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SPECIFICITY AND STRUCTURAL RELATIONSHIPS OF STEROIDS WHICH AFFECT PHOSPHOLIPASE/ PROSTAGLANDIN SYNTHETASE, *IN VIVO*: A POSSIBLE RELATION TO BLOOD PRESSURE

DONALD J. FRETLAND, EDWARD A. BROWN and PETER S. CAMMARATA*
G. D. Searle & Co., 4901 Searle Parkway, Skokie, IL 60077, U.S.A.

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Summary—Fifty-two steroids, structurally related to spironolactone, were tested for their ability to alter excretion of urinary prostaglandin-E metabolites (U-PGE-M). We found that steroids which are associated with elevation or depresssion of blood pressure will elevate or depress basal levels of U-PGE-M in the rat. Structural requirements for elevation or depression of metabolites are narrow and sensitive to slight conformational changes in either the C-17 side chain or the steroid nucleus. Metabolite-elevating steroids share a common basic conformation, and metabolite-depressing steroids share a different common basic conformation. These two basic conformations differ chiefly in the C-17 side chain. The conformational requirements are analogous to the specificity shown by hormone receptors or by enzymes. A strong association of urine volume with U-PGE-M ($r = -0.93$) was demonstrated in rats treated with spironolactone. A possible explanation relating these results to alteration of blood pressure was presented.

INTRODUCTION

We previously have shown that spironolactone, a steroid with hypotensive activity, inhibits the excretion of urinary prostaglandin-E metabolites (U-PGE-M) in a linear, dose–response relation [1, 2] at doses far below those required to demonstrate diuretic activity. We also have shown that aldosterone, at doses less than required for sodium retention, and angiotensin, at doses less than required for pressor activity, stimulated U-PGE-M excretion in a linear dose–response relation. Stimulation of excretion by both pressor agents could be inhibited by spironolactone, and the inhibition of angiotensin-stimulated excretion could be demonstrated in the aldosterone-depleted rat. These results raised the possibility that aldosterone, angiotensin, spironolactone, and prostaglandin-E, might be acting at the same locus in a highly associated mechanism related to blood pressure.

In order to explore the specificity of spironolactone's activity on these metabolite-stimulatory pressor agents, we tested fifty-two structurally related steroids, some of which have been shown to be associated with blood pressure changes *in vivo* [3]. We report studies showing that only spironolactone and three other hypotensive steroids, out of fifty-two analogs, inhibit U-PGE-M excretion, while aldosterone, deoxycorticosterone, cortisol and progesterone, hormonal steroids associated with blood pressure elevation, stimulated U-PGE-M excretion. Structural requirements for either inhibitory or stimulatory activity were very narrow and stereospecific, reminiscent of the structural specificity shown by receptors or enzymes for specific agonists.

EXPERIMENTAL

Chemicals

Aldosterone, progesterone, estradiol, and testosterone propionate were purchased from Calbiochem-Behring (La Jolla, CA), hydrocortisone from Sigma Chemical Co. (St Louis, MO), deoxycorticosterone from J. T. Baker Chemicals (Phillipsburg, NJ) and 17-alpha-hydroxy-; 17-alpha-20-alpha-dihydroxy-; and 17-alpha-20-beta-dihydroxy; progesterones from Steraloids Inc. (Wilton, NH). The remaining steroids were synthesized in the chemical research department of G. D. Searle & Co. Prior to release for testing in any biological assay, newly synthesized compounds must have a demonstrated purity of at least 95% and possess chemical and spectroscopic data supporting the assigned structure. Spironolactone (Aldactone®) is a product of G. D. Searle & Co., (Skokie, IL, U.S.A.).

In vivo *experiments*

Five rats were used to test each compound. Tests were conducted in groups of 35 rats per group. Spironolactone was retested at the same time to insure comparison of results on different days. Aldosterone (0.125 mg/kg), progesterone (40 mg/kg), deoxycorticosterone (1.25 mg/kg), cortisol (1.0 mg/kg), estradiol (0.1 mg/kg), and testosterone propionate (1.0 mg/kg) were tested at 5× the minimal biologically active dose, while the remaining steroids were tested at a dose of 50 mg/kg, which is 225× the minimal active dose for spironolactone.

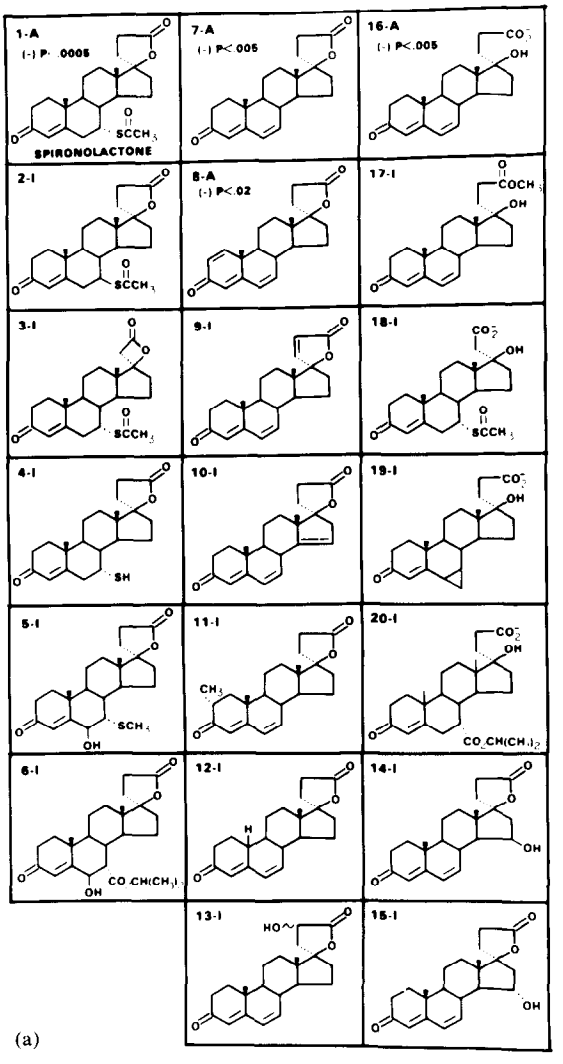
*To whom correspondence should be addressed.

Table 1. Means and tests of significance of U-PGE-M excretion and urine volumes

Test No.	Dose* (mg/kg)	Response (24 h)	Means ± SEM Control	Test	*t*	D.F.	*P*
1	5	U-PGE-M	127 ± 6	107 ± 7	−2.33	18	$P < 0.05$
		u.v.	13.1 ± 1.0	20.6 ± 1.0	5.48	18	$P < 0.005$
2	5	U-PGE-M	129 ± 5.0	111 ± 3	−3.41	18	$P < 0.005$
		u.v.	12.9 ± 0.8	15.4 ± 0.1	1.75	18	$P < 0.05$
3	50	U-PGE-M	129 ± 5.0	97 ± 7.0	−3.59	23	$P < 0.005$
		u.v.	12.5 ± 0.9	15.2 ± 0.9	1.96	23	$0.05 < P < 0.1$
4	50	U-PGE-M	123 ± 2.0	86 ± 8.0	−4.22	18	$P < 0.0005$
		u.v.	13.2 ± 0.7	17.8 ± 1.3	3.09	18	$P < 0.01$
5	50	U-PGE-M	123 ± 2.5	82.7 ± 8.6	−4.54	18	$P < 0.0005$
		u.v.	12.8 ± 0.5	16.1 ± 0.5	4.24	18	$P < 0.0005$
6	50	U-PGE-M	123 ± 3.7	101 ± 4.2	−3.77	23	$P < 0.005$
		u.v.	12.9 ± 0.6	14.7 ± 0.6	2.30	23	$P < 0.05$
7	50	U-PGE-M	121 ± 2.8	94 ± 7.7	−3.41	18	$P < 0.005$
		u.v.	13.8 ± 0.6	17.0 ± 0.9	2.66	18	$P < 0.02$

t = Students *t* value. D.F. = Degrees of freedom. *P* = Significance level. U-PGE-M = Urinary-Prostaglandin-E Metabolites (ng/24 h). u.v. = Urine volume (cc/24 h). Dose*: Spironolactone.

Male, Sprague–Dawley rats, 180–200 g, were placed in individual metabolism cages and kept in a quiet, constant temperature, 12-h light, 12-h dark, environment. Two 24-h control-urine samples were collected, after which rats were injected subcutaneously with a single dose of compound suspended in 1.0 ml of 0.9% saline. It had previously been shown [1] that neither the stress of injection nor the vehicle significantly affect U-PGE-M excretion. Three 24-h test-urine samples were then collected. Urine excre-



(a)

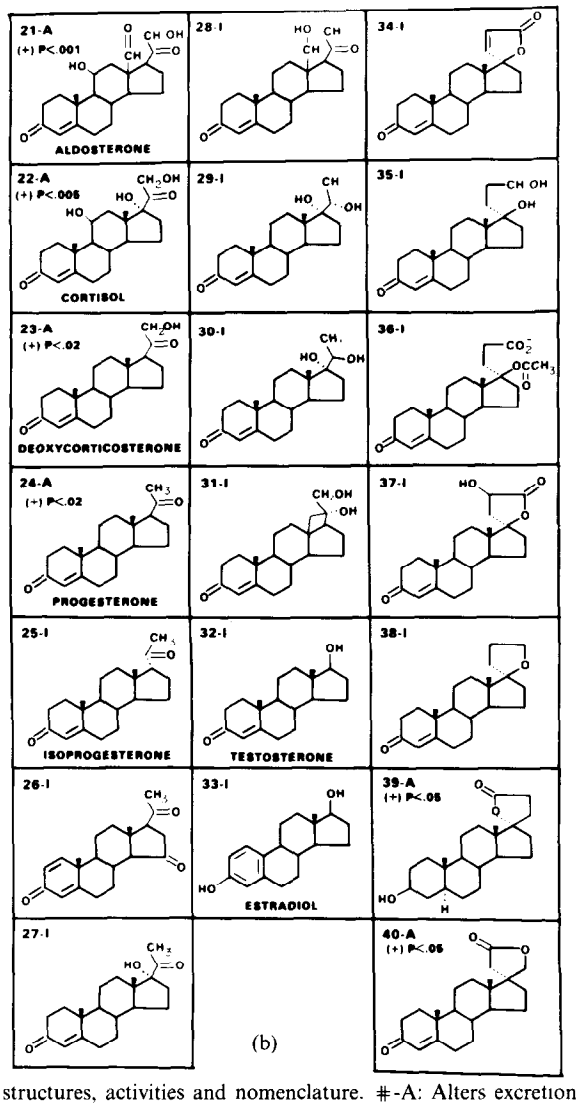


Fig. 1. Summary of structures, activities and nomenclature. #-A: Alters excretion of U-PGE-M. #-I: No effect on U-PGE-M. (+): Stimulates excretion of U-PGE-M, $P < 0.05$. (−): Inhibits excretion of U-PGE-M, $P < 0.05$. **1.** 7α-(Acetylthio)-17-hydroxy-3-oxo-17α-pregn-4-ene-21-carboxylic acid, γ-lactone. **2.** 7β-(Acetylthio)-17-hydroxy-3-oxo-17α-pregn-4-ene-21-carboxylic acid, γ-lactone. **3.** 7α-(Acetylthio)-17-hydroxy-3-oxo-17α-pregn-4-en-20-oic acid, β-lactone. **4.** 17-Hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid, γ-lactone. **5.** 6β,17-Dihydroxy-7α-(methylthio)-3-oxo-17α-pregn-4-ene-21-carboxylic acid, γ-lactone. **6.** 6β,17-Dihydroxy-3-oxo-17α-pregn-4-ene-7α,21-dicarboxylic acid, γ-lactone, (l-methylethyl) ester. **7.** 17-Hydroxy-3.oxo-17α-pregna-4,6-diene-21-carboxylic acid, γ-lactone. **8.** 17-Hydroxy-3-oxo-17α-pregna-1,4,6-triene-21-carboxylic acid, γ-lactone. **9.** 17-Hydroxy-3-oxo-17α-pregna-4,6,20-triene-21-carboxylic acid, γ-lactone. **10.** 17-Hydroxy-3-oxo-17α-pregna-4,6,14-triene-21-carboxylic acid, γ-lactone. **11.** 17-Hydroxy-2α-methyl-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid, γ-lactone. **12.** 17-Hydroxy-3-oxo-19-nor-17α-pregna-4,6-diene-21-carboxylic acid, γ-lactone. **13.** 17,21-Dihydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid, γ-lactone. **14.** 15β,17-Dihydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid, γ-lactone. **15.** 15α,17-Dihydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid, γ-lactone. **16.** 17-Hydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid monopotassium salt. **17.** Methyl 17-hydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylate. **18.** 7α(Acetylthio)-17-hydroxy-3-oxo-17α-pregn-4-en-20-oic-acid. **19.** 6α,7α-Dihydro-17-hydroxy-3-oxo-3′H-cyclopropa[6,7]-17α-pregna-4,6-diene-21-carboxylic acid, monopotassium salt. **20.** 17-Hydroxy-3-oxo-17α-pregn-4-ene-7α,21-dicarboxylic acid, 7-(1-methylethyl)ester, monopotassium salt. **21.** 11β,21-Dihydroxy-3,20-dioxopregn-4-ene-18-al. **22.** 11β,17,21-Trihydroxypregn-4-ene-3,20-dione. **23.** 21-Hydroxypregn-4-ene-3,20-dione. **24.** Pregn-4-ene-3,20-dione. **25.** 17α-Pregn-4-ene-3,20-dione. **26.** Pregna-1,4-diene-3,15,20-trione. **27.** 17-Hydroxy-pregn-4-ene-3,20-dione. **28.** 18-Hydroxypregn-4-ene-3,20-dione. **29.** 17,20S-Dihydroxypregn-4-en-3-one. **30.** 17,20R-Dihydroxypregn-4-en-3-one. **31.** 20S-Hydroxy-20-(hydroxymethyl)-13,21-cyclo-18-norpregn-4-en-3-one. **32.** 17β-Hydroxyandrost-4-ene-3-one. **33.** Estra-1,3,5,(10)-triene-3,17α-diol. **34.** 17-Hydroxy-3-oxo-17α-pregna-4,20-diene-21-carboxylic acid, γ-lactone. **35.** 17β-Hydroxy-17-(3-hydroxypropyl)-androst-4-en-3-one. **36.** 17-(Acetyloxy)-3-oxo-17α-pregn-4-ene-21-carboxylic acid. **37.** 17,21-Dihydroxy-3-oxo-17α-pregn-4-ene-21-carboxylic acid, γ-lactone. **38.** 4′,5′-Dihydrospiro[androst-4-ene-17β,2′(3′H)-furanl-3-one. **39.** 3β,17-Dihydroxy-5α-pregnane-21-carboxylic acid, γ-lactone. **40.** 17-(Hydroxymethyl)-3-oxo-17α-pregn-4-en-21-oic acid, γ-lactone.

tion was measured daily, and aliquots were centrifuged and stored frozen until assayed for U-PGE-M by radioimmunoassay [2]. For each compound, the mean values of U-PGE-M for all rats on two control days ($n = 10$), the mean value for all rats on three test days ($n = 15$), and group t-tests [4] for significant difference of means were calculated.

In order to assess reproducibility and accuracy, data from seven separate tests with spironolactone were assembled in Table 1. A regression analysis of urine volume vs U-PGE-M excretion was then conducted, using the Minitab statistical program [5]. Thirteen data points were used in the regression analysis, seven of which were derived from control and six derived from test urines.

RESULTS

The results of tests of forty steroids on U-PGE-M excretion, their structures, and significance of results are summarized in Fig. 1. The compounds fell into three categories according to their effect on U-PGE-M excretion; 4 inhibitors, 6 stimulators, and 30 inactive compounds. Twelve additional steroids were tested and found inactive. Since they were simple derivatives of the structures shown, they were omitted.

Inhibitory steroid activity was restricted to a narrow range, exhibited by compounds **1, 7, 8** and **16**. Compounds **7** and **16** are metabolites of spironolactone, and compound **8** is a synthetic, unsaturated analog of compound **7** which is the main metabolite of spironolactone (for review on metabolism of spironolactone, see [6]). Two other metabolites of spironolactone, compounds **4** and **5,** were inactive. Since both compound **1** and **16** are metabolized *in vivo* to form compound **7,** compounds **7** and **8** are the only two of fifty-two closely related steroids that possess intrinsic inhibitory activity.

Rigid requirements for nuclear conformation, chirality, and ease of ring closure contribute to the narrow specificity observed. For example, compound **2,** the beta-isomer of compound **1,** is totally inactive. Compound **3,** identical to compound **1** except for the smaller, 4-atom, C-17, strained lactone ring, is inactive because of a rigid requirement for correct size of the lactone ring. Compounds **4, 5** and **6,** where enzymatic activity required to form compound **7** is prevented by a thiol, a thioether, and an isopropyl ester substitution at C-7, are also inactive.

Rigid conformational constraints around compound **7** are demonstrated by compounds **9, 10, 11, 12, 13, 14** and **15,** where C-17 lactone ring unsaturation, D-ring unsaturation, 2-methyl substitution, C-10 demethylation, hydroxylation of the C-17 lactone ring, or hydroxylation of the D-ring, respectively, produce conformational changes which are associated with loss of inhibitory activity of compound **7**.

Both conformational constraints and the requirement for a critical C-17 lactone ring structure are illustrated by compounds **17, 18, 19** and **20.** Compound **17,** the methyl ester of compound **16** (which *in vivo* is in equilibrium with compound **7**), cannot lactonize and is inactive as a result. Similarly, compound **18,** which lactonizes only with great difficulty because of the strain inherent in a four membered ring, is also inactive. Compounds **19** and **20,** although they can lactonize easily, are also inactive, probably because B-ring derivatization prevents enzymatic formation of B-ring unsaturation, with resultant formation of compounds **16** and **7**.

The range of active structures which stimulate U-PGE-M excretion seems somewhat broader. Nevertheless, this class of stimulatory steroids again demonstrates a rigorous requirement for correct configuration of the C-17 side chain and conformation of the nucleus. It is well established that aldosterone, deoxycorticosterone, cortisol, and progesterone, compounds **21–24,** interact *in vivo* with at least three different specific endogenous receptors to produce hormonal effects [7]. Despite having different receptor targets, they all share a common ability to stimulate U-PGE-M excretion.

Of the four stimulatory natural steroids, progesterone can be regarded as the prototype of activity. Allowed variants on this basic structure are a C-21 hydroxyl group, a C-17 alpha-hydroxyl group with a 11-hydroxy group, and a C-18 hydroxyl group. These modifications can be viewed as progesterone derivatives which do not seriously perturb a requisite prototypic conformation. If this assumption is correct, then the following analogs should be inactive, which indeed they are.

Compound **25,** isoprogesterone, epimeric with progesterone at C-17, is inactive, illustrating a requirement for correct configuration. Compound **25** also eliminates membrane-perturbing, non-specific liphophylicity as a possible explanation of the stimulatory action. Compound **26** is inactive, probably because A-ring unsaturation, and a C-15 oxo group changes conformation of a requisite nucleus. Compound **27,** 17-alpha-hydroxyprogesterone and compound **28,** 18-hydroxy-progesterone, which can be regarded as rough analogs of cortisol or of aldosterone respectively, are also inactive, probably because they violate a required configuration of the C-17 side chain as did compound **25.** Compounds **29, 30,** and **31** are also inactive, probably because they lack the requisite C-20 oxo function, and because of an altered side chain configuration. Compounds **32** and **33,** testosterone and estradiol respectively, are inactive as expected since they lack the requisite C-17 side chain.

Although very close analogs of aldosterone and cortisol were not available, compounds **34, 35, 36, 37** and **38** possess the same A-ring, the same nucleus, and at least two oxygen atoms in reasonable proximity in space to where they are found in aldosterone and cortisol. This same series served as a test for

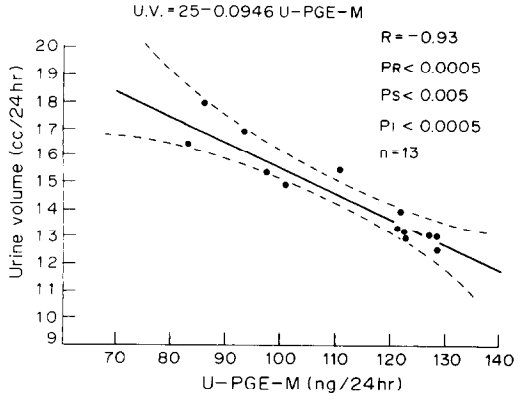


Fig. 2. Relation of urine volume (cc/24 h) to U-PGE-M (ng/24 h) excretion. ● = Experimental. (– – – –) = 95% Confidence belt. R = Correlation coefficient. P_r = Significance of overall regression. P_s = Significance of slope. P_i = Significance of intercept. n = No. of data pairs.

activity of analogs of spironolactone which do not possess a C-7 substituent. None of these five close structural analogs were active, illustrating the sensitivity to side chain conformation required for stimulatory activity.

Compound **40,** an analog of compound **7** with oxygen atoms of the lactone ring displaced by one carbon atom, and compound **39,** the reduced oxo and ring analog in which the C-17 oxygen atom is alpha rather than beta, are both active as stimulators of U-PGE-M excretion. Thus, when the critical lactone ring required for inhibitory activity is slightly altered, either a loss or a reversal of activity from inhibition to stimulation occurs.

In order to permit assessment of both reproducibility and accuracy of the assay, the means of U-PGE-M and urine volumes from seven separate spironolactone control tests have been gathered in Table 1. It is evident that measurement of basal levels of U-PGE-M excretion are reproducible from group to group with small SEM. The coefficient of variation for the seven control means was 2.9%, while that of the test means was 6.8%.

The regression analysis of 24-h urine volume versus 24-h U-PGE-M excretion, shown in Fig. 2, yields a correlation coefficient of −0.93 indicating that both prior to treatment, and after injection of spironolactone, the volume of urine, and the total quantity of metabolite excreted in a 24 h period are strongly associated. A test of significance of overall regression yielded $P < 0.0005$, and the coefficients of the slope ($P < 0.005$) and intercept ($P < 0.0005$) were also significant. Of thirteen points used in the analysis, all but one lie on, or well within, the 95% confidence belt.

DISCUSSION

These studies demonstrate that steroids associated with blood pressure elevation stimulate the excretion of prostaglandin metabolites, while steroids associated with blood pressure depression inhibit excretion of metabolites. Because neither the precursor fatty acids nor prostaglandins are found free in tissue, stimulation or inhibition of phospholipase/prostaglandin synthetase activity has probably occurred. Both stimulation and inhibition are critically dependent on steroid conformation, highly reminiscent of the specificity exhibited by natural steroids for their endogenous receptors. It should be emphasized that reference to a receptor is strictly an analogy, since this *in vivo* study was necessarily obscured by steroid metabolism, and no attempt was made to satisfy necessary criteria for identification of receptors [7].

The simplest of four structures with inhibitory activity was compound **7**. Since it is the metabolic product of two other active compounds (**1, 16**) *in vivo*, compound **7** is the prototype of steroids interacting with the inhibitory locus. Any modification of this prototype which significantly changed the conformation of the basic steroid nucleus, changed the configuration of the C-17 side chain, changed the size of the lactone ring, or prevented formation of the lactone ring resulted in loss of inhibitory activity or caused reversal of activity. Thus, only 2 of 52 closely related structures (**7, 8**) have intrinsic activity as inhibitors. It may be significant that these same compounds are the only diuretic/hypotensive agents [6] of the 52 tested.

The simplest structure with stimulatory activity was that of progesterone. As with inhibitory activity, stimulatory activity was a sensitive function of side chain structure and nuclear conformation. This is perhaps best illustrated by the total inactivity of isoprogesterone, an epimer of progesterone, and is further illustrated by the inactivity of eleven other closely related steroids carrying side chain or nuclear modifications.

Since this is a new steroid bioassay, it was necessary to present evidence of validity and reproducibility of the data. This investigation was conducted over a 6 month period, during which time spironolactone was tested seven times (5 rats/test) as a control substance, each test separated by at least a 2 week period. Despite variation with time and dose, basal levels of control and test U-PGE-M excretion were reproducible, as evidenced by the small coefficients of variation. The same reproducibility was exhibited by urine volumes.

The *in vivo* nature of the study precludes a rigorous explanation of the results at the molecular level, but two hypothesis relating to receptor mechanisms were considered and guided the choice of steroids for testing. The first was the hypothesis [8] that hormone–receptor interaction somehow activated a lipid-derived factor, which then transferred information from the complex to an "amplifier" in another part of the cell (for review, see [9]). In this hypothesis prostaglandins might act as the putative second mes-

senger. Some support is the observation that aldosterone stimulates phospholipid acylation-deacylation [10]. By competing for the aldosterone receptor, spironolactone in this hypothesis would be expected to inhibit the activation process and formation of prostaglandins acting as second messenger.

In order to test this possibility rigorously, natural steroid hormones which react with specific receptors had to be included in the test, even though they could not be regarded as analogs of spironolactone. Aldosterone, deoxycorticosterone, cortisol, and progesterone did activate phospholipase/prostaglandin synthetase, and spironolactone inhibited, in accordance with the hypothesis. However, estradiol and testosterone did not activate the enzyme system. This failure could be rationalized by numerous metabolic considerations, but clearly further work is required before this explanation can be seriously considered.

The second hypothesis, based on one proposed in prior work [12], is partially related to the first but more restricted. According to it, steroids which are hypertensive or hypotensive have blood pressure regulating activities which are modulated by prostaglandins in a process closely associated with the primary mechanism of action of the particular steroid but separate from it. This would explain the large correlation coefficients of U-PGE-M with dose of pressor agent (angiotensin = 0.80 and aldosterone = 0.77) reported earlier [1]. According to this second hypothesis, neither estradiol or testosterone should have significant effects on U-PGE-M excretion since neither have shown chronic blood pressure regulating activity. Thus, seven biologically active steroids, despite having different overall physiological activities, fulfill the predictions for effects on U-PGE-M excretion made by this second hypothesis. Minor discrepancies are presented by compounds **27** and **29,** reported to be hypertensive in sheep [11], but since neither concomitant measurements of blood pressure nor metabolic studies were made, reasons other than failure of the hypothesis could explain their inactivity.

Some experimental support for the second hypothesis is provided by results shown in Fig. 2. Direct effects of steroids on fluid regulation or smooth muscle activity have never been shown, although indirect effects have long been known. A hypothesis of prostaglandin modulation of blood pressure effects of steroids is made attractive by the fact that direct effects of prostaglandins on blood pressure are well documented [13]. The surprisingly strong correlation (−0.93) between U-PGE-M excretion and urine volume is partial evidence supporting the hypothesis. Despite the fact that the data are derived from untreated and treated rats, they all lie on the same regression line, indicating that under both basal and steroid intervention conditions, prostaglandins may be a determinant of urine volume, and indirectly a determinant of both total body fluid and long term blood pressure [14].

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