Comparison of Methods for Quantitating Melanophore Responses.* (23017)

S. DEUTSCH, E. T. ANGELAKOS, AND E. R. LOEW
Department of Physiology, Boston University School of Medicine, Boston, Mass.

Development of methods for quantitation of melanophore responses followed a proposal by Sulman(1) that the corticotropin molecule has inherent melanophore stimulating activity. The relationship between the melanophore stimulating (MS) and adrenal ascorbic acid depleting activities of corticotropin is still controversial. Recent evidence of a similarity in sequence of amino acids in both intermedin and corticotropin may possibly explain the MS activity of corticotropin preparations(2,3). The report of Lerner and coworkers(4) indicating an ability of intermedin preparations to stimulate melanin pigmentation in man has also increased the need for methods of assaying intermedin activity in physiological media. Recent reports(5,6) indicate that there is an increased melanophore stimulating activity of the blood in certain clinical conditions such as Addison's disease, Cushing's syndrome, rheumatoid arthritis, postoperative stress, pregnancy, and others. Because of the variability of melanophore responses(7), it is felt that only objective quantitative methods which yield data that can be treated statistically are acceptable for purposes of bioassay. Qualitative methods utilizing arbitrary scales having no mathematical basis, thus precluding statistical analysis, have been criticized as methods of bioassay(8). Methods for quantitating melanophore responses may be divided into 2 broad groups: those utilizing intact animals, and those which make use of isolated strips of adult frog or tadpole skin. Photoelectric measurement of the changes in light reflected from the surface of intact frogs or transmitted through isolated skin has been employed in quantitating the responses of frog melanophores. Schizume and coworkers(9) have measured the light reflected from isolated strips of frog skin in quantitating melanophore responses.

* Supported by grant from Parke, Davis and Co., Detroit, Mich.

The following study was carried out in order to compare an *in vivo* method previously reported(7) with an *in vitro* method first reported by Wright(10) and developed by Frieden and coworkers(11). Because the methods of preparation of pituitary extracts differ widely in various laboratories it was felt that only a comparison of the two methods using the same hormone preparation would be a true test of the precision, sensitivity, and reliability of the two methods.

Materials and methods. A detailed description of the *in vivo* method has been presented(7). For the *in vitro* method, frogs (*Rana pipiens*) were pithed, and the skin from both legs placed in Ringer's solution pH 7.13 at 23°C. Strips of skin were mounted between 2 pieces of plastic having holes 18 mm in diameter, through which light transmission could be measured. It was found that the Ringer's solution had to be changed twice at 20 minute intervals following removal of the skins from the animals. If this was not done the pigment within the melanophores dispersed after a short period of concentration. A transmission of 25 on the densitometer was chosen as the minimum transmission that would be allowed as a starting point. The melanophores were considered maximally contracted if, after preliminary washings, 2 transmission measurements taken 15 minutes apart did not differ by more than 2 units. Following the control measurements, a range of concentrations of Armour ACTH lot no. L60311 was added, and transmission measurements were taken 10, 20, and 40 minutes later. The skins were then washed twice at 15 minute intervals with Ringer's solution, and immersed in a "standard" concentration of ACTH. Transmission measurements were made 40 minutes after exposure to the "standard" solution. Groups of frog skins were immersed in a range of concentrations of 0.001 μg/ml to 0.05 μg/ml in a volume of 30 ml. The work was carried out in 2 series.

TABLE I. Effect of Corticotrophin (ACTH) on Light Transmission in Isolated Frog Skin.

ACTH (μg/ml)	Light transmission readings: Initial	40 min.	"Standard" .025 μg/ml	Δ_1/Δ_2*
		Series 1		
.001	† 32.3	28.1	24.4	.53
	‡ 4.6	5.3	3.9	.23
.005	32.4	29.3	24.2	.34
	6.9	5.6	5.6	.52
.025	34.4	30.8	28.5	.59
	7.7	5.3	6.1	.30
.125	36.5	22.2	25.0	1.71
	8.4	10.0	7.1	1.01
Control	32.7	31.3		
	8.9	5.8		
		Series 2		
			.250 μg/ml	
.125	40.5	27.1	24.3	.81
	4.1	8.7	9.2	.16
.250	38.6	21.0	18.1	.73
	2.4	4.9	7.0	.34
.500	40.3	21.0	20.3	.97
	3.3	8.9	10.2	.17
Controls	43.0	41.0		
	7.8	7.4		

* Δ_1 = Starting transmission, 40 min. response to dose. Δ_2 = Starting transmission, 40 min. response to "standard" dose.
† Mean. ‡ Stand. dev.

One series of skins was subjected to concentrations of 0.001, 0.005, 0.025, and 0.125 μg/ml. A concentration of 0.025 μg/ml was used as the "standard" for this series. The second series of skins was treated with doses of 0.125, 0.250, and 0.50 μg/ml. The "standard" concentration for this series was 0.250 μg/ml. Groups of skins were placed in 30 ml of Ringer's solution and served as controls.

Results. Table I contains a summary of the data obtained. Mean starting values, mean transmission 40 minutes after exposure of the first concentration of ACTH, and the mean transmission 40 minutes after the second "standard" concentration of ACTH are presented. The difference in the light transmission before and after exposure to the first concentration (Δ_1) and between the starting transmission and the response to the second concentration (Δ_2), can be expressed as a ratio, (Δ_1/Δ_2), following the method of Frieden *et al.*(11). Table I shows the mean Δ_1/Δ_2 ratios. The results with Δ_1/Δ_2 ratios when the first concentration is the same as the "standard" concentration do not agree with those of Frieden *et al.*(11), who reported a mean ratio 0.95 ± 0.04 under such conditions. Generally it was found that using the same concentration, the response to the second exposure was higher than that obtained on first exposure even after repeated washings between exposures. In the first series the second response to 0.025 μg/ml was 1.7 times greater than the first response. Similarly a ratio of 1.3 between second and first exposures was obtained in the second series with 0.25 μg/ml. Since the variability was increased by expressing the response as the ratio, it was decided to use the 40 minute transmission measurements as the responses.

A log dose-response line was drawn between the concentrations of 0.025 to 0.250 μg/ml. Application of Bartlett's test indicated a homogeneity of the variances of these groups. It was concluded from this that the variance was independent of the response. Analysis of the variance was performed. Equation of the line was found to be $Y_x = 13.0\text{-}11.1 \log X$. The precision index lambda was calculated to be 0.60.

Discussion. A statistical comparison of the *in vitro* and *in vivo* methods is presented in Table II. With respect to the sensitivities of the two methods, the one using intact animals is twice as sensitive as that using isolated frog skin. When one considers the total amount of hormone needed for the *in vitro* method (30 ml) the *in vivo* method is 60 times more sensitive. The former method is less specific (12) and more susceptible to changes in pH, temperature, and ionic concentration(13). Similar considerations regarding sensitivity

TABLE II. Statistical Comparison of *In Vivo* and *In Vitro* Methods of Quantitating Melanophore Responses.

	In vivo	*In vitro*
Sensitivity (μg)	.064	.125
Range of response (μg)	.064–6.4	.125–.250
Mean coef. of variation	10.5	35.7
Regression line		
Slope	4.0	11.1
Variance of error	2.82	44.6
Lambda	.39	.60

and specificity would apply to the *in vitro* method reported by Schizume and associates (9).

The dose range for submaximal responses was 100 fold in the method using intact frogs. With the isolated preparations the range was only 2 fold. A long range for submaximal responses is considered desirable for a bioassay since it permits comparison between standard and unknown even though their potencies may vary considerably.

As a measure of the variability of the response to a given dose, the mean coefficient of variation was 3.4 times greater in the method using isolated frog skin.

With respect to the regression line, the slope obtained with the *in vitro* method was greater than that with the *in vivo* method. However the variance of error of the latter method was considerably less than that of the former. Thus, lambda, the precision index, was greater for the *in vitro* method, and approximately 2.4 times as many determinations would have to be carried out using this method in order to obtain an error of 10% or less.

Conclusions. It is concluded that the method employing intact frogs is superior to that in which strips of frog skin are used for assay of melanophore stimulating hormone.

1. Sulman, F. G., *Lancet,* 1952, v2, 247.
2. Harris, J. I., and Roos, P., *Nature,* 1956, v178, 90.
3. Geschwind, I. I., Li, C. H., and Banafi, L., *J. Am. Chem. Soc.,* 1956, v78, 4494.
4. Schizume, K., and Lerner, A. B., *J. Clin. Endocrinol.,* 1954, v14, 1491.
5. Lerner, A. B., Schizume, K., and Bunding, I., *J. Clin. Endocrinol. and Metab.,* 1954, v14, 1463.
6. Sulman, F. G., *ibid.,* 1956, v16, 755.
7. Deutsch, S., Angelakos, E. T., and Loew, E. R., *Endocrinology,* 1956, v58, 33.
8. Emmens, C. W., Principles of Biological Assay, Chapman and Hall Ltd., 1948.
9. Schizume, K., Lerner, A. B., and Fitzpatrick, T. B., *Endocrinology,* 1954, v54, 553.
10. Wright, P. A., *J. Cell Comp. Physiol.,* 1948, v31, 111.
11. Frieden, E. H., Fishbein, J. W., and Hisaw, F. L., *Arch. Biochem.,* 1948, v17, 183.
12. Landgrebe, F. W., Ketterer, B., and Warina, H., in "The Hormones", G. Pincus and Thiaman. Ed. Academic Press, N. Y., 1955, v3, Chap. IX.
13. Sulman, F. G., *Acta Endocrinol.,* 1952, v11, 1.

Received January 22, 1957. P.S.E.B.M., 1957, v94.

Biliary Excretion of Bile Acids and Cholesterol in Bile Fistula Rats. Bile Acids and Steroids.*† (23018)

STEN ERIKSSON (Introduced by O. Wintersteiner)
Department of Physiological Chemistry, University of Lund, Sweden.

It has been demonstrated that bile acids are the main metabolic end products of cholesterol in the rat (Byers and Biggs(1), Bergström(2), Chaikoff and Dauben(3)). Rat bile contains taurocholic and taurochenodesoxycholic acid (Bergström and Sjövall(4)). Several authors have studied excretion of bile acids and cholesterol in the bile duct cannulated rat. Friedman, Byers and Michaelis (5) found that excretion of cholic acid and cholesterol in bile gradually decreased during the first 2 days following cannulation. Thompson and Vars(6a,b) however, studying the influence of altered thyroid activity upon excretion of cholic acid found that the biliary level of this substance rose following cannulation and reached a fairly constant plateau on third or fourth postoperative day. Recently Wysock, Portman and Mann(7) reported studies on nutritional effects upon biliary excretion of cholic acid and dihydroxycholanic acids. Their results, however, were based entirely upon determinations of

* Preliminary report was read at meeting of the Swedish Biochem. Soc., Uppsala, Jan. 27, 1956(10).

† This work has been supported by grant from Medical Faculty, University of Lund.