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Regulation of the 11β-Hydroxysteroid Dehydrogenase in the Rat Adrenal

Decrease Enzymatic Activity Induced by ACTH

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Patients with ectopic ACTH syndrome often develop hypertension and hypokalemic alkalosis with an abnormal increase in the ratio of plasma cortisol to cortisone, indicating that 11β-hydroxysteroid dehydrogenase (11βHSD) activity is inhibited. Inhibition of 11βHSD allows access of cortisol or corticosterone to the mineralocorticoid receptor where it act as a mineralocorticoid. Two isozymes, 11βHSD-1 and 11βHSD-2, have been cloned and characterized. The rat adrenal expresses the mRNAs for 11βHSD-2 and, in lesser amounts, 11βHSD-1. We investigated the effect of ACTH on the 11 11βHSD-2 activity in the rat adrenal. Rat adrenal cells zone fasciculata (ZF) were dispersed and incubated separately with increasing concentrations of ACTH for 90 min, and secretion of corticosterone (B) and 11-dehydrocorticosterone (A) in the media was measured by enzyme-linked immunoabsorbent assays (ELISA). The conversion of [^{3}H]B to [^{3}H]A in the presence of 0.5 m*M* NAD^+ was evaluated in microsomes prepared from dispersed cells preincubated for 30 min with cyanoketone and metyrapone followed by incubation for 30 min with the same inhibitors, with and without 10 n*M* ACTH. The dispersed cells of the ZF produced significant amounts of A which increased with ACTH. The basal B/A ratio was 0.97 ± 0.05. ACTH caused a concentration-dependent increase in the ratio of B/A with a maximum ratio of 9.58 ± 0.20. ACTH also inhibited the conversion of [^{3}H]B to [^{3}H]A in microsomes in which endogenous B production was inhibited by cyanoketone and metyrapone. ACTH did not change the K_m for B conversion, but the V_{max} was reduced significantly (1.73 ± 0.43 pmol/min . mg protein), indicating that ACTH suppressed the 11βHSD-2 in a noncompetitive fashion. Dibutyryl cyclic AMP (dcAMP) also produced a concentration-dependent increase in the B/A ratio, but various concentrations of calcium did not affect the enzyme activity. In summary, adrenal cells treated with ACTH results in a significant increase in the ratio of B/A in the ZF owing a noncompetitive inhibition of the 11βHSD-2 via the ACTH receptor.

Key Words: 11β-hydroxysteroid dehydrogenase; ACTH; rat adrenals; ectopic ACTH syndrome.

Introduction

The mineralocorticoid receptor (MR) has a similar affinity for the glucocorticoids corticosterone (B) and cortisol (F) as for the mineralocorticoid aldosterone *(1)*. Cortisol and corticosterone have very minor antinatriuretic activities in vivo under physiologic conditions, although they are 100–1000 times as abundant as aldosterone in plasma. The selectivity of the mineralocorticoid receptor for aldosterone is believed to be conferred by the presence of 11β-hydroxysteroid dehydrogenase (11βHSD) in aldosterone target tissues, which rapidly converts B and F to the inactive forms 11-dehydrocorticosterone (A) and cortisone (E), respectively, before they bind the MR *(2,3)*.

At least two isozymes of the 11βHSD have been characterized and cloned. The type 1 isozyme (11βHSD-1) is $NADP^+$-dependent, has a high K_m of 1–3 μ*M* for B and F, is bidirectional, functioning primarily as a reductase in vivo, and does not colocalize with the MR in the kidney *(4)*. The type 2 isozyme (11βHSD-2) is NAD^+-dependent, unidirectional (dehydrogenase activity only), has a low K_m of 4–14

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n*M* for B and F, which is relevant to circulating levels of free glucocorticoids, and colocates with the MR in aldosterone target tissues *(5)*. 11βHSD-2 has been cloned from sheep *(6)*, human *(7)*, rabbit *(8)*, mouse *(9)*, and rat *(10)* renal cDNA libraries. *In situ* hybridization of the 11βHSD-2 mRNA indicates expression in the female reproductive system and the adrenals in rats, as well as in classic epithelial aldosterone target tissues *(11–13)*. The 11βHSD activity has been shown to modulate glucocorticoid binding to the glucocorticoid receptor *(14,15)*. This is particularly crucial in the placenta, where 11βHSD-2 maintains normal fetal blood levels in the face of elevated maternal glucocorticoids *(16,17)*, and in the hypothalamus, where it modulates access of F and B to the hypothalamo–pituitary–adrenal feedback system *(18)*. *In situ* hybridization indicates that mRNA expression signal is moderately abundant in the zone fasciculata (ZF), weak in the medulla, and absent in the zone glomerulosa *(13)*. However, a role for the enzyme in adrenal function is unknown *(13)*.

Patients with ectopic ACTH syndrome often develop hypertension and hypokalemic alkalosis *(19)*. The urinary ratios of tetrahydrocortisol (THF) plus allotetrahydrocortisol (5αTHF) to tetrahydrocortisone (THE) and plasma ratios of F to E in the patients with ectopic ACTH syndrome are increased, suggesting that the 11βHSD activity is inhibited *(20–22)*. It has been postulated that ACTH inhibits the 11βHSD by producing a substrate excess resulting in saturation of the enzyme *(20–22)*. It is also known that B or F can produce substrate inhibition of the enzyme *(23)*.

Activity and mRNA for the 11βHSD enzymes have been observed in adrenal glands of humans, sheep, rats, mice, and hamsters, but the functional significance is not clear *(13,24–27)*. The rat adrenal expresses both 11βHSD-1 and 11βHSD-2 mRNA *(13)*. We are reporting results of studies of the effect of ACTH on the B and A production in the rat adrenal cells.

Results

Production of B and A from Dispersed Cells of Adrenal ZF

11-Dehydrocorticosterone was secreted from the ZF cells. ACTH caused a significant concentration-dependent increase in A and B in ZF cells as shown in Fig. 1A. A and B production by ZF cells was not altered by A-II or potassium. ACTH produced a significant increase in the ratios of B/A in a concentration-dependent manner in the media of ZF cells. The maximum B/A ratios was 9.58 ± 0.20 for the ZF cells incubated with 10 n*M* ACTH. A-II and potassium had no effect on the ZF ratio (Fig. 1B).

Concentration-Dependent Effect of ACTH on the 11βHSD-2 Activity of the ZF Cells

We confirmed that the rat adrenal has much higher 11βHSD-2 than 11βHSD-1 activity *(13)*, so we only studied the effect of ACTH on the 11βHSD-2 activity. The 11βHSD-2 activity was inhibited by incubation for 30 min with ACTH in a concentration-dependent manner as shown

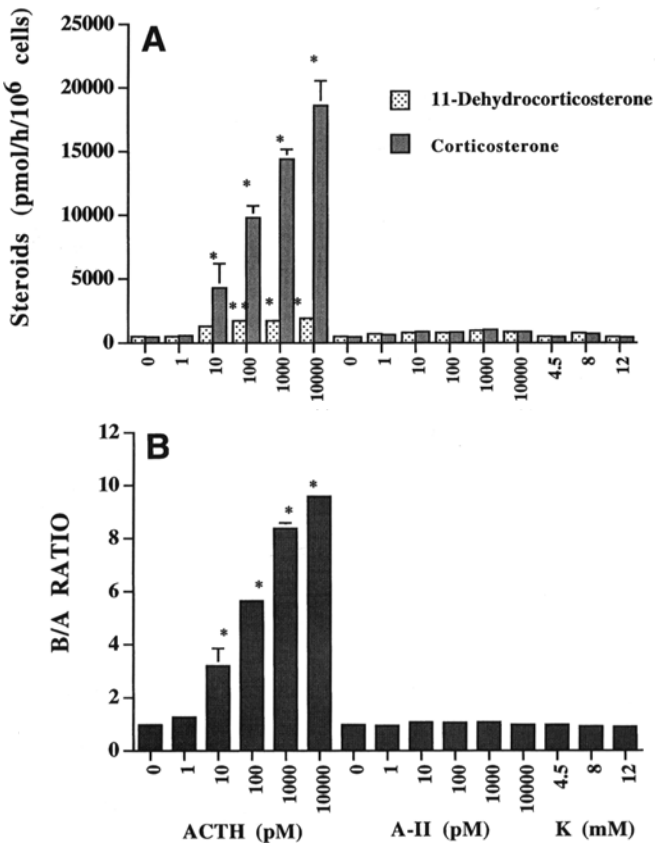

Fig. 1. A. Effect of ACTH, A-II, and potassium on the secretion of A and B from dispersed cells of the ZF. The cells were incubated with increasing concentrations of ACTH, A-II or potassium for 90 min and the steroids; in the media measured by ELISA. Results are presented as the mean production of the steroids (pmol/h . 106 cells) + SEM. (*$P < 0.01$ and **$P < 0.05$ different from the control). B. Effect of ACTH, A-II and potassium on the ratio of B/A from dispersed cells of the ZF. (*$P < 0.01$ different from the control.) Data were calculated from Fig. 1A.

Effect of Various Calcium Concentrations on the 11βHSD-2 Activity

No effect on the enzyme activity was found with incubation with various calcium concentrations between 0 and 1.8 m*M* (data not shown). One micromolar of calcium ionophore suppressed the activity to 56.5 ± 2.2% ($P < 0.05$) for the ZF cells compared with the controls.

Discussion

Eighty percent of patients with Cushing's syndrome are hypertensive; the percentage is even greater in those with ectopic ACTH syndrome *(28,29)*. The marked hypercortisolism of ectopic Cushing's syndrome is associated with hypertension and hypokalemic alkalosis *(21,22)*. The urinary ratio of THF + 5αTHF/THE *(21)*, a measure of 11βHSD activity, is elevated in patients with Cushing's syndrome and is significantly higher in patients with ectopic ACTH syndrome than in those with Cushing's disease *(21,22)* and is inversely correlated with serum potassium,

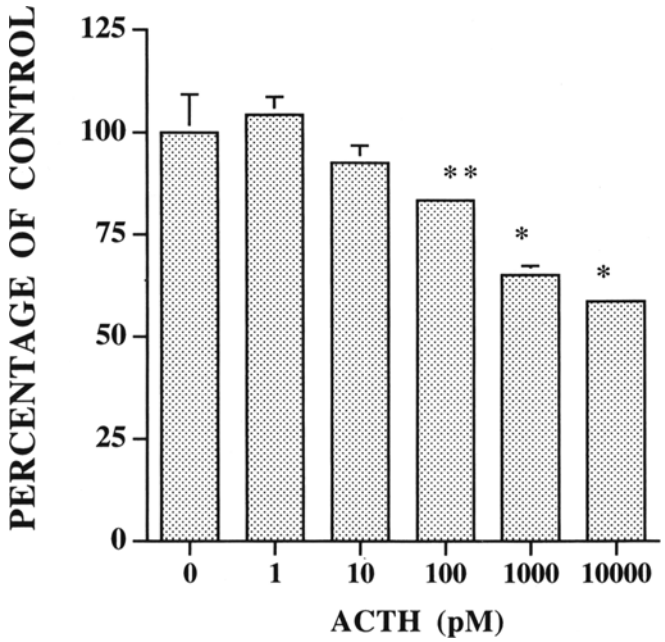

Fig. 2. Concentration dependent effects of ACTH on 11βHSD-2 activity in the rat adrenal. The ZF cells were preincubated with 10 μ*M* cyanoketone and 25 μ*M* metyrapone for 30 min followed by incubation with the same inhibitors and increasing concentrations of ACTH for 30 min. Microsomes were prepared by centrifugation at 100,000*g* for 60 min. The microsomes were incubated for 15 min with 200,000 dpm of [^{3}H]B and 0.5 m*M* NAD^+. Converted [^{3}H]A and residual [^{3}H]B were separated by TLC. Results are presented as the mean percentage of control (0 p*M* ACTH) ± SEM. (*$P < 0.01$ and **$P < 0.05$ different from the control.)

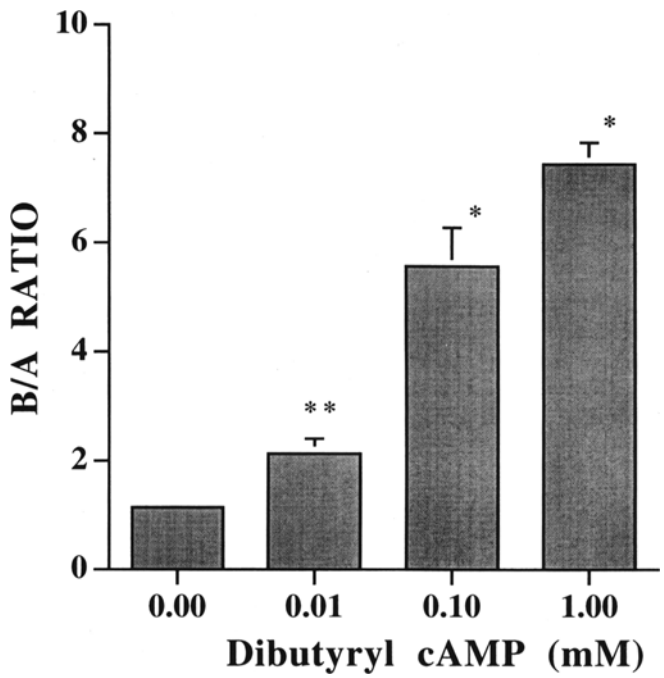

Fig. 3. Effect of dcAMP on the B/A ratio. The dispersed adrenal cells were preincubated with 10 μ*M* cyanoketone and 25 μ*M* metyrapone for 30 min followed by incubation with the same inhibitors and increasing concentrations of dcAMP for 30 min. Adrenal microsomes were incubated with 200,000 dpm of [^{3}H]B and 0.5 m*M* NAD^+ for 15 min. (*$P < 0.01$ and **$P < 0.05$ different from the control.)

in Fig. 2. Ten nanomolars of ACTH significantly suppressed the activity to 58.8 ± 1.8 % of the controls. The cyanoketone and metyrapone preincubation successfully inhibited ACTH-dependent production of B, since mean concentrations of B in the cytosols measured by ELISA were 0.94, 1.13, 1.31, 1.02, and 1.09 n*M* for 0, 10, 100, 1000, and 10,000 p*M* ACTH, respectively. Incubation of microsomes with similar amounts of ACTH did not inhibit the conversion of [^{3}H]B to [^{3}H]A, indicating that ACTH did not have a direct effect on the 11βHSD-2 enzyme, as has also been shown by others *(27)*.

*Inhibition of the 11βHSD-2 Activity by 10 n*M *ACTH in the Adrenal ZF Cells*

The mean K_m for B conversion to A and V_{max} of the 11βHSD-2 in cells treated with 10 n*M* ACTH compared to untreated is listed in Table 1. Ten nanomolars of ACTH did not change the K_m, but reduced the V_{max} suppressing the activity to 60.1% in the ZF cells.

Effect of cAMP on the Secretion of A and B

cAMP caused a significant increase in B, A (data not shown) and the B/A ratio in a concentration-dependent manner in ZF as ACTH did (Fig. 3).

Table 1
Effect of ACTH on the Mean K_m and V_{max} of the 11βHSD-2 in the Rat Adrenal[a]

K_m, n*M*, for B		V_{max}, A pmol/min/mg protein	
Control	10 n*M* ACTH	Control	10 n*M* ACTH
24.6 ± 3.7	21.9 ± 4.8	4.34 ± 1.35	1.73 ± 0.43*b*

Dispersed adrenal cells were preincubated with 10 μ*M* cyanoketone and 25 μ*M* metyrapone for 30 min followed by incubation with the same inhibitors with or without 10 n*M* ACTH for 30 min. The cells were homogenized and crude microsomes were prepared by centrifugation and incubated with increasing concentrations of [^{3}H]B and 0.5 m*M* NAD^+ for 15 min. The conversion of [^{3}H]B to [^{3}H]A was measured by TLC. The K_m and the V_{max} were obtained from double-reciprocal plots. Data were expressed as the mean ± SEM of 5 experiments. *$P < 0.05$ vs the control.

indicating a reduction in 11βHSD-2 activity *(22)*. The marked elevation of cortisol is caused by increased adrenal secretion of cortisol and by defective inactivation of cortisol by the 11βHSD enzyme *(20–22)*. The cause of the defective 11βHSD activity in Cushing patients is unclear. The possibilities include: cortisol inactivation overload or substrate inhibition *(21)*; ACTH inhibition of the renal conversion of cortisol to cortisone *(20)*, and, as shown in the present study, a disproportionally smaller increase of

the 11-keto in comparison to the 11-hydroxy steroid when the adrenal is stimulated by ACTH. Plasma levels of cortisone increase significantly when exogenous cortisol is administered, but do not increase when plasma cortisol is increased by ACTH stimulation either from endogenous or exogenous sources *(20)*.

These studies demonstrated that ZF cells produced a significant amount of A, further extending perfusion studies *(27)*. ACTH treatment resulted in a significant increase in the ratio of B/A, but stimulation with A-II or potassium did not, suggesting that inhibition of the 11βHSD is specific to ACTH stimulation. ACTH also inhibited the 11βHSD-2 activity in a noncompetitive fashion in adrenal cells in which the formation of 11β-hydroxylated steroids had been inhibited by cyanoketone and metyrapone before adding tritiated B.

ACTH had no direct effect when incubated with the rat adrenal microsomes *(27)*. The inhibitory action was mediated via the ACTH receptor coupled to the adenylate cyclase, because cAMP also caused an significant increase in the B/A ratio. Reduction of the V_{max} without a change in the K_m by ACTH suggest that 11βHSD-2 activity is decreased by suppression of the expression of enzyme mRNA or by promotion of enzyme degradation.

In conclusion, the present studies indicated that rat adrenal 11βHSD-2 activity, as assessed by A production, exists primarily in ZF cells and is inversely affected by ACTH concentrations. ACTH suppressed the enzyme activity in a noncompetitive fashion independently of its effects on ACTH-dependent steroid synthesis. Cushing syndrome owing to ectopic ACTH has a marked increase in the cortisol:cortisone ratio not only because of overwhelming the 11-HSD enzyme, but also because of an inhibitory effect of ACTH on the 11-βHSD-2 enzyme in the adrenal and maybe elsewhere.

Materials and Methods

Materials

Male outbred Sprague-Dawley rats were purchased from Harlan Industries (Indianapolis, IN). Deoxyribonuclease (DNase) I, bovine serum albumin (BSA), angiotensin II (A-II), metyrapone, dibutyryl cyclic AMP (dcAMP), calcium ionophore A23187, NAD^+ and unlabeled steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase type 1 and ACTH-(1-24) were purchased from Worthington Biochemical Co. (Freehold, NJ) and Organon Inc. (West Orange, NJ), respectively. Cyanoketone was generously provided from Sanofi Pharmaceutical Laboratories (Sterling, NY). [1,2-3H]-corticosterone (SA, 25 Ci/mmol) was tritiated by Amersham Corp. (Arlington Heights, IL). Channeled thin layer chromatography (TLC) plates (silica gel 60 A) and reagent grade solvents were purchased from Whatman Inc. (Clifton, NJ) and Fisher Scientific (Medford, MA), respectively. Ham's F- 12 and Hank's BSS without calcium and magnesium were supplied by the Cytology Campus Core Resource.

Preparation of the Isolated Rat Adrenal Cells

Six to 12 male SD rats were sacrificed by carbon dioxide narcosis and asphyxiation. The adrenals were quickly removed and cleaned. The ZF were manually separated from the zone glomerulosa (ZG). The cores containing the ZF, zone reticularis, and medulla were digested with 2 mg/mL collagenase and 0.5 mg/mL DNase I in Ham's F-12 containing 5 mg/mL of BSA for 40 min at 37°C to obtain isolated cells. The calcium and potassium concentrations in the Ham's F-12 were adjusted at 1.8 and 4.5 m*M* with addition of 1 M CaCl2 and KCl, respectively. When the effect of calcium on the 11βHSD-2 was investigated, Ham's F-12 containing 0.3 m*M* calcium was used for the dispersion. The dispersed cells were washed three times with Ham's F-12 to remove the collagenase and reconstituted in the same medium. Protein content was determined by a BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

Endogenous Production of B and A from the Rat Adrenal Cells

The dispersed ZF cells (200,000 cells/well) were incubated with increasing concentrations of ACTH (0–10 n*M*), A-II (0–10 n*M*), or potassium (4.5, 8, and 12 m*M*) in 500 μL of the Hams' F-12 containing 2 mg/mL of BSA for 1.5 h at 37°C using 24-well plates. The incubation was terminated by cooling on ice, and the supernatants were frozen until assayed. The concentrations of B and A were measured by respective ELISA.

Effect of ACTH on the 11βHSD Activity of the Adrenal Cells

Dispersed adrenal cells (1 million cells) were preincubated with 10 μ*M* of cyanoketone (3β-hydroxysteroid dehydrogenase inhibitor) and 25 μ*M* of metyrapone (11β-hydroxylase and aldosterone synthetase inhibitor) in Ham's F-12 containing 2 mg/mL BSA for 30 min at 37°C, followed by an incubation of another 30 min at 37°C with the inhibitors, with or without 10 n*M* ACTH in the Ham's F-12. The cells were then homogenized in ice-cold buffer (50 m*M* Tris-HCl, 1 m*M* MgCl2, and 0.25 *M* sucrose, pH 8.0) with a Teflon-glass homogenizer. A 100,000*g* for 60 min at 4°C centrifugation was done to prepare microsomes. The pellet was reconstituted in ice-cold incubation buffer (50 m*M* Tris-HCl and 1 m*M* MgCl2, pH 8.0). Twenty to 60 μg of the protein were incubated for 15 min at 37°C with increasing amounts of [3H]B in 500 μL of the incubation buffer in the presence of 0.5 m*M* NAD^+. For evaluation of ACTH concentration-dependency, the cells were incubated with increasing concentrations of ACTH (0–10 n*M*), and the microsomes were incubated with 200,000 dpm of [^{3}H]B and 0.5 m*M* NAD^+. The incubation was terminated by the addition of 5 mL of methylene chloride, 20 μg unlabeled B and A were added as markers, and the steroids were separated by TLC in acetone-methylene chloride (18:82, vol/vol). Areas corresponding to the steroids were located

under UV light, scraped, eluted with 500 μL isopropanol, and counted by liquid scintillation spectrometry. To verify that the inhibitors successfully inhibited B production, concentrations of B in the cytosols were determined by the ELISA.

Effect of dcAMP on the 11βHSD-2 Activity

The dispersed cells were preincubated with 10 μ*M* of cyanoketone and 25 μ*M* of metyrapone for 30 min at 37°C followed by incubation with the same inhibitors and 0, 0.01, 0.1, or 1 m*M* dcAMP for 30 min. The concentrations of B and A in the media were determined by the respective ELISAs to verify inhibition. The cells were then homogenized and the microsomes were incubated with [^{3}H]B and NAD^+ as above.

Effect of Various Calcium Concentrations on the 11βHSD-2 Activity

The dispersed cells were preincubated for 30 min at 37°C in Hank's BSS without calcium containing 10 μ*M* cyanoketone, 25 μ*M* metyrapone, and 2 mg/mL BSA. They were then incubated in Hank's BSS containing 200,000 dpm [^{3}H]B, 2 mg/mL BSA, and 0, 0.3, 0.5, 0.7, 1.0, 1.5, or 1.8 m*M* calcium or 1 μ*M* calcium ionophore for 10 min at 37°C. The cells were homogenized and the enzyme activities were determined as mentioned above.

Enzyme-Linked Immunosorbent Assays (ELISA)

ELISAs for B and A were done as described *(30)* using antibodies raised in sheep against corticosterone-3-carboxymethoxylamine-chicken serum albumin conjugate and 11-dehydrocorticosterone-3-carboxymethoxylamine-chicken serum albumin conjugate. The anticorticosterone antibody has a crossreactivity of 0.83% with A, 2.7% with F, 12.5% with DOC, 2.5% with progesterone, and 0.083% with 18-OH-DOC. The antibody against anti-11-dehydrocorticosterone has a crossreactivity of 0.81% with B, <0.09% with F, 0.75% with DOC, 0.11% with cortisone, and 0.1% with progesterone.

Statistical Analysis

All the experiments were performed in duplicate. All values are expressed as the mean ± SEM. Differences in the measured variables between control and ACTH-treated samples were evaluated by ANQVA or Wilcoxon's signed rank test, where appropriate. Enzyme kinetic data (K_m and V_{max}) were obtained from double-reciprocal plots (Lineweaver-Burk plots).

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