstene-3α : 11β-diol-20-one. X_6, X_7, urinary ketosteroids.

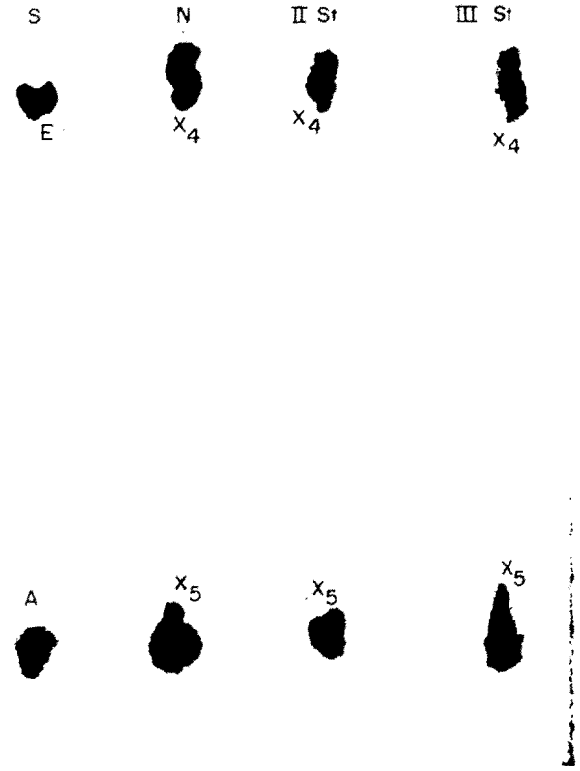

FIG. 2. Fractionation of urinary 17-ketosteroids in 96-hr chromatogram from: N; normal rats; II St, rats under 30 days treatment with morphine; III St, rats under 140 days treatment with morphine S, standard steroids, i.e. E, etiocholanone, A, androsterone. X_4, X_5, urinary 17-ketosteroids.

The behaviour of the animals was characterized by reduced activity, loss of weight, ipotermia and unusual incidence of abscesses in skin, lung and peritoneum. Urinary steroid fractionation on the thirtieth day of the treatment by paper chromatography has given a steroid pattern similar to the controls but with lower colour intensity of the developed spots (Figs. 1–3).

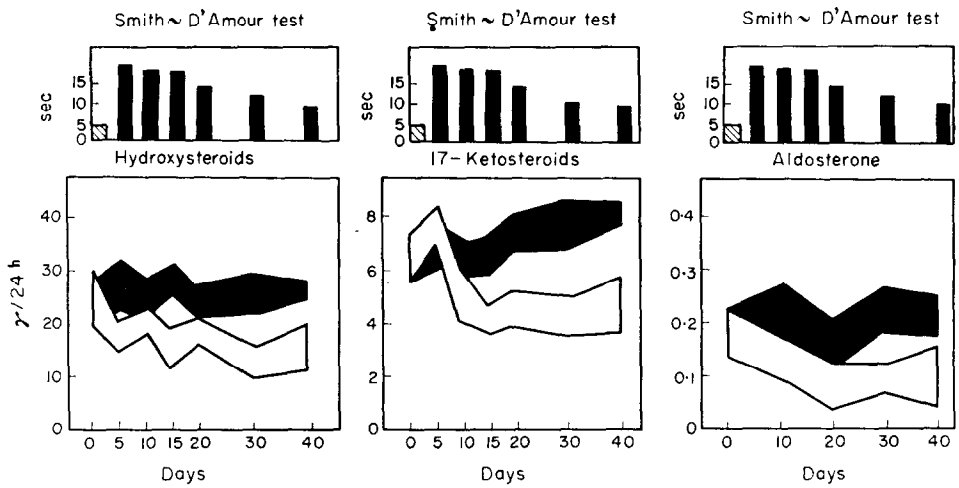

FIG. 4. Range of urinary steroids in rats during a treatment with morphine (5 mg/kg body weight daily). Solid area; controls. Open area; rats under morphine.

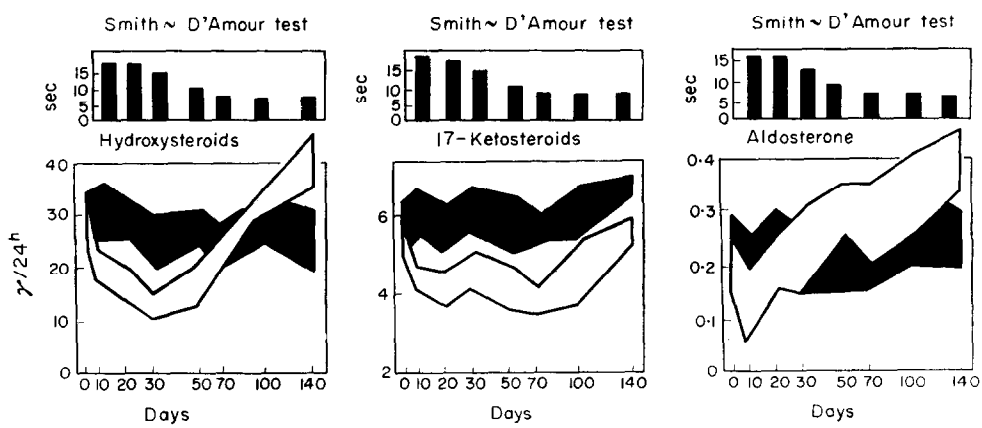

FIG. 5. Range of urinary steroids in rats during a long term treatment with morphine (10 mg/kg body weight daily). Solid area: controls. Open area: rats under morphine.

Third stage (Table 3; Fig. 5). Repeated administration of morphine at the same dose levels (no more stabilizing) induced in rats an aggressive behaviour and eventually cannibalism, while the reaction time to painful stimulus appeared to approximate more and more to the normal values. At this stage the 17-ketosteroids have increased to

TABLE 3. URINARY STEROIDS IN RATS DURING A TREATMENT WITH MORPHINE (10 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr $\pm$ s.d.)					17 Ketosteroids (μg/24 hr $\pm$ s.d.)				Aldosterone (μg 24/ hr $\pm$ s.d.)				
			P (t test)				P (t test)					P (t-test)	
Days	Controls	Treated	Respecting controls	Between days	Controls	Treated	Respecting controls	Between days	Days	Controls	Treated	Respecting controls	Between days
0	29·2 ± 3·61	29·1 ± 3·50		(0–I) >0·05	5·7 ± 0·40	5·6 ± 0·56		(0–I)	0	0·27 ± 0·02	0·26 ± 0·07		
8–10 (I)	29·5 ± 3·16	24·5 ± 5·68	>0·05	(I–II) <0·05	5·7 ± 0·42	4·4 ± 0·46	<0·001	<0·01 (I–II)	3–10 (I)	0·24 ± 0·03	0·15 ± 0·05	<0·01	(O–I) <0·01
18–20 (II)	29·7 ± 3·10	17·8 ± 4·38	<0·001	(II–III) <0·05	5·6 ± 0·49	3·9 ± 0·81	<0·001	<0·01 (II–III)					(I–III) <0·01
28–30 (III)	26·1 ± 3·16	12·4 ± 4·15	<0·001	(III–IV) <0·001	5·9 ± 0·57	3·3 ± 0·54	<0·001	<0·001 (III–IV)					
68–70 (IV)	26·3 ± 2·90	24·9 ± 1·41	>0·05	(IV–V) <0·001	5·7 ± 0·31	3·8 ± 0·41	<0·001	>0 05 (IV–V)	63–70 (II)	0·25 ± 0·33	0·27 ± 0·05	>0·05	
98–100 (V)	27·5 ± 2·66	32·4 ± 3·16	<0·01	V–VI <0·05	6·0 ± 0·65	4·5 ± 0·47	<0·001	<0·01 (I–VI)					
138–140 (VI)	26·1 ± 3·16	39·8 ± 7·50	<0 001		6·1 ± 0·74	5·2 ± 0·67	<0·05	>0·05	133–140 (III)	0·25 ± 0·09	0·36 ± 0·13	<0·05	

TABLE 4. URINARY EXCRETION OF STEROIDS IN MORPHINE TREATED RATS (15 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr $\pm$ s.d.)					17 Ketosteroids (μg/24 hr $\pm$ s.d.)				Aldosterone (μg/24 hr $\pm$ s.d.)				
			P (t-test)				P (t-test)					P (t-test)	
Days	Controls	Treated	Respecting controls	Between days	Controls	Treated	Respecting controls	Between days	Days	Controls	Treated	Respecting controls	Between days
0	27·30 ± 5·29	25·33 ± 4·57	>0·05	(0–I)	6·06 ± 2·28	6·11 ± 0·77	>0·05	(0–III)	0	0·19 ± 0·05	0·19 ± 0·03		(0–I)
3–5(I)	25·83 ± 2·56	33·66 ± 1·26	<0·001	<0·001	6·11 ± 0·61	5·06 ± 0·47	<0·01	<0·001	3–10(I)	0·22 ± 0·04	0·13 ± 0·10	>0 05	>0·05
18–20(II)	28·83 ± 2·40	22·83 ± 1·84	<0·001		6·25 ± 0·41	4·53 ± 0·66	<0·001						
				(0–III)									(0–II)
38–40(III)	29·16 ± 2·04	19·33 ± 2·44	<0 001	<0 05	6·50 ± 0·55	4·01 ± 0·55	<0·001		33–40(II)	0·22 ± 0·03	0·10 ± 0·03	<0·01	<0·01

reach about the normal level whereas the hydroxysteroids and the aldosterone increased above the starting levels.

Paper chromatograms of steroids on the 140th day of treatment have given a modified pattern. Chromatograms of hydroxysteroids have shown a paler X_1 spot and an increase in colour and size of the X_2 and X_3 spots. Ultra violet analysis of NaOH-sprayed chromatograms revealed a new fluorescent spot of small size near the lower

TABLE 5. URINARY HYDROXYSTEROIDS AND 17-KETOSTEROIDS IN MORPHINE TREATED RATS (20 MG/KG BODY WEIGHT DAILY)

	Hydroxysteroids (μg 24/hr)*		17-Ketosteroids (μg/24 hr)*	
Days	Normal	Treated	Normal	Treated
0	31·5±4·53	29·25±5·56	6·37±0·94	6·57±0·43
3–5	27·5±1·26	35·73±2·88	6·62±0·85	4·37±0·74
18–20	31·5±4·63	21·75±5·56	7·00±0·91	3·85±0·62
38–40	30·2±4·19	20·00±4·07	7·12±0·94	3·55±0·40

* ± s.d.

end of chromatograms. The NaOH fluorescence of the spot reveals 3-keto grouping but its steroid structure remains unknown. Chromatograms of 17-ketosteroids have shown X_4–X_5 spots similar to those of the second stage. Moreover the size and colour intensity of X_6 have increased while those of the X_7 have decreased, the size and colour intensity of X_6 having increased (Figs. 1–3).

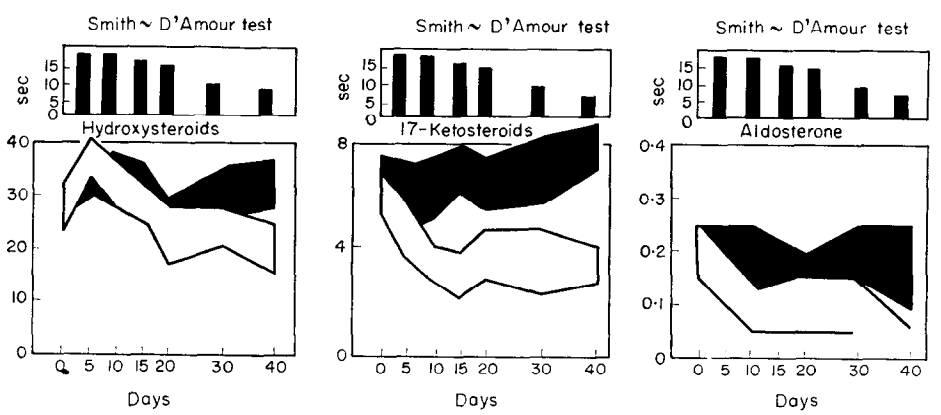

FIG. 6. Range of urinary steroids in rats during a treatment with morphine (20 mg/kg body weight daily). Solid area: controls. Open area: rats under morphine.

(c) *Urinary excretion of steroids in rats during abstinence syndrome (Tables 8 and 9)*

In the second stage of treatment the withdrawal of stabilizing doses of morphine so as nalorphine injection have induced a tremendous increase of hydroxysteroids and possibly of aldosterone in rat urine, whereas the 17-ketosteroids have attained normal levels.

TABLE 6. URINARY STEROIDS IN RATS DURING A TREATMENT WITH MORPHINE (25 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr ± s.d.)*					17-Ketosteroids (μg/24 hr ± s.d.)*				Aldosterone (μg/24 hr ± s.d.)†				
			P (t test)				P (t-test)					P (t-test)	
Days	Controls	Treated	Respecting control	Between days	Controls	Treated	Respecting controls	Between days	days	Controls	Treated	Respecting controls	Between days
0	28·5 ±6·63	27·2±3·5		(0–I) < 0·001	6·40±0·34	6·8±1·1		(0–I) < 0·001	0	0·24±0·03	0·23±0·02		(0–I) > 0·05
8–10(I)	26·6 ±4·48	35·4±2·6	< 0·01	(I–II) < 0·001	7·07±0·44	5·2±0·4	< 0·001	(I–II) < 0·001	3–10(I)	0·22±0·22	0·16±0·17	> 0·05	
18–20(II)	29·3 ±3·90	26·3±3·7	> 0·05		7·01±0·51	3·9±0·5	< 0·001						
38–40(III)	28·5 ±3·71	14·4±1·8	< 0·001	(II–II) < 0·001	7·11±0·90	4·3±0·3	< 0·001	(II–III) > 0·05	33–40(II)	0·25±0·03	0·10±0·03	< 0·001	(0–II) < 0·001

* Ten autocontrol rats.
† Eight autocontrol rats.

TABLE 7. URINARY STEROIDS IN RATS DURING A TREATMENT WITH MORPHINE (75 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr ± s.d.)*					17-Ketosteroids (μg/24 hr ± s.d.)*				Aldosterone (μg/24 hr ± s.d.)†				
			P (t-test)				P (t-test)					P (t-test)	
Days	Controls	Treated	Respecting controls	Between days	Controls	Treated	Respecting controls	Between days	Days	Controls	Treated	Respecting controls	Between days
0	27±2·2	28·1 ±3·50		(0–I)	6·71±1·5	6·85±1·1		(0–I)	0	0·21±0·03	0·22±0·02		(0–I)
3–5(I)	26±1·8	38·6 ±2·80	< 0·001	< 0·001 (I–II)	7·32±1·1	7·10±0·3	> 0·05	> 0·05 (I–II)	3–10(I)	0·22±0·24	0·16±0·09	> 0·05	> 0·05
18–20(II)	26±1·6	15·4 ±1·79	< 0·001	< 0·001 (II–III)	6·88±1·7	4·30±0·7	< 0·001	< 0·01 (II–III)					
38–40(III)	27±2·0	10·9 ±3·60	< 0·001	< 0·001	7·17±1·4	3·90±0·6	< 0·05		33–40(II)	0·22±0·24	~0·03‡		

* Ten autocontrol rats.
† Eight autocontrol rats.
‡ Measures about the method sensibility limit.

On withdrawal, or soon after nalorphine, rat abstinence syndrome[20] has developed rapidly, animals were depressed, voided and frequently excreted semiliquid evacuations. During withdrawal, tolerance to the analgesic effect of morphine was slowly lost over a period of about 10 days, whereas after nalorphine injection tolerance disappeared rapidly (a few days). After withdrawal, urinary levels of hydroxysteroids and

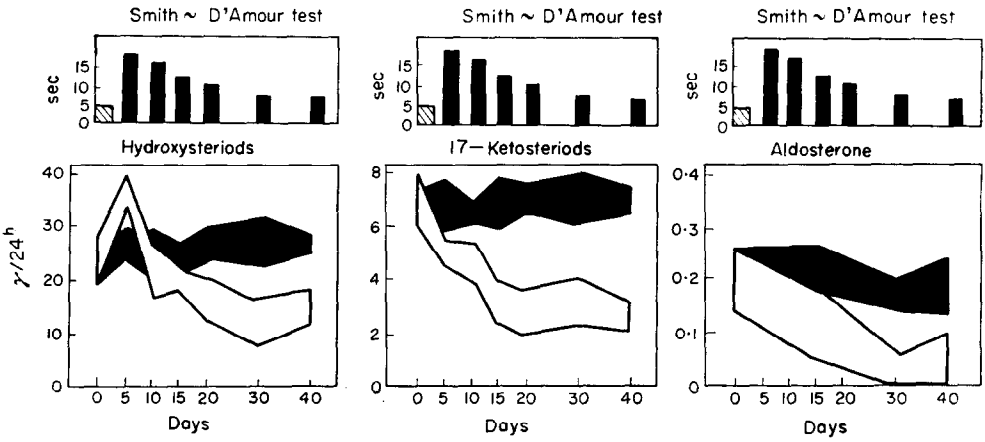

FIG. 7. Range of urinary steroids in rats during a treatment with morphine (30 mg/kg body weight daily). Solid area: controls. Open area: rats under morphine.

TABLE 8. URINARY EXCRETION OF STEROIDS IN RATS ON WITHDRAWAL OF MORPHINE*

Morphine	Days of treatment	Days of withdrawal	No. of samples†	Hydroxysteroids (μg/hr)‡	17-Ketosteroids (μh/24 hr)	No. of samples‡	Aldosterone (μg/24 hr)
—	—	—	3	29·2 ± 3·6	6·4 ± 0·3	1	0·25
30	40	—	2	15·3 ± 2·8	2·8 ± 0·8	—	—
30	40	3	2	80·3 ± 13·2	12·6 ± 2·8	—	—
30	40	5	2	65·4 ± 9·6	8·5 ± 1·8	—	—
30	40	8	2	55·6 ± 7·4	7·4 ± 1·7	—	—
30	40	13	2	41·3 ± 6·5	6·7 ± 1·8	—	—
75	40	—	3	13·2 ± 3·0	2·2 ± 0·9	1	0·1
75	40	3	3	102·5 ± 22·4	8·3 ± 2·2	1	0·5
75	40	5	3	90·3 ± 11·2	10·2 ± 2·6	1	0·4
75	40	8	3	70·5 ± 9·6	7·6 ± 1·8	1	0·3
75	40	13	3	55·7 ± 7·5	6·9 ± 1·8	1	0·3

* average daily excretion ± s.d.
† One sample = reunited 24-hr samples of urine from three rats.
‡ One sample = reunited 24-hr samples of urine from eight rats.

aldosterone remained high and attained normal values on approximately the thirteenth to fifteenth day, whereas after nalorphine injection complete steroid recovery was accomplished on about the fifth day.

TABLE 9. URINARY EXCRETION OF STEROIDS IN MORPHINIZED RATS ON ABSTINENCE INDUCED BY NALORPHINE

Dose of morphine mg/kg per hr	Days of treatment	Dose of nalorphine (mg/kg)	Days of abstinence	No. of samples*	Hydroxy-steroids (μg/24 hr $\pm$ s.d.)	17-Keto-steroids (μg/24 hr $\pm$ s.d.)	No. of samples†	Aldosterone (μg/24 hr $\pm$ s.d.)
—	—	—	—	4	28·5 ± 6·6	6·2 ± 0·4	1	0·3
25	40	—	—	4	10·4 ± 1·8	4·3 ± 0·3	1	0·1
25	40	50	1	4	105·5 ± 24·2	10·5 ± 3·5	1	0·7
25	40	50	3	4	38·2 ± 12·9	8·7 ± 1·6	1	0·3
25	40	50	5	4	25·3 ± 8·7	9·2 ± 1·8	1	0·2
75	40	—	—	1	12·2	4·1	—	—
75	40	50	1	1	85·5	10·4	—	—
75	40	50	3	1	45·3	7·5	—	—
75	40	50	5	1	31·5	5·3	—	—

* One sample = reunited 24 hr samples of urine from three rats.
† One sample = reunited 24 hr samples of urine from eight rats.

(d) *Effects of ACTH and cold on urinary excretion of adrenocortical steroids in morphinized rats*

Table 10 records urinary levels of hydroxysteroids aldosterone and 17-ketosteroids from morphinized rats exposed to low ambient temperature (5 °C for 5 hr).

In rats acting as untreated controls, the cold markedly increased the urinary excretion of hydroxysteroids and slightly that of the 17-ketosteroids; levels of aldosterone appear to be unaffected.

TABLE 10. EFFECT OF COLD ON URINARY STEROIDS IN RATS CHRONICALLY TREATED WITH MORPHINE (25 DAYS)

Morphine	Temperature (°C)	No. of samples*	Hydroxysteroids (μg/24 hr $\pm$ s.d.)	17-Ketosteroids (μg/24 hr $\pm$ s.d.)	No. of samples†	Aldosterone (μg/24 hr $\pm$ s.d.
	20	4	28·70±3·22	5·71±0·48	1	0·20
	5	4	82·80±7·25	9·12±0·76	1	0·22
15	20	2	15·02±2·10	3·15±0·78	—	—
	5	4	47·85±3·75	5·35±1·62	—	—
25	20	4	17·01±3·21	2·82±0·74	1	0·15
	5	4	28·92±2·45	3·42±0·74	1	0·15

* One sample = reunited 24-hr samples of urine from three rats.
† One sample = reunited 24-hr samples of urine from eight rats.

Morphine did not inhibit the effects of cold on steroid excretion, but the increase of urinary levels of hydroxysteroids and 17-ketosteroids achieved in rats to which morphine was given and which were exposed to cold, did not attain the high values obtained from the untreated controls (Table 11).

TABLE 11. INCREASE OF URINARY EXCRETION OF HYDROXYSTEROIDS + 17-KETOSTEROIDS IN NORMAL AND MORPHINIZED RATS (25 MG/KG BODY WEIGHT) AFTER TEMPERATURE LOWERING

Samples*	Treatment	Increase†	*P* (*t*-test)
4	—	57·0±6·16	<0·001
4	Morphine	12·2±4·39	

* Reunited 24-hr samples of urine from three rats.
† μg/24 hr $\pm$ s.d.

Table 12 depicts the rise of urinary hydroxysteroids and 17-ketosteroids we have found in normal rats injected with ACTH. In morphinized rats the same dose of ACTH has also increased the urinary hydroxysteroids and the 17-ketosteroids although at very lower levels than in normal animals (Table 13). Results suggested that the low levels of urinary corticosteroids achieved in morphinized rats exposed to cold may be more strictly related with the lessened response of adrenals to ACTH than with a decrease in hormone released from hypophysis.

TABLE 12. EFFECT OF ACTH ON URINARY EXCRETION OF STEROIDS IN RATS CHRONICALLY TREATED WITH MORPHINE (28 DAYS)

Morphine (mg/kg)	ACTH (15 U/kg i.p.)	No. of samples*	Hydroxy-steroids (μg/24 hr ± s.d.)	17-Keto-steroids (μg/24 hr ± s.d.)	No. of samples†	Aldosterone (μg/24 hr ± s.d.)
—	—	4	29·60 ± 3·10	5·80 ± 0·62	1	0·25
—	0·1	4	176·17 ± 25·85	22·72 ± 6·16	1	0·28
15	—	2	14·85 ± 3·04	3·10 ± 0·84	—	—
15	0·1	4	88·90 ± 8·91	9·60 ± 1·55	—	—
25	—	4	15·15 ± 2·18	3·25 ± 0·74	1	0·15
25	0·1	4	50·52 ± 4·71	11·97 ± 5·40	1	0·15

* One sample = reunited 24-hr samples of urines from three rats.
† One sample = reunited 24-hr samples of urine from eight rats.

TABLE 13. INCREASE OF URINARY EXCRETION OF HYDROXYSTEROIDS + 17-KETOSTEROIDS IN NORMAL AND MORPHINIZED RATS (25 MG/KG BODY WEIGHT) AFTER ACTH

Samples*	Treatment	Increase†	*P* (*t*-test)
4	—	163·44 ± 58·55	<0·01
4	Morphine	44·15 ± 4·81	

* Reunited 24 hr samples of urine from three rats.
† μg/24 hr ± s.d.

TABLE 14. URINARY HYDROSTEROIDS AND 17-KETOSTEROIDS IN MORPHINE PLUS NALORPHINE TREATED RATS (20 MG/KG BODY WEIGHT RESPECTIVELY DAILY)

	Hydroxysteroids (μg/24 hr ± s.d.)		17-Ketosteroids (μg/24 hr ± s.d.	
Days	Normal	Treated	Normal	Treated
0	31·20 ± 2·51	30·25 ± 2·88	6·30 ± 0·48	6·37 ± 2·00
3–5	30·10 ± 3·00	26·25 ± 2·88	5·30 ± 0·50	5·25 ± 0·60
18–20	27·25 ± 2·07	26·25 ± 1·73	6·45 ± 3·31	5·25 ± 0·45
38–40	30·25 ± 3·31	24·50 ± 3·09	6·57 ± 2·75	4·02 ± 0·68

(e) *Urinary excretion of steroids in rats given nalorphine alone or morphine plus nalorphine*

Only a slight decrease of steroids appeared after a long term treatment with nalorphine alone (Fig. 8). Rats given morphine (20 mg/kg) plus nalorphine (20 mg/kg) (Table 14, Fig. 9) excreted hydroxysteroids, 17-ketosteroids and aldosterone at higher levels than animals under morphine alone (Table 5, Fig. 6). Moreover the increase of hydroxysteroids previously observed in the first stage of treatment with morphine, at the same dose, was no longer noted after combined administration of morphine and nalorphine. During combined treatment the analgesic effect of morphine appeared to be lessened.

DISCUSSION

Although many researchers have attentively studied the complex problem of the influence of morphine on adrenal and gonad functions, few and conflicting data have been reported on the subject of production, metabolism and excretion of hormonal steroids under morphine.

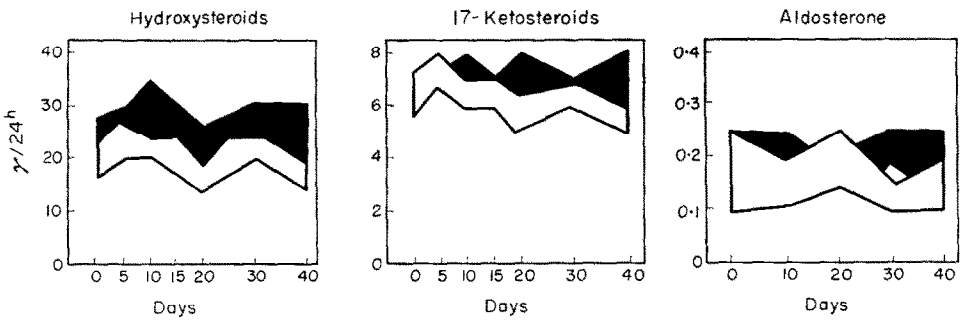

FIG. 8. Range of urinary steroids in rats during a treatment with nalorphine (50 mg/kg body weight). Solid area: controls. Open area: rats under nalorphine.

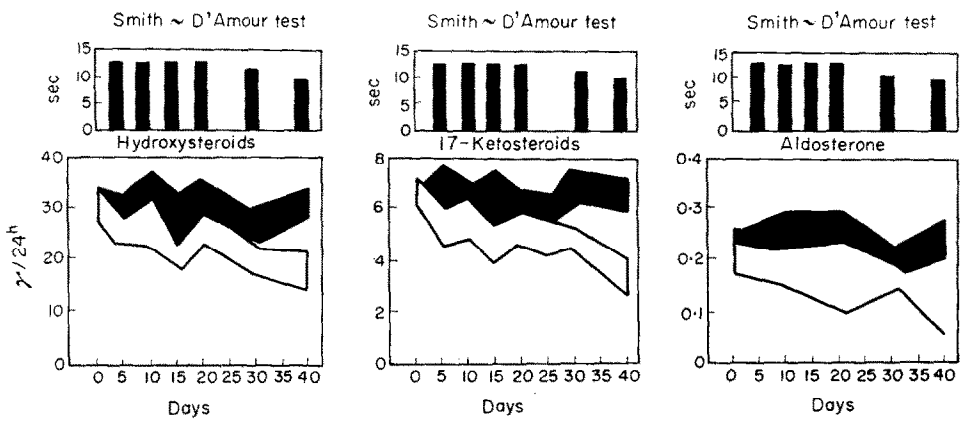

FIG. 9. Range of urinary steroids in rats under a combined treatment with morphine plus nalorphine (20 mg/kg and 20 mg/kg body weight, respectively). Solid area: controls. Opens area: rats under combined treatment.

Single doses of morphine increase in the cat the excretion of 17-ketosteroids.[21] In post-addict men[22] single subcutaneous injections of morphine as large as 45–80 mg induced a decrease of urinary 17-ketosteroids ranging from 35 to 40 per cent; higher doses, however, achieved lesser depressant effects and sometimes "erratic responses" on 17-ketosteroid excretion, indicating an adrenocortical stimulation indeed of a depression. No data are reported on the effects of analgesic doses of morphine on steroids.

The reports on the urinary pattern of glyco and mineralcorticoids after a single dose of morphine are scanty. In man an increase as well as a decrease of blood and urine 17-hydroxysteroids has been occasionally observed.[22, 23] No report is available on the production and metabolism of testicular and adrenal steroids under chronic morphine in animals.

In the case of two patients, 17-hydroxycorticosteroids[23] were found to decrease during a cycle of addiction and to increase after withdrawal. In nine volunteers addicted to morphine through increasing doses of the drug,[22] stabilizing doses of 240 mg–360 mg produced an average decrease of 50 per cent in 17-ketosteroid excretion. Withdrawal accomplished an average increase of 17-ketosteroids in the urine. Some subjects, however, showed no increase nor decrease of urinary steroids.

According to our previous work,[4] injection of high doses of morphine in untreated rats may induce an increase of urinary hydroxysteroids. Lower doses produced lower effects on steroids. With regard to the effect of doses these results agree quite well with the observations of George and Way[24] through ascorbic acid fall test in rats. Moreover all the applied doses have reduced without exception the urinary excretion of 17-ketosteroids.

The increase of hydroxysteroids observed in the first stage of treatment may well be the result of a stimulatory effect of morphine on hypophysis eventually stressing in nature, since it is found to be lacking after small doses of the drug.

With reference to the depression of urinary steroids observed in the second stage of treatment by "stabilizing" doses of morphine the opinion of a reduction of urinary adrenal steroids as due exclusively to an impairment of hypophysis by morphine, is hardly sustainable in the rat, since either ACTH injection or the exposure to cold failed to have the same effect on urinary steroids as in untreated animals. This suggests that under morphine the target glands are less responsive to proper corticotropic stimulation. On the contrary the responsibility of ACTH may be invoked to explain the urinary increase of hydroxysteroids observed shortly after withdrawal or nalorphine injection in morphinized rats. The problem of the function of the aforementioned hormonal derangement with reference to the biological disposition to tolerance, addiction and abstinence is quite intricate. Selye[25] reports that adrenalectomy does not impair the ability of rats tolerant to morphine to withstand otherwise lethal doses of the drug. Tanabe and Cafruny[26] observed acquired tolerance to increasing doses of morphine in hypophysectomized rats. The course of withdrawal appeared not to be modified with respect to normal rats.

Moreover, in human addicts Fraser and Isbell[27] have found that a treatment with cortisone or ACTH tends to hasten and aggravate the appearance of withdrawal symptoms. Winter and Flataker[5] have shown an increase of antagonistic effects of nalorphine by cortisone in rats. In the present work the increase of urinary corticosteroids was found to parallel the course of abstinence syndrome.

It may be noted that an increase of urinary steroids also occurs when the injected doses of morphine are not "stabilizing". However, though still obscure as are most of the biological events affected by morphine, the relations between the course of experimental morphinism and hormonal derangements induced by the drug appear worthy of further investigations.

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