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FLUMETHASONE INDUCTION OF LIVER TYROSINE AMINOTRANSFERASE ACTIVITY IN INBRED STRAINS AND OBESE MUTANT MICE*

ROBERT L. BLAKE

The Jackson Laboratory, Bar Harbor, Me. 04609, U.S.A.

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Abstract—The activity of mouse liver L-tyrosine-2-oxoglutarate aminotransferase can be induced to high levels in 4 to 6 hr by the administration of the synthetic difluoro glucocorticoid, flumethasone (6α, 9α-difluoro-11β, 17α 21-trihydroxy-16α-methyl-pregna-1, 4-diene-3, 20-dione). A single injection of flumethasone to adrenalectomized C57BL/6J mice at a dose of 10^{-8} moles (0·4 μg) per kilogram of body weight resulted in a 10-fold induction of enzyme activity in approximately 4 hr. In comparison, the administration of hydrocortisone sodium succinate (Solu-Cortef) at doses of $5{\cdot}0 \times 10^{-5}$ (24·2 mg) and 10^{-6} moles (0·48 mg) per kilogram of body weight resulted in a 4-fold and no detectable elevation of enzyme activity, respectively, after 4 hr. The effect of enzyme induction was maintained for a longer period of time by flumethasone than by the hydrocortisone derivatives tested. Dietary status markedly influenced the extent of the induction by flumethasone as demonstrated by the enhancement of enzyme induction by fasting in DBA/2J mice or by a controlled feeding schedule in C57BL/6J mice. Studies on the glucocorticoid regulation of liver enzyme synthesis in C57BL/6J-*ob* (obese mutant) mice demonstrated that flumethasone was significantly more effective than hydrocortisone derivatives as an inducer of liver tyrosine aminotransferase activity.

THE GLUCOCORTICOID activity of adrenal cortical steroids has been determined extensively by measuring the enhancement of glycogen deposition in the liver of adrenalectomized rats.[1–6] The basis for this effect is thought to be primarily the result of an increase in the rate at which amino acids are converted into glucose and glycogen. In this conversion process, transamination is frequently an initial reaction of the gluconeogenic mechanism. Studies on the role of hormonal enzyme induction in glucocorticoid action demonstrate that one of the earliest effects on enzyme-forming systems of the liver[7] is an elevation of the activity of tyrosine aminotransferase, TA, (EC 2.6.1.5, L-tyrosine: 2-oxoglutarate aminotransferase). Studies on the mechanism of glucocorticoid action showed that the elevation of enzyme activity was because of an increased rate of enzyme synthesis[8] rather than a decreased rate of enzyme degradation. Enzyme activity can also be increased *in vitro* in the perfused liver,[9, 10] in fetal liver in organ culture,[11] and in hepatoma cells in tissue culture.[12, 13]

During the course of our studies on the effectiveness of hydrocortisone to cause an increase in mouse liver TA activity in various inbred strains, F_1 hybrids, and mutant mice, it was observed that both hydrocortisone acetate and hydrocortisone sodium succinate (Solu-Cortef) were considerably less effective in C57BL/6J-*ob* (obese

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mutant) mice than in the normal littermate controls. In an attempt to characterize further the basis for this apparent difference in the hydrocortisone sensitivity of TA it was decided to test certain synthetic analogs of corticosteroids for their enzyme-inducing potency in the C57BL/6J-*ob* mice. A wide variety of synthetic glucocorticoids are now available for experimental purposes in basic research since the discovery nearly 20 years ago that cortisone offered relief to sufferers of rheumatoid arthritis. Some of the most active synthetic glucocorticoids are the 9α-halogenated steroids such as triamcinolone (9α-fluoro-16α hydroxyprednisolone diacetate) and dexamethasone (9α-fluoro-16α methyl prednisolone).

The results presented in this report deal with the induction of mouse liver TA activity by the synthetic difluoro glucocorticoid, flumethasone (6α, 9α-difluoro-11β, 17α, 21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione). This compound is structurally identical with dexamethasone except for another fluoro substitution in the 6α position (Fig. 1). Flumethasone may be one of the most active glucocorticoids

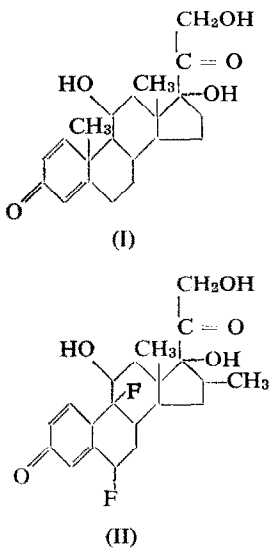

FIG. 1. Structural formulae of hydrocortisone (I) and flumethasone (II).

synthesized.[14] In a list[15] of approximately 300 synthetic glucocorticoids tested for their capacity to promote liver glycogen deposition in the rat and/or mouse, flumethasone acetate was reported with the highest potency, about 677 times greater than hydrocortisone. The glucocorticoid activity of dexamethasone has been reported at 180[16] to 265[15] times greater than hydrocortisone. In studies on the growth-inhibitory effects of adrenal cortical hormones,[17] flumethasone was reported as approximately 1000 times more effective than hydrocortisone in causing thymus atrophy and involution of the bursa of Fabricius in 1-day-old cockerels. In this same test system, flumethasone was approximately 40 times more active than dexamethasone. In a method of testing steroid-induced corticotrophin (ACTH) suppression in rats,[18] flumethasone at doses of 3 to 6 μg per 100 grams body weight, was approximately four times more active than dexamethasone. Flumethasone apparently has not been tested previously for its enzyme-inducing potency.

MATERIALS AND METHODS*

General procedure. Inbred strains and mutant mice were procured from the Production Department of The Jackson Laboratory, Bar Harbor, Me. The obese mutant mice used in this investigation were from the C57BL/6J-*ob* strain. Mice were fed *ad lib.* a pelleted standard mouse diet manufactured by the Emory Morse Company of Guilford, Conn., containing 19% protein and 6% fat. Drugs and hormones were given to mice by intraperitoneal injection. Bilaterally adrenalectomized mice, maintained on regular diet and 0·9% NaCl solution, were used for experimental purposes 1 week postoperatively.

The mice were killed by cervical dislocation. The livers were rapidly excised, washed in an ice-cold 0·14 M KCl solution, blotted, frozen, and stored overnight at −20°. A 10% liver homogenate (w:v) in 0·14 M KCl was prepared with either a teflon-glass homogenizer operated for 2 min or a Brinkmann Polytron Disperser operated for 30 sec. No significant differences were detected in either the enzyme activity or protein content of the supernatant fluid from the homogenates prepared by these two techniques. The homogenate was centrifuged for 20 min at 12,000 *g* in the SS-34 rotor of a Sorvall RC-2 automatic refrigerated centrifuge at 0°. Subsequently, a sample of the supernatant fluid was carefully removed with a disposable Pasteur capillary pipet, and chilled in ice until use for the enzyme assay or protein determination.

Analytical. TA activity was assayed by the enol borate method of Lin and Knox[19] adapted to an end-point determination in which the concentration of *p*-hydroxyphenylpyruvate was calculated from the absorption differences at 310 mμ between the arsenate–borate mixture and the arsenate blank. Arsenate was used in place of the keto-enol tautomerase to accelerate the equilibration of the keto and enol tautomers with the enol borate complex.[20]

The liver supernatant fluid, varying in aliquot volume from 0·25 to 1·0 ml, was pipetted into 5-ml Sorvall cellulose nitrate tubes (10 × 80 mm) containing the following amounts of reaction components in 0·57 M Trizma Base (Tris) buffer (final pH 8·1):0·25 μmoles of pyridoxal-5-phosphate, 12 μmoles of L-tyrosine, and 10 μmoles of diethyldithiocarbamic acid (DDC). Distilled water was added to make a 3·0-ml preincubation volume.

All reaction components were chilled in an ice bath during the preparation of the assay system. The resulting mixture was preincubated in a water bath at 25° for 10 min. Subsequently, the reaction tubes were placed in an ice bath for 10 min and 300 μmoles of α-keto-glutarate was added to initiate the enzyme reaction. The complete reaction system in a final volume of 3·5 ml was incubated in a water bath at 25° for 20 min. The reaction was stopped by the addition of 1·0 ml of 40% trichloroacetic acid (TCA).

The TCA-protein mixture was centrifuged at 12,000 *g* for 10 min in the SS-34 rotor of a Sorvall RC-2 automatic refrigerated centrifuge at 0°. A 0·5-ml aliquot of the supernatant fluid was added to 3·0 ml of 2·0 M arsenate, pH 6·5, (keto sample) and another 0·5-ml aliquot was added to 3·0 ml of 1·0 M borate in 2·0 M arsenate, pH 6·5, (enol borate sample). After 15 min the absorption of the enol-borate solution at 310 mμ was determined in a Zeiss PMQ II spectrophotometer, using the arsenate keto

* The principles of laboratory animal care as promulgated by the National Society of Medical Research are observed in this Laboratory.

solution as its blank. Enzyme units are expressed as μmoles of *p*-hydroxyphenylpyruvate accumulated per gram of liver supernatant protein per minute at 25°.

The protein concentration of the liver supernatant fluid was determined by the biuret method[21] with 0·1% potassium iodide added to the reagent. Bovine serum albumin was utilized as the protein standard for the biuret determinations.

Materials. Cycloheximide, pyridoxal-5-phosphate, α-ketoglutarate (free acid), L-tyrosine, and Tris(hydroxymethyl)aminomethane (Trizma Base) was purchased from the Sigma Chemical Co., St. Louis, Mo. Diethyldithiocarbamic acid (DDC) (sodium salt) was purchased from Eastman Organic Chemicals, Rochester, N. Y. Hydrocortisone sodium succinate (Solu-Cortef) was purchased from the Upjohn Company, Kalamazoo, Mich. Hydrocortisone acetate was purchased from the Mann Research Laboratories, New York, N. Y.

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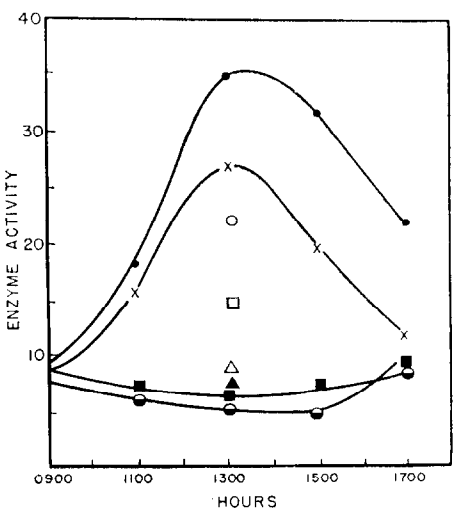

FIG. 2. Induction of tyrosine aminotransferase activity in C57BL/6J and DBA/2J mice by Solu-Cortef and the inhibition by cycloheximide. C57BL/6J and DBA/2J male mice of 8 to 10 weeks of age were injected intraperitoneally with (Solu-Cortef) hydrocortisone sodium succinate at a dose of $5·0 \times 10^{-5}$ moles per kilogram of body weight. Values at each time point represent the mean for analyses on five to six mice. Symbols refer to the following experimental conditions: ●, Solu-Cortef to DBA/2J mice; X, Solu-Cortef to C58BL/6J mice; ○, cycloheximide and Solu-Cortef to DBA/2J mice; □, cycloheximide and Solu-Cortef to C57BL/6J mice; Δ, cycloheximide to DBA/2J mice; ▲, cycloheximide to C57BL/6J mice; ■, untreated DBA/2J mice; and ◒, untreated C57BL/6J mice. Enzyme activity was expressed as μmoles of *p*-hydroxyphenylpyruvate accumulated per gram of supernatant liver protein per minute at 25°.

Standard errors of the mean:

0900	1100	1100HC	1300	1300HC	1500	1500HC	1700	1700HC
0·5	1·2	2·2	0·7	1·5	1·2	1·8	0·6	4·4
0·6	0·5	1·8	0·6	1·7	0·9	1·5	0·6	0·7

* Generic name = Flumethasone; systematic chemical nomenclature = 6α,9α-difluoro-11β, 17α,21-trihydroxy-16α-methyl-pregna-1, 4 diene-3, 20-dione; $C_{22}H_{28}F_2O_5$; mol. wt. = 410·44.

RESULTS

Comparative studies with flumethasone and Solu-Cortef. The induction of liver TA activity by glucocorticoids has been accomplished in several investigations by the administration of hydrocortisone either as the free alcohol, or as liposoluble (acetate) and hydrosoluble (hemisuccinate) derivatives. The results presented in Fig. 2 demonstrate the effects of hydrocortisone sodium succinate (Solu-Cortef) administration on the activity of mouse liver TA in the C57BL/6J and DBA/2J inbred strains. At 0900 hr, mice received a single intraperitoneal injection of Solu-Cortef at a dose of $5{\cdot}0 \times 10^{-5}$ moles per kilogram of body weight (about 25 mg/kg). The mice were killed at 2-hr intervals in an 8-hr induction period. The enzyme activity increased rapidly in both strains reaching a maximum about 4 hr after the injection. At the induction maximum, the TA activity in the C57BL/6J and DBA/2J strains was increased about 3·6- and 4·2-fold, respectively, above the 0900-hr level. Cycloheximide inhibited the increase in enzyme activity in both strains by about 50 per cent when given by intraperitoneal injection at 0900 and 1100 hr at a dose of 1 μg per gram of body weight. Solu-Cortef was slightly but consistently more effective as an inducer of TA activity in the DBA/2J mice.

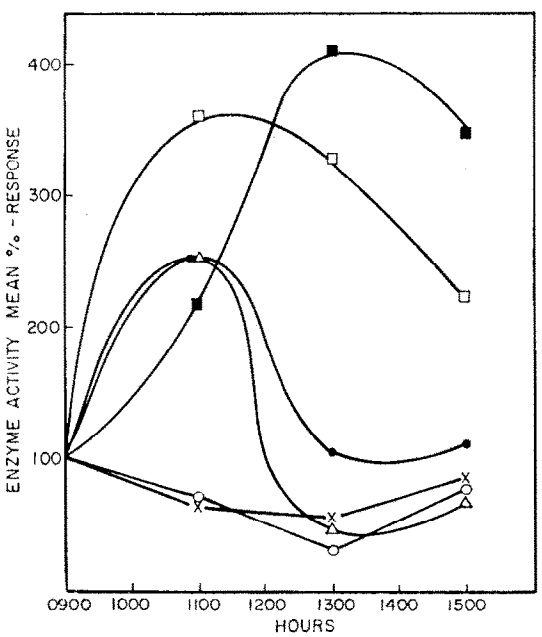

FIG. 3. Kinetics of tyrosine aminotransferase induction by flumethasone and hydrocortisone derivatives in C57BL/6J mice. C57BL/6J male mice of 8 weeks of age were injected intraperitoneally at 0900 hr with one of the following compounds: flumethasone (free alcohol), ■; hydrocortisone (free alcohol), □; 95% ethanol, ●; and Solu-Cortef, △. Drugs were given at a dose of 10^{-6} moles per kilogram of body weight. Flumethasone and hydrocortisone were dissolved in ethanol. Ethanol was given at a dose of 0·4 ml per kilogram of body weight. Sham injections, ○, consisted of a puncture of the abdominal wall with the hypodermic needle. Mice were killed at 2-hr intervals for determination of enzyme activity. The results are presented as the mean per cent response relative to the enzyme activity at 0900 hr which was expressed as 100 per cent. The basal enzyme activity in untreated mice, X, decreases from 0900 to about 1300 hr.

Solu-Cortef was also effective as an inducer of mouse liver TA activity at lower doses, although the induction response was considerably less. As shown in Fig. 3, the administration of Solu-Cortef to C57BL/6J mice at a dose of 10^{-6} moles per kilogram of body weight resulted in approximately a 2·5-fold induction of enzyme activity in 2 hr. Subsequently, the enzyme activity declined rapidly so that 4 hr after the injection the enzyme level was the same as in the untreated mice. When hydrocortisone was given as the free alcohol at a dose of 10^{-6} moles per kilogram of body weight the enzyme activity increased approximately 3·4-fold in 2 hr. However, the intraperitoneal administration of 95% ethanol, the solvent used to dissolve the hydrocortisone, also resulted in an increase in enzyme activity. The combination of hydrocortisone (free alcohol) and ethanol resulted in a longer period of induced enzyme activity as compared to ethanol alone. When flumethasone (free alcohol) was given at a dose of 10^{-6} moles per kilogram of body weight, the induction response was slower at first compared to hydrocortisone, but reached higher levels (4·2-fold in 4 hr), and the enzyme activity

TABLE 1. FLUMETHASONE INDUCTION OF LIVER TYROSINE AMINOTRANSFERASE ACTIVITY IN C57BL/6J MICE*

Injection	Enzyme activity	
	Mean	Individual values
Normal mice		
Untreated (0900)	5·1 ± 0·6 (5)	4·7, 2·9, 4·7, 7·9, 5·3
Solu-Cortef ($5·0 \times 10^{-5}$ moles)	27·1 ± 1·7 (8)	28·3, 25·8, 20·0, 28·9, 24·2, 26·8, 36·7, 25·8
Ethanol	11·4 ± 2·0 (7)	5·7, 7·1, 10·7, 14·1, 20·5, 7·2, 14·7
Flumethasone		
(10^{-6} moles)	26·7 ± 1·5 (6)	31·2, 29·8, 27·4, 22·2, 22·6, 26·8
(10^{-7} moles)	26·7 ± 1·1 (6)	25·8, 28·7, 29·2, 27·4, 26·8, 22·0
(10^{-8} moles)	15·7 ± 3·3 (6)	16·8, 4·6, 13·0, 25·6, 11·3, 22·6
(10^{-9} moles)	23·0 ± 4·2 (7)	29·9, 24·3, 4·2, 31·4, 13·1, 35·7, 22·2
Adrenalectomized mice		
Untreated (0900)	2·2 ± 0·04 (5)	2·2, 2·6, 2·4, 1·8, 2·2
Ethanol	2·0 ± 0·24 (5)	1·3, 1·8, 2·0, 2·1, 2·8
Flumethasone		
(10^{-6} moles)	20·7 ± 1·7 (4)	23·4, 21·4, 15·7, 22·0
(10^{-8} moles)	24·5 ± 2·9 (4)	30·2, 26·0, 23·4, 18·6

* At 0900 hr, C57BL/6J male mice 8 to 10 weeks of age and fed *ad lib.* were injected intraperitoneally with one of the following compounds: hydrocortisone monosodium succinate (Solu-Cortef), $5·0 \times 10^{-5}$ moles per kilogram of body weight; 95% ethanol, 0·4 ml per kilogram of body weight; and flumethasone (free alcohol) at various doses per kilogram of body weight dissolved in 95% ethanol. The adrenalectomized mice were used 1 week postoperatively. At 1300 hr, the mice were killed and the liver enzyme activity determined as described in Methods. Enzyme activity was calculated as μmoles of *p*-hydroxyphenylpyruvate accumulated per gram of supernatant protein per minute. The results are expressed as the mean ± the standard error. Individual enzyme activities are enclosed in parentheses.

was maintained at an induced level for a longer period of time. After 6 hr, the enzyme activity in the flumethasone-, hydrocortisone- and ethanol-treated mice was about 3·5-, 2·0- and 1·2-fold higher, respectively, than the zero time level.

Flumethasone was also effective as an inducer of liver TA at doses 100- and 1000-fold lower than the starting dose of 10^{-6} moles per kilogram of body weight. The results presented in Table 1 demonstrate the induction of TA by flumethasone at various doses in adrenalectomized and intact mice. Since the glucocorticoid potency of flumethasone has been estimated at about 700 times that of hydrocortisone, initially

flumethasone was tested at doses approximately 500 times lower than the "standard dose" of hydrocortisone used to induce TA ($5{\cdot}0 \times 10^{-5}$ moles per kilogram of body weight). At a dose of 10^{-7} (moles/kg), a single injection of flumethasone caused a 4- to 6-fold increase in enzyme activity in 4 hr. At a dose of 10^{-6} (moles/kg), flumethasone produced about the same induction in 4 hr. Larger doses of glucocorticoids maintain the induced enzyme activity for longer periods of time. The drug was consistently effective at 10^{-7} moles per kilogram of body weight. The induction response at 10^{-8} and lower doses of flumethasone was variable. The results at the very low doses (10^{-8}, 10^{-9}) suggested that flumethasone may potentiate the action of glucocorticoids which are endogenously released as a result of a nonspecific stress from either handling or injection of the mice or from the solvent. Accordingly, if there is no endogenous release of glucocorticoids, then an induction effect of flumethasone is not observed at the very low doses. When adrenalectomized mice were injected with flumethasone a significant induction was observed even at the 10^{-8} dose level.

Influence of dietary status. Dietary status can markedly influence the extent of TA induction by flumethasone, as demonstrated by the results in Table 2. When DBA/2J

TABLE 2. INFLUENCE OF DIETARY STATUS ON THE FLUMETHASONE INDUCTION OF LIVER TYROSINE AMINOTRANSFERASE ACTIVITY*

Experimental condition	Time (hr)	Enzyme activity: DBA/2J (Fasted)	Enzyme activity: C57BL/6J (Controlled feeding)
Untreated	0900	6·5, 2·4, 4·4, 3·6, 5·3	3·8, 2·4, 3·5, 6·7
Flumethasone	1100		4·3, 8·9, 5·6, 16·4† (2)
	1100		10·6, 11·8, 13·3, 14·7 (2)
	1300	46·6, 54·2, 47·6, 57·4 (1)	16·1, 23·9, 20·6, 21·4† (4)
	1300	45·7, 67·5, 36·1, 61·6 (2)	38·8, 52·9, 34·9, 39·2 (4)
	1300	69·9, 66·3, 48·0, 49·3 (3)	
	1500		13·9, 4·6, 13·9, 4·7† (4)
	1500		56·2, 31·6, 30·8, 24·0 (4)
Ethanol	1300	39·4, 31·4 (3)	3·3, 2·3, 4·4, 2·5 (4)

* DBA/2J male mice of approximately 22 weeks of age were fasted for 24 hr prior to experimentation. At 0900 hr, they received intraperitoneal injections of flumethasone (free alcohol) dissolved in 95% ethanol at a dose of 10^{-6} moles per kilogram of body weight per injection. Ethanol was given at a volume body weight ratio of 0·4 ml per kilogram of body weight. Numerals enclosed in parentheses refer to the number of injections. The second and third injections were given at 1030 and 1130 hr. The mice were killed for enzyme analyses at 2-hr intervals.

In the controlled feeding experiments, C57BL/6J male mice of 8 weeks were fasted for 39 hr starting at 0900 hr. At 2400 hr, pellets were placed in the hoppers and the mice were allowed free access to food until 0800 hr. At 0900 hr, the mice received intraperitoneal injections of flumethasone dissolved in 95% ethanol at doses of either 10^{-9} or 10^{-6} moles per kilogram of body weight per injection. Numerals enclosed in parentheses refer to the number of injections.

† Refer to the 10^{-9} dose regimen.

mice were fasted for 24 hr and then received a single intraperitoneal injection of the drug at a dose of 10^{-6} moles per kilogram of body weight, the enzyme activity increased rapidly from about 4·4 units at 0900 hr to approximately 51·4 units at 1300 hr. The average 4-hr induction response was not significantly greater after two or three injections of flumethasone. Three intraperitoneal injections of ethanol also increased the enzyme activity. The combined effects of flumethasone and ethanol in a multiple injection schedule were to increase the extent of enzyme induction in several of the

fasting DBA/2J mice up to 65·0 to 70·0 units. In comparison the extent of enzyme induction in fed DBA/2J mice (Fig. 2) at 1300 hr after a single injection of Solu-Cortef ($5{\cdot}0 \times 10^{-5}$ moles per kilogram of body weight) was approximately 35·0 units, about a 4·2-fold induction response in 4 hr. Single injections of flumethasone (10^{-6} dose) and Solu-Cortef ($5{\cdot}0 \times 10^{-5}$ dose) are approximately equivalent in the 4-hr induction response in DBA/2J mice fed *ad lib.*

The results presented in Table 2 also demonstrate how modifications of the dietary status can markedly influence the extent of TA induction by flumethasone in C57BL/6J mice. In the controlled feeding period, mice were first fasted for 39 hr starting at 0900 hr and then were re-fed from 2400 to 0800 hr. At 0900 hr, the mice were injected intraperitoneally with flumethasone at doses of either 10^{-6} (single injection) or 10^{-9} (4 hourly injections) moles per kilogram of body weight. Mice were killed at 1100, 1300 and 1500 hr. After a controlled feeding period, the enzyme activity at 0900 hr was low (3·8, 2·4 and 3·5 units) compared to basal activities at the same time (Fig. 2) in mice fed *ad lib.* Upon administration of flumethasone, the enzyme activity increased rapidly in mice treated with either the 10^{-6} or the 10^{-9} dose regimens. Although the 4-hr induction response was greater in mice treated with the 10^{-6} dose, approximately a 7-fold induction occurred in mice receiving 4 injections of flumethasone at 10^{-9} moles per kilogram. Four intraperitoneal injections of the solvent (95% ethanol) did not cause the TA activity to increase after 4 hr.

Induction of enzyme activity in obese mice. As described previously, during the course of our studies on the effectiveness of hydrocortisone to cause an increase in mouse liver TA activity in various inbred strains, F_1 hybrids, and mutant mice, it was

TABLE 3. COMPARATIVE EFFECTS OF HYDROCORTISONE DERIVATIVES AND FLUMETHASONE AS INDUCERS OF LIVER TYROSINE AMINOTRANSFERASE ACTIVITY IN OBESE MUTANT MICE*

Treatment	Enzyme activity	
	Obese mutant (C57BL/6J-*ob*)	Normal littermates (+/+ and +/*ob*)
Group I (35–38 days)		
Untreated	2·7, 4·0	3·8, 1·3
Solu-Cortef	5·0, 3·9, 4·7, 6·1 (4·9)	12·7, 10·0, 12·1, 6·3 (10·3)
Flumethasone	9·6, 9·0, 9·6, 9·7 (9·5)	17·9, 16·1, 12·1, 17·6 (15·9)
Group II (98–107 days)		
Untreated	2·6	1·6
Solu-Cortef	3·5, 2·9, 3·1, 3·6 (3·3)	9·7, 7·6, 6·3, 5·6, 9·1 (7·7)
Hydrocortisone Acetate	3·9, 4·4, 4·0, 3·7 (4·0)	11·2, 7·5, 8·9 (9·2)
Group III (394–404 days)		
Untreated	6·3, 3·1, 2·4 (3·9)	1·3, 3·3, 3·4 (2·7)
Solu-Cortef	2·7, 5·9, 5·7, 3·1 (4·4)	6·0, 4·3, 7·6, 6·9 (6·2)
Flumethasone	6·3, 11·6, 6·5, 7·3 (7·4)	9·8, 11·0, 6·5, 8·9 (9·1)
Hydrocortisone Acetate	4·3, 5·8	10·8, 9·5

* At 0900 hr, C57BL/6J-*ob* and "normal" littermates (males) of three age groups (indicated in parentheses) were injected intraperitoneally with one of the following compounds: Solu-Cortef, hydrocortisone acetate, ethanol, hydrocortisone (free alcohol), and flumethasone (free alcohol). Steroids (except Solu-Cortef) were dissolved in 95% ethanol. The compounds were given four times at hourly intervals at a dose of 10^{-5} moles per kilogram of body weight per injection. Ethanol was given at a dose of 0·4 ml per kilogram of body weight. All mice were killed at 1300 hr and the liver enzyme activity determined as explained in Methods. Enzyme activity was expressed as μmoles of *p*-hydroxyphenylpyruvate accumulated per gram of liver supernatant protein per minute at 25°. Data enclosed in parentheses refer to the average 4-hr enzyme induction response. Normal littermates included both the homozygous (+/+) and heterozygous (+/*ob*) mice.

observed that both hydrocortisone acetate and hydrocortisone sodium succinate (Solu-Cortef) were considerably less effective in C57BL/6J-*ob* (obese mutant mice) than in normal littermate controls. Subsequently, it was decided to test certain synthetic analogs of corticosteroids for their enzyme-inducing potency in the C57BL/6J-*ob* mice. The results presented in Table 3 demonstrate the effects of the intraperitoneal administration of Solu-Cortef, hydrocortisone acetate, hydrocortisone (free alcohol), and flumethasone on the liver TA activity after a multiple injection schedule. Starting at 0900 hr, mice were injected at hourly intervals with one of the four compounds at a dose of 10^{-6} moles per kilogram of body weight per injection. The mice were killed at 1300 hr and the enzyme activity determined. The obese mice were tested at three age groups: I = 35–38 days; II = 98–107 days; and III = 394–404 days. The body weights of the obese mutant mice averaged about 30·0, 50·0, and 70·0 g for Groups I, II and III respectively. The body weights of the littermate controls averaged about 20·0, 28·0 and 40·0 g. The results are expressed in terms of the 4-hr induction response. Although Solu-Cortef, hydrocortisone acetate and hydrocortisone (free alcohol) caused significant increases of TA activity in the normal littermates, only flumethasone was effective in the obese mutant mice.

In the obese mice, the flumethasone induction response was about 50 per cent of the response observed in the normal littermates (calculated on the basis of increase in enzyme activity above the untreated values; Group I, obese to controls, 29·15:15·9; Group III, 7·4:9·1). The extent of the enzyme induction by flumethasone was greater in the young mice, particularly in the normal littermates. Hydrocortisone acetate and Solu-Cortef were approximately equivalent in their enzyme-inducing potencies in the obese mice, although both the solvent and the derivative were different (a liposoluble acetate derivative as compared to the hydro-soluble succinate compound). In normal C57BL/6J mice (Fig. 3), hydrocortisone (free alcohol) produced a greater induction of TA activity than Solu-Cortef.

DISCUSSION

Research on the development of compounds more potent than cortisol has resulted in the production of numerous synthetic analogs of the adrenal cortical steroids. Analyses of the structural requirements for enhanced biological activity demonstrated that halogen substitution of the steroid nucleus at the 6α and 9α positions greatly increased the activity. Introduction of a Δ^1-double bond also enhanced the biological activity of corticosteroids which includes anti-inflammatory action, ACTH suppression, gluconeogenic action, thymolytic and eosinopenic activity, tumor-growth inhibition and teratogenic effects. Flumethasone, with fluoro substituents at both the 6α and 9α positions, as well as a Δ^1-double bond (Fig. 1), has been tested for several of these biological activities and found to be very active.[14, 15, 17, 18] The present report demonstrates the potency of flumethasone as a synthetic hormone in the induction of specific liver enzyme synthesis, a biological response that requires a relatively short test period (2–4 hr) in comparison to most other assays of corticosteroid activity. Induction of TA activity is one of the earliest consequences of glucocorticoid action upon enzyme-forming systems of the liver.[7] Since liver TA acts as a regulatory enzyme in the tyrosine catabolic pathway, studies on the glucocorticoid control in various inbred strains and mutant mice provides an experimental approach to investigate the role of steroid hormones as "effectors" of genetic expression in the control of mam-

malian metabolism. Studies on TA induction in tissue culture[22] indicate that glucocorticoids act at both transcriptional and translational levels of enzyme induction. In the transcriptional phase, during the synthesis of messenger RNA, actinomycin D blocks the enzyme induction process if given simultaneously or soon after the inducer. Later in the induction cycle, when the TA templates become stabilized, enzyme synthesis continues even in the presence of actinomycin D. However, TA synthesis decreases in this phase of the induction cycle when the inducer is removed. There is a requirement for continuous presence of the inducer, although the messenger RNA is stable, suggesting a translational role for glucocorticoids in enzyme induction. Tomkins *et al.*[22] have proposed that glucocorticoids may inhibit the action of a cytoplasmic repressor.

The induction of mouse liver TA activity by flumethasone given at 10^{-8} and 10^{-9} moles per kilogram of body weight corresponds to doses of approximately 0·1 and 0·01 μg of the drug, respectively, per 25 g mouse. Drug inhibitors of mammalian enzyme induction, such as actinomycin D, puromycin and cycloheximide, have been used to block liver protein synthesis *in vivo* in rats at intraperitoneal doses of 18·5 μg,[23] 2·5 mg,[24] and 25 μg[25] per 25 g of body weight. The administration of flumethasone, at the 10^{-8} dose, to adrenalectomized C57BL/6J mice resulted in approximately a 10-fold elevation of enzyme activity in 4 hr with an increase from the 0900-hr endogenous level of 2·2 units to 24·5 units at 1300 hr (Table 1). It is interesting that the 4-hr induction response after the administration of flumethasone at a dose of 10^{-6} moles per kilogram of body weight (10 μg/25 g mouse) was not significantly higher although the dose was 100-fold greater. This may imply that the amount of glucocorticoid required to initiate the enzyme induction response (transcription of messenger RNA) is considerably less than the amount of glucocorticoid required to maintain the induced enzyme activity at the translational level by a continuous synthesis *de novo* of enzyme. While the length of the actinomycin D sensitive period appears to be relatively independent of the dose of the glucocorticoid inducer, the duration of induced TA activity is very much dependent upon the dose of the inducer. Presumably, the induced enzyme activity declines as the concentration of exogenously administered glucocorticoid decreases to "normal" physiological levels. Once the enzyme induction cycle is initiated, the primary effect of metabolism of the inducer may be to modify the translational control of TA synthesis.

In contrast to the action of flumethasone, the induction of mouse liver TA activity by Solu-Cortef (a drug preparation frequently used to induce rat liver TA activity *in vivo*) required considerably larger doses of the glucocorticoid to obtain similar levels of induced enzyme activity. The Solu-Cortef induction of enzyme activity to 25·0 units in 4 hr in C57BL/6J mice was accomplished with a dose of 625 μg per 25 g mouse (Fig. 2). At a dose of 12·5 μg of Solu-Cortef per 25 g of body weight, the enzyme activity increased about 2·5-fold in 2 hr, and after 4 hr returned to essentially the same level as in the untreated mice (Fig. 3). A possible explanation for the marked differences in enzyme-inducing potency of flumethasone and Solu-Cortef may be differences in the rates at which the synthetic steroid and the hydrocortisone sodium succinate derivate are inactivated or removed from the body. During the period of induced enzyme activity, the concentration of the inducer is constantly changing as a result partially of the metabolic activity of drug detoxification mechanisms. Flumethasone may be metabolized at a much slower rate than hydrocortisone sodium succinate. Studies on

the metabolism of synthetic corticosteroids *in vitro*[26, 27] demonstrated that 1,2-dehydrogenation and substitution of a fluorine atom in the 9α position inhibit reduction of ring A. However, Todd and Hechter[28] reported that under conditions *in vitro* fluorocortisol was metabolized at a rate only slightly lower than that of cortisol. In addition, Florini and Buyske[29] concluded that the enhanced biological activity of hydrocortisone derivatives, such as prednisolone and triamcinolone could not be attributed solely to their slower metabolism, since they found no direct correlation between glucocorticoid activity and rate of metabolism in rat liver slices. In contrast, studies *in vivo* on the rate of removal of corticosteroids from body fluids do indicate a rapid turnover rate.[30] The rapid turnover rate of steroids may be one reason why rather excessive amounts of natural glucocorticoids (pharmacological doses of 10^{-5} and 10^{-6} moles per kilogram of body weight) are frequently used to elevate the level of inducible liver enzyme activity *in vivo*. Saturating the enzyme induction mechanisms results in more consistent induction maximums among treated animals and a longer period of induced enzyme level.

Information on the tissue concentration of glucocorticoids in various species, including the mouse or rat, are difficult to obtain for different physiological conditions.[30] Knox *et al.*[31] concluded, on the basis of their comparative studies on the induction of tryptophan pyrrolase in rat liver by hydrocortisone derivatives and by stressful stimuli, that the amount of glucocorticoid secreted at physiological levels in response to certain "stress" conditions was in the order of 0·25 mg per 100 g of body weight. That is, the extent of enzyme induction in response to a stressful stimuli was equivalent in its effect to a dose of at least 0·25 mg per 100 g of body weight of administered hydrocortisone.

As an additional explanation for the marked differences in the enzyme-inducing potencies of flumethasone and Solu-Cortef, it is possible that the enhanced activity of synthetic corticosteroids may result from differences in binding affinity to various plasma proteins such as "transcortin"[32] or to tissue specific steroid "receptors". Both nuclear[33–35] and cytoplasmic[36, 37] steroid receptors have been proposed as possible sites of hormone action. Flumethasone may act more effectively on steroid receptors of the liver enhancing the induction mechanism of the glucocorticoid inducible enzymes.

The results presented in this report also demonstrate how the basal level of TA activity, as well as the extent of enzyme induction by a synthetic glucocorticoid hormone, can be markedly influenced by various dietary conditions including fasting, controlled feeding, and the physiologically uncontrolled feeding of obese mice. Mice of the C57BL/6J-*ob* strain are compulsive eaters, consuming considerable quantities of food and gaining weight rapidly up to 60 to 80 g in the adult animals. The dietary and metabolic status of the obese mutant mouse differs greatly from that of the normal lean littermates. When hydrocortisone was given to obese mice (Table 3) of three age groups, the enzyme induction response was significantly less than that observed in the normal littermate controls. The demonstration of a decrease sensitivity of a metabolic control mechanism in obese mice to exogenously administered glucocorticoid raises the question whether this system, and possibly other metabolic controls, are less responsive to glucocorticoids secreted endogenously. It is of interest that abnormal production of glucocorticoids has been implicated as a possible causal factor in Cushing's syndrome,[38] a disease associated with obesity and metabolic abnormalities

characteristic of prediabetic or diabetic states. Yates and Urquhart[30] have concluded that the control of plasma cortisol and corticosterone concentrations is accomplished by a closed-loop (negative-feedback) system with primary regulatory components in the central nervous system.

In addition to the condition of obesity in the mutant mice, the presence of insulin resistance[39] and increased liver glycogen content are suggestive of excessive adrenocortical secretion as a significant factor in disturbed carbohydrate metabolism. Hellman[40] has described a hyperplasia of the adrenal cortex of mice suffering from the hereditary obese-hyperglycemic syndrome. If there is a hypersecretion of glucocorticoids in C57BL/6J-*ob* mutant mice, then it is not reflected in a marked elevation of the basal level of liver TA activity (Table 3). However, the absence of an elevated TA activity in the C57BL/6J-*ob* mice may be indirect evidence that the enzyme induction system, as well as other metabolic regulatory components, are indeed less responsive to endogenously secreted glucorticoids. An elevation of TA activity by excessive adrenocortical secretion could occur early in the development of obesity as a transitory phase of disturbance in metabolic regulation. The continued secretion of glucocorticoids during the development of obesity may cause liver enzyme adaptation resulting in an increased rate of steroid metabolism. There is experimental evidence that in the obese human patient exogenously administered corticosteroids are removed at a faster rate than in the normal person.[41, 42]

While the dietary and metabolic balance of the obese mice appears to decrease the effectiveness of glucocorticoids to cause liver TA induction, the dietary conditions of fasting and of fasting followed by a controlled feeding period enhance the liver enzyme induction process in normal mice by the synthetic glucocorticoid, flumethasone. Greengard and Baker[43, 44] demonstrated that fasting also enhanced the induction of liver TA activity in the rat by hydrocortisone. Enhancement by starvation resembles the action of glucagon which selectively potentiates the induction effect of hydrocortisone. The enhancing effect of the controlled feeding period may resemble the effect on glucose 6-phosphate dehydrogenase. An "exaggerated" synthesis of glucose 6-phosphate dehydrogenase is known to occur in rat liver after the animals are fasted 2 to 3 days and then re-fed a balanced diet. Potter *et al.*[45] demonstrated that fasting, re-fed adapted rats have accentuated oscillations in enzyme activity and different periodicities as compared with rats fed *ad lib.* It may be worth studying the obese mutant mice in a fasting, re-fed adaptation cycle to determine whether it enhances the glucocorticoid sensitivity of liver TA.

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