reason for this discrepancy is not known but it probably results from differences in the intestinal flora of the rats used in the two investigations. When a bile fistula is made on a rat from the strain used in this investigation only conjugated bile acids are excreted, mostly turocholic acid. This is in agreement with previous investigations.

The distribution of radioactivity between the 3 main bile acids found in the small intestine and blood is shown in Table II. The proportion of unconjugated bile acids is much higher in the blood than in the small intestine. The 2 rats (No. 5 and 6) having only small amounts of free bile acids in the small intestine also have a definitely lower percentage of free acids in the blood than the other animals. The results seem to indicate a preferential removal by the liver of the conjugated bile acids present in the portal blood.

Summary. Rats were given tritium labelled cholic acid intraperitoneally or orally and killed by exsanguination after 24 or 48 hours. The concentration of "total cholic acid" in whole blood was calculated from the distribution of isotope in intestine and blood and previous figures for intestinal "cholic acid." A value of about 0.08 mg/100 ml was found. Chromatographic analysis showed that more unconjugated cholic acid was present in the systemic blood than in the small intestine.

1. Sobotka, H., *Physiological Chemistry of the Bile,* Baltimore, Williams and Wilkins, 1937.
2. Mac Intyre, I., Wootton, I. D. P., *Ann. Rev. Biochem.,* 1960, v29, 635.
3. Rudman, D., Kendall, F. E., *J. Clin. Invest.,* 1957, v36, 530.
4. Osborn, E. C., Wootton, I. D. P., Da Silva, L. C., Sherlock, S., *Lancet,* 1959, vII, 1049.
5. Carey, J. B., Jr., *Science,* 1956, v123, 892.
6. ———, *J. Clin. Invest.,* 1958, v37, 1494.
7. Byers, S. O., Friedman, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 425.
8. Olivecrona, T., Sjövall, J., *Acta Physiol. Scand.,* 1959, v46, 284.
9. Wilzbach, E. E., *J. Am. Chem. Soc.,* 1957, v79, 1013.
10. Bergström, S., Lindstedt, S., *Acta Chem. Scand.,* 1957, v11, 1275.
11. Norman, A., *Acta Physiol. Scand.,* 1954, v32, 1.
12. Norman, A., Sjövall, J., *J. Biol. Chem.,* 1958, v233, 872.
13. Gustafsson, B., Norman, A., Sjövall, J., *Arch. Biochem. Biophys.,* 1960, v91, 93.
14. Portman, O. W., Murphy, P., *ibid.,* 1958, v76, 367.
15. Eriksson, S., *Acta Physiol. Scand.,* 1960, v48, 439.

Received March 20, 1961. P.S.E.B.M., 1961, v107.

Application of Physical Concepts to Dose-Response Relationships of Mixed Estrogens and Mixed Androgens. A Mathematical Analysis.* (26611)

HERBERT S. STRICKLER, ELEANOR L. SAIER AND ROBERT C. GRAUER
Department of Research in Endocrinology and Metabolism, William H. Singer Memorial Research Laboratory, Allegheny General Hospital, Pittsburgh, Pa.

The application of physical concepts to biological responsiveness with a view toward achieving a better understanding of biological phenomena has intrigued many workers in the field. We originally reported(1) on the biological responsiveness to topical application of varying mixtures of androsterone and dehydroepiandrosterone to chick combs. We recently reported(2) the results of injecting mixtures of estrone, estriol and estradiol 17β on biological response of the uteri of immature rats. Our results and discussions were based on conventional dose-response relationships. Our present aim was to see if our data would lend themselves to the concept of surface adsorption as set forth in the Langmuir(3) adsorption isotherm, especially since topical application involves direct ap-

* Supported in part by a grant-in-aid from U. S. Public Health Service.

plication of hormones to the target organ. Hitchcock(4) first pointed out the formal identity of the simplest Langmuir adsorption isotherm with an equation he derived from the law of mass action as applied to a reversible homogeneous reaction. Lineweaver and Burk(5), Levvy and Marsh(6) and more recently Stetten(7) have applied similar transformations to other biological data with good results.

For this presentation the formulations of Lineweaver and Burk(5), dealing with velocities of reaction, have been transformed into equations dealing with biological responses as follows:

$$\text{Response} = R = \frac{R_\infty C}{K_s + C} \quad (I)$$ or in reciprocal form $y = bx + a$ (II) where R_∞ is the response at infinite concentration C_∞, K_s is the dissociation constant of the surface-substrate complex, a is $1/R_\infty$, y is $1/R$, x is $1/C$ and b is K_s/R_∞. (*cf.* also Stetten(7)).

In applying the above concept of surface† adsorption of surface-substrate complexes to our problem of biological responsiveness we think of the target organ as containing one or more active sites which become covered with the hormone. As the concentration approaches infinity the response of the target organ levels off, approaching a maximum. If this is true, a straight line function can be obtained from this type of data by plotting $1/R$ against $1/C$ wherein R is response of the animal to the dose and C is dose or concentration. The equation of this line ($y = bx + a$) can then be determined by the method of least squares and with this information we can evaluate the original equation (I). The limiting factor R_∞ is defined as the reciprocal of the intercept, a, of this straight line and it can readily be seen from this that a large intercept infers a poor response. The constant b which is equal to K_s/R_∞ is defined by the slope of this line. The affinity constant (a/b) of the hormone for the surface may be interpreted as the reciprocal of K_s, the dissociation constant, which measures the ease of loosening the steroid from the enzyme.

When applying the above concepts to data of a biological nature one should always examine such data by plotting. This is to be done before an equation is derived by least squares since the lowest dose has the largest moment, and it can alter the fit of the equation markedly. For example, the fit of this reciprocal type of plot to the data for androsterone was excellent except at 1 gamma, which point was then discarded for the least squares calculation. The type of plot used by Stetten(7) (that usually employed for adsorption data) does spread the points out more evenly but in view of the behavior of the data in the terrace point region(Huggins (8)) we feel that our procedure is to be preferred for dose response data. Lineweaver and Burk(5) also favor the type of plot that we employ.

Results. Calculations using this formulation with our data for pure estrogens and androgens are presented in Table I. It is noted that estrone, which is less hydrophyllic than estradiol or estriol, has a much smaller affinity coefficient than either of the others. This concept, however, neglects biological interchange at the surface and assumes the same mode of action for all 3 estrogens. Note that b is equal to the dissociation constant K_s, measuring the ease of loosening the steroid from the enzyme, divided by the maximum biological power of the steroid, R_∞. This value for estradiol is markedly different from that of estrone and estriol (Table 1). The basic dose response curves for the estrogens using this concept are shown in Fig. 1. Androsterone and dehydroepiandrosterone, on the other hand, do not show much difference in their affinity constants, as would be expected from the fact that they are both 3-OH, 17-ketosteroids. However, b differs markedly, being much smaller for the more active androsterone. The dose response curves for androsterone and dehydroepiandrosterone are presented in Fig. 2. The remarkably good fit of these steroids to this concept is noteworthy since the method of

† In this paper the term "surface" means the net system contributing to the observed (macro) effect; only diffusion into cells and enzyme activity at a mitochondrial site may be involved, or several stages may be utilized.

TABLE I. Constants of Langmuir Adsorption Equations Calculated from Experimental Data on Estrone, Estradiol 17β, Estriol, Androsterone and Dehydroepiandrosterone.

Hormone	Slope b	Intercept a	a/b affinity constant	a/b (calc.)	Limits of response: 1/a (Stetten Q)	1/a + control	Highest observed value
E_1 Estrone	.0176	.0104	.59	—	96.1	117.6	103.5
E_2 Estradiol	.0019	.0078	4.11	—	128.2	149.7	94.1
E_3 Estriol	.0018	.0514	4.36	—	19.5	41.8	45.3
% by weight $E_1 + E_3$							
90 + 10	.0122	.0093	.76	.97	107.5	130.4	109.4
75 + 25	.0079	.0112	1.42	1.53	89.2	112.2	106.8
50 + 50	.0134	.0125	.93	2.47	80.0	102.5	100.2
25 + 75	.0153	.0186	1.22	3.42	53.5	76.0	101.7
10 + 90	.0120	.0282	2.35	3.99	35.5	58.0	63.0
% by weight $E_2 + E_3$							
1.64 + 98.36	.0099	.0375	3.78	4.36	26.7	51.6	58.4
4.76 + 95.24	.0114	.0137	1.20	4.35	73.0	96.0	45.3
13.04 + 86.96	.0037	.0149	4.03	4.33	67.1	89.1	85.6
% by weight $E_1 + E_2$							
99.45 + .55	.0453	−.0063	—	.61	—	—	—
98.36 + 1.64	.0415	−.0068	—	.65	—	—	—
95.24 + 4.76	.0247	−.0016	—	.76	—	—	—
86.76 + 13.04	.0083	+.0098	—	1.05	—	—	—
68.97 + 31.03	.0119	−.0102	—	1.68	—	—	—
Androsterone	3.525	.635	.180	—	1.57	1.85	1.71
Dehydroepiandrosterone	10.124	1.507	.149	—	.66	.93	.88
% by weight A + D							
75A + 25D	4.208	.881	.210	.172	1.13	1.39	1.45
60A + 40D	6.784	.827	.122	.168	1.21	1.58	1.48
50A + 50D	4.534	.770	.170	.164	1.30	1.60	1.70
40A + 60D	5.406	.911	.169	.161	1.10	1.42	1.43
25A + 75D	4.655	.835	.179	.157	1.20	1.50	1.54

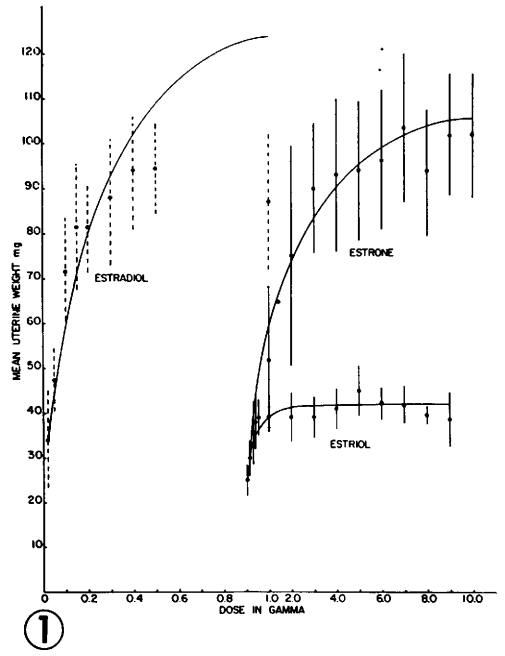
dosage presents a direct physiological pathway to reach the target organ, as contrasted with the parenteral mode of administration of the estrogens.

For the *mixtures,* equation (I) may again be plotted in the same fashion as was done for the pure hormones, *i.e.* 1/R versus 1/C or $y = bx + a$, where b and a, of course, do not have precisely the same meanings as for the single steroids. This was done for the various mixtures and the results have been summarized and included in Table I. Note that C is now the *total* concentration (*i.e.* the sum of the concentrations of the components). The proof for this procedure comes from a consideration of the Langmuir(3) relation for 2 gases competing for a surface.

Whence $\frac{1}{\text{Response}} = \frac{1}{R_\infty} + \frac{1}{R_\infty\left[\frac{B\rho + B'}{\rho + 1}\right]}$

$\frac{1}{C}$; $\rho = \frac{C_1}{C_2}$; $y = a + bx$ where R_∞ is maximum response for the particular mixture of estrogens, etc. in constant ratio ρ, C is total concentration and B and B′ are constants. Hence, a/b is $[(B\rho + B')/(\rho + 1)]$. On evaluating the constants B and B′, a/b becomes $\frac{a_1\rho/b_1 + a_2/b_2}{\rho + 1}$ (III). Hence, the affinity constant K = a/b may again be calculated for each mixture from the constants of the responses to the pure hormones. Comparisons of such calculated and observed ratios are also given in Table I.

Limits of response have been calculated, for pure compounds and all mixtures, directly from the equation (I) and compared to observed values. The results in most all cases are quite good. The fit of the equation (I) to the data for pure estradiol 17β (Table I) is not as good, however, as desired at the limits of responsiveness. To fit the data used in computation of the effect of mixtures, it was necessary to favor the dose range before



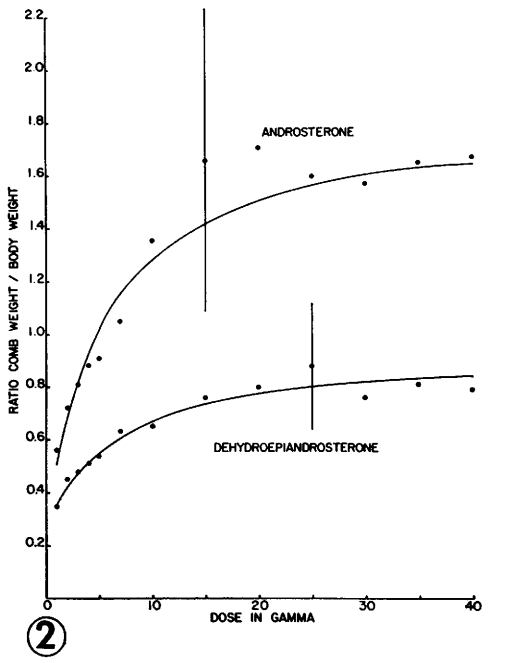


FIG. 1. Basic dose response data for estradiol 17β, estrone and estriol fitted by Langmuir adsorption concept (equation I) together with standard deviations (parenteral administration).

FIG. 2. Basic dose response data for androsterone and dehydroepiandrosterone fitted by Langmuir adsorption concept (equation I) together with the maximum standard deviation for each (topical application).

the threshold region. There is some indication in the original data(1,2) of a decreased response at the highest dosages. This, of course, is not predicted in the present theoretical work, which yields maximum response only at infinite dose.

To make further comparisons additive responses were calculated for each constituent of the dose in the mixture from the appropriate equation for the pure estrogen involved (Table I). These values were added and the sums then compared with the smoothed values obtained from the equations derived from the experimental data for each mixture (Table I). The results of these evaluations with each of the mixtures are discussed separately as follows:

Estrone-Estriol. Additivity was apparent through the entire dose range in the mixtures 90%-10% and 75%-25% but fell off at 8γ in 50%-50%, 7γ in 25%-75% and at 6γ in the 10%-90% mixture. Comparisons were made by taking into consideration the standard deviation for each dose response of the mixture.

Example: In the mixture 10% estrone-90% estriol(2) where the total dose was $C = 1.5\ \gamma$ ($C_1 = 0.15\ \gamma$ estrone + $C_2 = 1.35\ \gamma$ estriol) the actual observed response was 48.6 $\pm$ 5.6 mg. The calculated response was as follows: $\frac{1}{R}$ for estrone $= y_1 = .0176 \left(\frac{1}{C_1}\right) + .0104 = .0176 \times 6.667 + .0104 = 7.8$. $\frac{1}{R}$ for estriol $= y_2 = .0118 \left(\frac{1}{C_2}\right) + .0514 = .0118 \times .7407 + .0514 = 16.6$. 7.8 + 16.6 + 23 (control) = 47.4 mg calculated additive uterine weight. The value for this point from the smoothing of the experimental data with the Langmuir type of plot is 50.5 mg and the experimental standard deviation is $\pm$ 5.6 mg indicating additivity in all respects. Above the region of additivity, the calculated additive responses are significantly higher than the observed responses, indicating a damping of the response of estrone by the highly hydrophilic estriol. The affinity constants for the mixtures rise with increasing estriol content and the

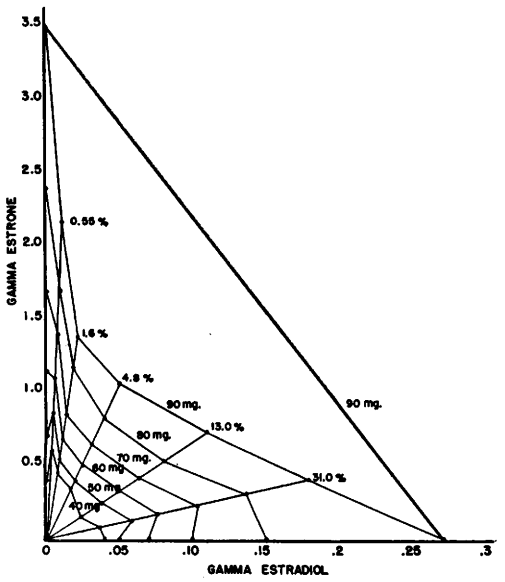


FIG. 3. Dose response curves for mixtures of estrone and estradiol plotted as an isobologram (Loewe(9)) showing synergism.

intercept value approached or was even less than either of the pure steroids (Table I). Agreement of a/b (affinity constant) calculated from equation III with those from the smooth (Langmuir) experimental data is only fair (columns 4 and 5, Table I). This may indicate that there is more than one active surface.

Estradiol-Estriol. In the combination of these 2 estrogens, additivity is found through the dose range 1.525 γ for mixtures 98.4% + 1.6% whereas in the mixture containing 94.2% + 4.8% additivity extends to the dose range of 3.675 γ. However, it falls again at 1.4376 γ dose in the mixture 86.96% + 13.04%. It is noted that a suggestion of synergism is seen in the response to dose in the 95.2% + 4.8% mixture. Good agreement of calculated and observed affinity constants (a/b) is observed in 2 out of the 3 mixtures. The mixture which is synergistic gives a sharply different affinity constant.

Estrone-Estradiol. This particular group of mixtures does not lend itself to the concept of surface adsorption. Inspection of Table I shows immediately the negative intercept. Additive responses were calculated, however, using the equation for the pure compounds and compared with the individual experimental points and their standard deviations. The results are as follows:

99.45% + 0.55% additivity over the entire dose range within limits of experimental error.

98.36% + 1.64% additivity to dose of 6.86 γ

95.24% + 4.76% additivity to dose of 3.675 γ

86.96% + 13.04% additivity to dose of 1.4376 γ

68.97% + 31.03% additivity to dose of 0.7250 γ

These mixtures do seem, however, to show evidence of synergism. We have, therefore, used a method of plotting as suggested by Loewe(9) known as an isobologram‡ (Fig. 3). The isobologram was constructed from data read off smoothed curves which were drawn empirically since the Langmuir type of equation did not fit this set of data. Note in Fig. 3 that straight lines drawn between points of equal response for the pure estrogens are found *above* the majority of the experimental points.

Androgens. The calculated affinity coefficients (a/b) for mixtures of androsterone and dehydroepiandrosterone agree fairly well with the experimental values. The slopes (b) and intercepts (a) hover much closer to pure androsterone than dehydroepiandrosterone (cf Table I). It is to be stressed that the hormonal mixtures of androgens were applied directly to the target organ, the chick comb. Hence, the complications inherent in remote injection, by which the estrogens were administered, are mainly avoided. It is true that diffusion into the circulation from

‡ Isobolograms illustrate changes which properties of mixtures undergo, if quantity and relative proportions of the mixture components are continuously changed. The rays show points of the same relative proportions, the isoboles are the connecting lines of those mix points which show a definite biological response (in this case) to the same degree. Synergism is indicated when a line drawn diagonally between 2 points of the coordinate axes (pure estrogens) giving the same response, has all points of the isobole under this line.

TABLE II. Predicted Uterine Response.

Ratio, estrone/estriol	.5 γ estrone γ estriol	.5 γ estrone Uterine wt (mg)	1.5 γ estrone γ estriol	1.5 γ estrone Uterine wt (mg)
100: 0	0	44.9	0	68.2
90:10	.05	54.3	.17	82.8
75:25	.16	65.6	.50	89.5
50:50	.50	61.1	1.50	82.1
25:75	1.50	60.5	4.50	70.1
10:90	4.50	55.6	13.50	57.4

the chick comb undoubtedly occurred and also back diffusion of metabolic products (from liver, etc.) but the effect of these would be expected to be minor in comparison with the direct impact of the hormone.

Discussion. The equation presented in the previous section now provides a "tool" which can be used in various ways to examine trends in data obtained from the actions of mixed estrogens and/or androgens. These equations provide a continuity not generally provided by experimental data and therefore it becomes possible to calculate responses with a fixed amount of one component and variable amounts of another by using the various ratios determined experimentally.

To illustrate this, we have taken our data for estrone and estriol and thus can predict what may occur if one constituent is kept constant and the other varied (Table II). When these data are plotted the pattern is very similar in the low dose range to the recent work of Huggins and Jensen(8) with estrone and 16 epi-estriol as illustrated in their Fig. 4.

These calculations based on surface concepts naturally breed speculations as to such effects in other systems. Dorfman *et al.*(10) have recently noted that when the 17α hydrogen is replaced by an alkyl group (17α methyl) in 2αmethyl-17-β-hydroxy-androstane 3-one, the anti-estrogenic effect is increased 25 times. We postulated that this may be due to the increased screening action thus afforded against estrogen attachment at an active surface.

Summary. 1. An equation, Response $= \frac{R_x C}{K_s + C}$, based on the concept of surface adsorption as set forth in the Langmuir adsorption isotherm has been derived as an aid in understanding biological response to steroids. 2. The equations provide a tool by which theoretical predictions can be correlated with experimental data. 3. The Langmuir concept is given added support since the equation is valid whether the steroid is applied topically as in the androgens or parenterally as in the estrogens.

1. Grauer, R. C., Starkey, W. F., Saier, E. L., *Endocrinology,* 1948, v42, 141.

2. Grauer, R. C., Saier, E. L., Strickler, H. S., Cutuly, E., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 308.

3. Langmuir, I., Phys. Rev., 1913, v2, 331 and 1915, v6, 79.

4. Hitchcock, D. L., *J. Am. Chem. Soc.,* 1926, v48, 2870.

5. Lineweaver, H., Burk, D., *ibid.,* 1934, v56, 658.

6. Levvy, G. A., *Science,* 1954, v119, 337.

7. Stetten, DeWitt, Jr., *ibid.,* 1956, v124, 365.

8. Huggins, C., Jensen, E. V., *J. Exp. Med.,* 1955, v102, 335.

9. Loewe, W. S., *Klin. Wochenschrift,* 1927, v6, 1078.

10. Dorfman, R. I., Kincl, F. A., Ringold, H. J., *Endocrinology,* 1961, v68, 17.

Received March 20, 1961. P.S.E.B.M., 1961, v107.