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EFFECTS OF STARVATION AND REFEEDING ON THE EXCRETION OF URINARY STEROID METABOLITES IN MICE WITH DIFFERENT GENETIC BACKGROUND

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SUMMARY

Gas chromatographic steroid metabolic profiling procedures have been applied to investigations of the effects of starvation and refeeding in mice. Urinary steroid metabolites were quantitatively followed during the starvation—refeeding experiments for mice with different genetic backgrounds. Some quantitative alterations were noted for certain congenic strains of mice. The metabolites which exhibited such quantitative variations were tentatively identified by means of combined gas chromatography—mass spectrometry.

INTRODUCTION

Various physiological and biochemical changes induced by starvation and subsequent refeeding processes have been extensively studied [1, 2] in both humans and animals. Among numerous studies, some chronological alterations in the secretion of steroid hormones have also been investigated. In particular, the adrenal gland appears to respond to the state of starvation by both enhanced secretion and an altered frequency of hormonal release. The enhanced gluconeogenesis seen in starvation is expected to be related to an increase in corticosteroid metabolism. Additionally, some physiological parallels exist between the states of starvation and refeeding and certain diabetic conditions [3, 4].

Observations by Takahashi and co-workers [5, 6], that the circadian rhythm of adrenocortical activity in rats is strongly affected by the food intake, have been corroborated by others [7—9]. Food deprivation for five days completely disturbed the circadian rhythm [5], measured as the amount of corticosterone (the major corticosteroid hormone of rodents). Diurnal cycling and increase of plasma cortisol levels were also observed in humans during a three-day fasting

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period [10]. More recently, Quigley and Yen [11] observed a dramatic mid-day surge of plasma cortisol with an onset concomitant with food intake; food deprivation appears to attenuate markedly both the magnitude and time course of this cortisol surge.

While the hormone levels in plasma have been commonly measured under the conditions of food deprivation, Kley et al. [8] have suggested that steroid measurements in urine are more advantageous than the corresponding plasma measurements while working with small animals. The advantages are due to the larger volume of urine sample available by obtaining a 24-hour urine sample and the avoidance of diurnal hormone fluctuations. In their work on the adrenal evaluations in rats, Kley et al. [8] reported that the adrenal stimulations through starvation increased the urinary concentration of free corticosterone three to four times, while the aldosterone excretion was significantly suppressed. However, little is known about the effect of starvation—refeeding on the numerous other urinary steroids.

While the value of multicomponent steroid determinations under different metabolic circumstances has been demonstrated in humans [12—15], similar procedures could be even more useful in the assessment of metabolic alterations in animal models of human diseases. The significant advantage of using animal models is the elimination of several variables which can not be controlled in humans, such as genetic background, diet, environment, etc.

This report deals with the effects of acute starvation on the urinary excretion of steroidal metabolites in mice. Selected metabolites have been quantitated by capillary gas chromatography (GC), while combined gas chromatography—mass spectrometry (GC—MS) has provided the essential means of identification.

Since some genetic variations in the hormonal secretion and steroid metabolism in mice can be expected [16], the starvation—refeeding model experiment reported here involved male mice of two distinctly different genotypes (BALB/c and C57BL). In addition, the profiles of congenic C57BL animals were quantitatively compared for three haplotypes (B10, B10-RIII, and B10-AKM), differing only in a small section of the major histocompatibility gene complex (H-2) on the seventeenth chromosome [17].

EXPERIMENTAL

Test animals were housed in groups of five to a cage. The experimental animals used were all male mice (13—14 weeks of age) of the following strains: BALB/cAnNHap BR, and three different haplotypes B10, C57BL/10SnJ, (H-2^b); B10.RIII (71Ns/Sn), (H-2^r); B10.AKM/Sn, (H-2^m) of the C57BL strain. All C57BL mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) while the BALB/c mice were obtained from Harlan Industries (Indianapolis, IN, U.S.A.). 24-hour urine samples were collected from different animal groups in standard metabolism cages with wire-mesh floors. The urine was directed into a collection cup placed on dry ice so that the samples were immediately preserved by freezing. The specimens of mouse urine were collected prior to the starvation—refeeding experiment (controls), during the following two days of starvation, and during two days of refeeding.

Water was provided ad libitum, while a rat chow (Purina, Indianapolis, IN, U.S.A.) was given prior to the starvation experiment and during refeeding.

Mouse urine aliquots (5 ml) were analyzed by a previously reported [15] procedure, except that C_{18} Sep-Pak cartridges (Waters Assoc., Milford, MA, U.S.A.) were used for the initial isolation of steroids instead of the XAD-2 resin. In the described procedure [15], subsequent sample treatment consists of enzymatic and solvolytic deconjugations, removal of interfering acids and bases through ion-exchange chromatography, and, finally, preparation of methoxime—trimethylsilyl derivatives for gas chromatography.

A modified Varian 1400 gas chromatograph, a preconcentration technique [18], and a 20 m × 0.25 mm I.D. glass capillary column (coated statically with SE-30 silicone gum) were employed to record the urinary profiles of steroids. A PEP-2 Perkin-Elmer data system performed the peak integrations. The glass capillary column was later directly coupled to the ion source of a Hewlett-Packard 5980A quadrupole mass spectrometer for identification work. All spectra were recorded at scan rates of 100 a.m.u./sec, ion source temperature 280°C, and 70 eV ionization energy.

RESULTS AND DISCUSSION

To our knowledge, no systematic multicomponent determination of mouse urinary steroids has been reported in the literature. Yet, the metabolic profiling approach seems highly attractive for various inquiries into physiology and genetics of the laboratory rodents that are used so often as various models of human conditions. A high-resolution chromatogram of mouse urinary steroid metabolites is shown in Fig. 1, while tentatively identified mixture constituents are listed in Table I.

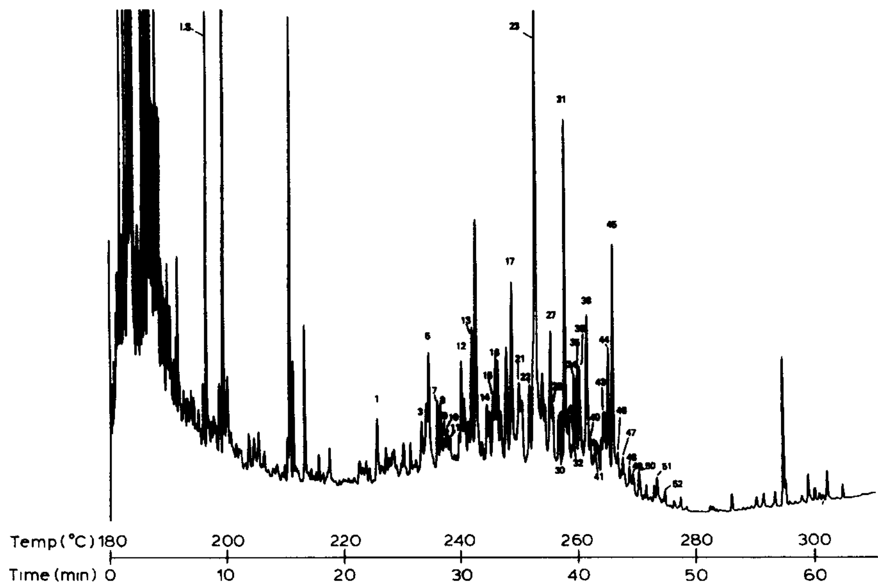


Fig. 1. Capillary gas chromatogram of methoxime—trimethylsilyl derivatives of mouse urinary steroids. Chromatographic conditions, see text. Tentative identifications are listed in Table I.

TABLE I

MOUSE URINARY STEROIDS

Peak No.	Derivatized MW	Identification	Principal *m/e*	Retention index (SE-30)
1	567	An androstanetriol-17-one	567, 536, 446, 356	2735
2	522	An androstenetriol	522, 507, 467, 432	
3	595	A pregnanetriol-20-one	564, 492, 474	2839
4	567	An androstanetriolone	536, 476, 446	2853
5	507	A pregnane-3,17-diol-20-one	507, 492, 476, 386	2863
6	567	An androstanetriol-16-one	536, 446, 356	
7	565	An androstenetriolone	534, 444, 354	2885
8	655	An androstanetetrolone	624, 565, 534, 475, 444	2895
9	550	A pregnanedioldione	550, 520, 417	2901
10	595	A pregnanetriolone	492, 402	2910
11	595	A pregnanetriolone	564, 474	2916
12	520	An androstadienetriol	520, 505, 431, 417	2949
13	595	A pregnanetriolone	580, 564, 474	2988
14	595	A pregnanetriolone	564, 492, 474, 384	3013
15	595	A pregnanetriolone	564, 474, 384	3034
16	683	A pregnanetetrolone	652, 562, 472	3041
17	655	An androstane-3,7,11,16-tetrol-17-one	624, 534, 444, 354	3072
18	683	A pregnanetetrolone	652, 562, 472	
19	595	A pregnanetriolone	580, 564, 474	
20	458	Cholesterol	368, 329	
21	624	An androstenetetrol-11-one	624, 534, 519, 444, 354	3106
22	609	A pregnanetriol-11,20-dione	609, 578, 506, 488	3107
23	683	A pregnanetetrol-20-one	652, 562, 472, 382	3141
24	683	A pregnanetetrolone	652, 562, 472, 382	
25	595	A pregnanetriolone	580, 564, 474	
26	683	A pregnanetetrol-20-one	652, 562, 472, 382	
27	683	A pregnanetetrol-20-one	652, 562, 472, 382	3180
28	654	A pregnanetetrol-11-one	461, 371	3186
29	566	A pregnanetriol-11-one	551, 461, 431, 371	
30	636	A pregnenetrioldione	605, 515, 425	
31	728	A pregnanepentol	535, 445, 355	3220
32	683	A pregnanetetrolone	494, 404	
33	683	A pregnanetetrolone	652, 562, 472	
34	683	A pregnanetetrolone	490, 400, 310	3244
35	636	A pregnenetrioldione	621, 605, 589, 469	
36	683	A pregnanetetrolone	490, 400, 310	3258
37	681	A pregnenetetrolone	488, 398, 308	
38	683	A pregnanetetrolone	652, 562, 472	3279
39	640	A pregnane-3,11,20,21-tetrol	486, 396, 381, 357	
40	728	A pregnanepentol	535, 445, 355	3293
41	683	A pregnanetetrolone	562, 472, 382	3306
42	683	A pregnanetetrolone	578, 488, 398, 308	
43	681	A pregnene-3,11,20,21-tetrol-16-one	488, 398, 308	3330
44	681	A pregnene-3,11,20,21-tetrol-16-one	578, 488, 398, 308	3340
45	728	A pregnane-3,11,16,20,21-pentol	535, 445, 355	3351
46	681	A pregnenetetrolone	578, 488, 398, 308	
47	728	A pregnanepentol	535, 445, 355	
48	593	A pregnenetriolone	593, 562, 472	
49	681	A pregnenetetrolone	488, 398, 308	
50	681	A pregnenetetrolone	488, 398, 308	3425
51	728	A pregnane-3,11,16,20,21-pentol	535, 445, 355	3482
52	728	A pregnane-3,11,16,20,21-pentol	535, 445, 355	3495

Although mass spectra of various steroid polyols were reported [19—26], positive identification of different isomers remains problematic. Mass spectrometry does not readily distinguish such isomers. There are two solutions to this problem: (1) acquisition of an extensive collection of various polyol samples, or (2) development of ancillary techniques that would be more powerful than mass spectrometry. Both approaches represent long-term goals. Until then, it is felt that qualitative information, as represented by Table I, is better

than none. In order to provide further documentation on these tentatively identified metabolites, retention indices relative to normal hydrocarbons are also included. Based on three determinations, variation in retention indices was found to be less than 0.4%.

Of particular interest are a number of late-eluting constituents: peaks 45, 51, and 52, apparently stereoisomers, whose mass spectra feature intensities at *m/e* 265, 355, 445, and 535; and peaks 43 and 44, also apparently stereoisomers, whose mass spectra feature *m/e* 308, 398, and 488. In none of these cases does there appear to be a molecular ion. Mass spectral information about steroid polyols provided by Gustafsson and co-workers [19—26], however, leads us to postulate that the first three substances are fully silylated pregnanepentols (MW 728), while the other two are silylated, methoximated pregnenetetrolones (MW 681).

From a biosynthetic point of view, it is reasonable to propose hydroxylation at positions 3, 11, 16, 20, and 21 for the supposed pregnanepentols. If so, an initial cleavage of the C_{20}—C_{21} bond would result in the loss of 103 mass units to give an ion of mass 625, from which the observed masses would be obtained by successive losses of trimethylsilanol (MW 90) units. Hydroxylation at C_{17} is unlikely, for a different set of mass peaks would thus be generated through C_{17}—C_{20} bond cleavage.

For the supposed pregnenetetrolones, a peak at *m/e* 276 suggests one oxo group and two hydroxyl groups at C_{16}, C_{20}, and C_{21}. Again, an initial loss of 103 mass units from an ion of mass 681, followed by successive losses of trimethylsilanol molecules, would yield the observed fragmentation pattern. The fact that these methoxime spectra are not dominated by the customary M—31, M—121, etc., peaks may indicate a structure significantly different from those of the common 20-oxosteroids. Tentatively, we propose 3,11,20,21-pregnenetetrol-16-one structures for constituents 43 and 44, with the position of the double bond unspecified.

As indicated above, multiple oxidations that occur at different sites of the basic steroidal skeleton present a challenge for identification studies. In this respect, it has become obvious that the steroid metabolites excreted into the urine of rodents are substantially different from those encountered in humans

TABLE II

EFFECTS OF STARVATION AND REFEEDING ON CONCENTRATION OF MOUSE URINARY STEROID CONSTITUENTS

S1 = first day of starvation; S2 = second day of starvation; R1 = first day of refeeding; R2 = second day of refeeding Concentrations are reported as percent of control.

Steroid	BALB/c				B10.AKM				B10				B10.RIII			
	S1	S2	R1	R2	S1	S2	R1	R2	S1	S2	R1	R2	S1	S2	R1	R2
17	49	96	104	166	25	18	114	150	43	11	103	99	41	19	119	99
23	41	50	50	57	78	43	65	117	144	57	147	122	86	58	76	119
31	50	114	64	72	80	60	113	139	104	59	151	93	137	78	95	134
43	105	169	128	133	266	109	85	133	182	127	190	118	98	70	57	105
44	128	134	151	103	25	16	59	104	80	40	154	93	97	31	77	172
45	68	137	100	119	38	34	62	106	110	60	195	173	155	140	124	245
51	43	301	58	49	138	64	96	132	132	65	109	132	118	68	84	97
52	54	303	57	80	152	56	86	148	92	42	45	71	107	201	108	174

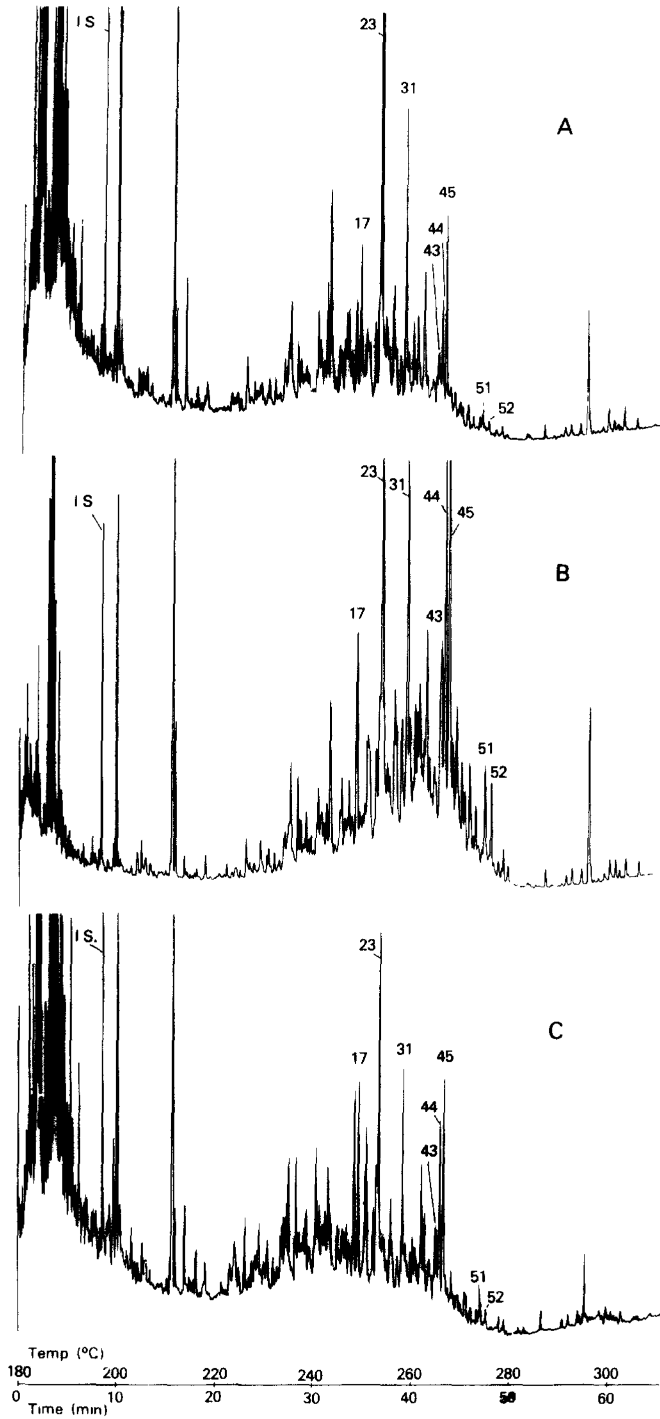


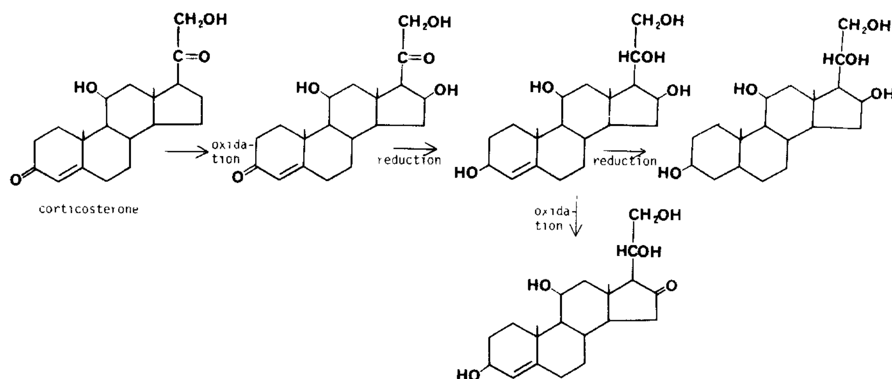
Fig. 2. Comparison of steroid metabolic profiles from the urine of BALB/c mice. (A) Control; (B) starvation; (C) refeeding. Chromatographic conditions, see text.

[15, 27]. Furthermore, the solvent extraction methodology commonly used in human studies [13, 27] fails to provide sufficient recoveries of very polar steroids from mouse urine. Fortunately, "solid extractions" [27] appear capable of extracting a sufficient amount of such steroids for reliable and reproducible analyses. While the absolute values of excreted mouse steroids are largely unknown (estimated at microgram amounts of corticosterone metabolites per 24 h), the coefficient of variation in our samples is estimated to be below 15%, enabling fairly reliable comparisons of mouse urinary steroid profiles. Individual variations of steroid excretion in different animals have been minimized through the urine collection from five animals per group.

The results of starvation—refeeding experiments are shown in Table II for those steroids that show significant alterations in urinary excretion and appear related to the physiological changes incurred during the experiment. A representative illustration of these effects is seen in Fig. 2, where the increases of certain profile components are evident for the starved animals (B) as compared to the controls (A); the profile of refed animals (C) is fairly comparable to the control. The values of Table II suggest, however, that a few profile components do not come back to their original value after the mice are refed. In some cases (e.g. peaks 23, 31, 51 and 52 in BALB/c mice) excretion rates below normal are observed.

Any results of metabolic profiling during starvation—refeeding experiments should be seen in view of various chronological alterations in the metabolism of various body fuels and the associated changes in the secretion of hormones. Of particular interest is that the starvation and refeeding processes evoke many of the same metabolic alterations that are observed in the mild forms of diabetes mellitus [3, 4]. It is known that the major known glucocorticoid in rodents, corticosterone, interacts with insulin and glucagon to effect the glycogenolytic and gluconeogenetic processes. Additionally, various glucocorticoids are known to participate in decreasing the responsiveness of muscle and adipose tissue to insulin-stimulated glucose uptake [29]. Starvation is expected to cause an elevation of corticosterone in rats with a return to normal levels during refeeding. However, if the starvation exceeds three or four days, the fat stores become depleted and the organism depends increasingly on the catabolism of body protein. Refeeding after such a prolonged fast may result in a period of carbohydrate intolerance and a less prompt return to the original hormonal conditions. Mlekusch et al. [30] reported that plasma concentrations of corticosterone in rats were increased at the initial period of starvation, but flattened off during the following two days of starvation, presumably due to an "adaptation" mechanism.

While the increases of corticosterone during the starvation of rodents are documented [5—9], virtually nothing is known about the numerous metabolites of corticosterone. The structures of steroids that show elevations in Table II are consistent with the general pathways of corticosteroid metabolism, i.e. biological oxidations taking place in the liver. Some increases in pregnenetetrolones (peaks 43 and 44) and pregnanepentols (peaks 45, 51 and 52) in BALB/c mice appear to be in agreement with the known corticosterone increases. It is conceivable that these compounds are biosynthesized as follows:



It must be pointed out, however, that the trends in metabolic excretions (Table II) are not uniform for all measured metabolites. While substantial increases of 200—300% of the original 24-h concentrations during starvation are observed in some instances (e.g. peaks 51 and 52 in BALB/c and peak 43 in B10.AKM mice), the levels of certain other steroids remain relatively less affected, while some show even decreases. It would be hard to explain such variations by a simple mechanism. The starvation process is known to induce a variety of liver enzymes [30]; some steroid oxidations could thus be viewed as non-specific metabolic interactions. Alternatively, some corticosterone products may have a metabolic role of their own; it has been reported [31] that similar compounds (e.g. synthetic glucocorticoids prednisolone and dexamethasone) may have contrasting effects on glucose uptake by rat adipocytes. Similarly, refeeding has shown variations that are not easily explained. It is, however, obvious that most metabolites show both positive and negative deviations from the control values.

Differences observed in steroid excretion among the different mouse strains indicate some genetic control over either the response of the adrenal gland to the starvation process, or alternatively, the liver metabolism of steroids. Variations observed with the H-2 congenic strains further support the notion that the major histocompatibility complex may exert a visible influence over the pathways of secondary metabolites [32—35]. While the biological reasons for this are presently unknown (the major biological role of H-2 is known to involve primarily the immune interactions), other "non-specific traits" of this gene complex have been observed [36, 37], including a linkage to the appearance of cortisone-induced cleft palate [38]. Although the liver is not generally considered in association with the immune response, an H-2 linkage with the liver metabolism was found for cAMP [33]. Moreover, glucagon binding to liver cell membranes appears also under some influence of the major histocompatibility complex [34].

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