A Rapid, Reproducible and Sensitive Levator Ani Test for Anabolic Activity.* (26742)

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It was recently demonstrated that an anabolic steroid could, within 39 hours after injection, induce a 4-fold increase in uptake of a labelled non-metabolizable amino acid by the levator ani muscle of the rat(1). The possibility of using this phenomenon as a rapid indicator of the anabolic potential of various steroids was therefore explored. The effect of 8 different steroids on uptake of the amino acid, alpha amino-isobutyric acid-1-C^{14} (AIB), was determined. The data indicate the new procedure to be more rapid than the standard levator ani test; it is also more sensitive and highly reproducible. Several other features of interest were noted, including the diminished excretion of AIB. It is suggested that excretion of AIB might also serve as an indicator of anabolic activity.

Method. Male rats of the Holtzman strain weighing approximately 150 g were kept in the laboratory on a diet of Rockland pellets until their weight was 200 ± 10 g. In each series of experiments the rats were divided into groups of 6 approximately equal in total weight. One group was given diluent only and served as the control, the second group was given testosterone propionate, 50 mg/kg, and served as the standard, and the other experimental group or groups were given the test steroids in the same dosage. Immediately after castration under ether anaesthesia the steroids or diluent were given intramuscularly. The animals were then subdivided into groups of 3 and placed in metabolic cages for collection of the pooled urine. Food was withheld but water was available. Thirty hours after castration each animal was given subcutaneously 1.0 ml of the AIB solution containing 550,000 ± 5,000 counts (specific activity 0.1 mC/mM). Nine hours later the animals were exsanguinated under ether anesthesia by direct cardiac puncture. The levator ani were excised, weighed, and individually homogenized with 3.0 ml of acetic acid (pH 3.5). The other organs and tissues were also excised, weighed and homogenized, in pools of 3 corresponding to the metabolism cage pools, with a proportional volume of acetic acid. After centrifugation the supernatants were plated in 0.5 ml aliquots and counted to a total of 1000 counts 3 times in a thin window gas flow counter with sample changer and printing timer.† One hundred ml of hot water was used to wash down the metabolism cages, added to the corresponding pooled urine, made up to a total volume of 200 ml and a 0.5 ml aliquot counted. Total nitrogen in the urine was determined by the microKjeldahl method. The individual blood serums were precipitated with tungstic acid and a 0.5 ml aliquot of the filtrate counted.‡

Results. The results of the 4 series of experiments are given in Tables I and II. All of the animals lost 15% in body weight as a result of castration and food deprivation. Nevertheless, all of the levator ani responded to the stimulation of the steroids in the 39 hours of experiment by an increase in weight and by an increase in uptake of AIB (Table I). Control levator ani weights ranged from an average of 63 to 87 mg and experimental ones from 110 to 134 mg. Ratios of experimental to control weights ranged from 1.35 to 2.13 and net increase in weight from 35 to 113% with an average of 50%. The uptake of AIB by control levators was 1,780 to

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† The sample changer and printing timer were purchased with a grant from the Ciba Co. through the courtesy of Dr. C. H. Sullivan.

‡ This filtrate was chosen because it gave a count recovery of 93%; serum plated directly gave a recovery of only 32% and often caused difficulty because of flaking and curling; trichloracetic acid filtrate was deliquescent when plated and recovery with it only 41%.

TABLE I. Levator Data.

Series	Steroid*	No. of rats	Avg body wt, g		Levator ani wt				Levator ani uptake			
			Original	Terminal	Avg mg	S.E., % of mean	Ratio: To control	Ratio: To test. prop.	Avg counts per g	S.E., % of mean	Ratio: To control	Ratio: To test. prop.
I	Methandrostenolone	6	195	168	108	4.7	1.72	.81	4,730	3.6	2.43	.62
	Testosterone propionate	6	196	165	134	2.2	2.13	—	7,590	2.8	3.89	—
	Control	6	200	163	63	12.4	—	.47	1,950	5.1	—	.26
II	Norethandrolone	6	211	178	125	8.9	1.44	1.01	5,750	4.8	3.23	.81
	Norethandrolone propionate	6	203	173	117	6.1	1.35	.95	6,660	4.4	3.74	.93
	Testosterone propionate	6	204	173	124	6.7	1.43	—	7,150	7.7	4.01	—
	Control	6	208	182	87	11.5	—	.70	1,780	3.6	—	.25
III	Nandrolone phenpropionate	6	198	171	115	5.4	1.44	.96	7,100	4.4	3.88	1.01
	Norpropandrolate	6	205	174	113	6.1	1.40	.94	7,110	5.9	3.88	1.01
	Testosterone propionate	6	205	171	120	5.1	1.48	—	7,040	4.7	3.86	—
	Control	6	197	171	81	5.3	—	.68	1,830	5.0	—	.26
IV	Testosterone	6	193	164	110	5.5	1.35	.95	8,270	4.0	4.35	1.08
	Testosterone enanthate	6	193	162	112	5.2	1.38	.97	7,630	3.9	4.02	.98
	Testosterone propionate	6	197	167	116	5.5	1.43	—	7,780	4.8	4.09	—
	Control	6	192	164	81	4.2	—	.70	1,900	5.0	—	.24

* Methandrostenolone: 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one;
Kindly supplied by Dr. C. H. Sullivan, Ciba Pharmaceutical Products, Inc., Summit, N. J.

Norethandrolone: 17α-ethyl-17β-hydroxy-19-norandrost-4-en-3-one;
Norethandrolone propionate: 17α-ethyl-4-estrene-3β,17-β-diol-3-propionate;
Norpropandrolate: 4-estrene-3β,17β-diol-3,17-dipropionate;
Kindly supplied by Drs. I. C. Winter, R. L. Craig, and T. H. Hayes, G. D. Searle and Co., Chicago, Ill.

Nandrolone phenpropionate: 19-nor-Δ^4-androstene-17β-ol-3-one-β-phenyl propionate;
Kindly supplied by Dr. H. A. Strade, Organon, Inc., Orange, N. J.

Testosterone enanthate: Testosterone-17-β-n-enanthate;
Kindly supplied by Mr. E. Grossman, E. R. Squibb and Sons, New York, N. Y.

Testosterone propionate and testosterone: Stock material from the hospital pharmacy; the latter compound was suspended in water, all other compounds were dissolved in oil.

TABLE II. Urine Data.

Series	Steroid	No. of rats	Urine nitrogen Avg mg/rat	Urine nitrogen Ratio To control	Urine nitrogen Ratio To test. prop.	Urine AIB Avg counts/rat	Urine AIB Ratio To control	Urine AIB Ratio To test. prop.
I	Methandrostenolone	6	227	1.23	1.08	3,500	.83	1.40
	Testosterone propionate	6	211	1.14	—	2,500	.60	—
	Control	6	185	—	.88	4,200	—	1.68
II	Norethandrolone	6	202	.78	1.01	3,700	.48	1.39
	Norethandrolone propionate	6	198	.76	.99	3,200	.42	1.20
	Testosterone propionate	6	200	.77	—	2,670	.35	—
	Control	6	259	—	1.30	7,670	—	2.87
III	Nandrolone phenpropionate	6	202	1.09	1.01	2,070	.36	.78
	Norpropandrolate	6	220	1.19	1.10	2,800	.49	1.05
	Testosterone propionate	6	200	1.08	—	2,670	.47	—
	Control	6	185	—	.93	5,700	—	2.13
IV	Testosterone	6	200	1.01	1.09	6,700	.47	1.33
	Testosterone enanthate	6	214	1.08	1.16	7,920	.56	1.58
	Testosterone propionate	6	184	.93	—	5,030	.36	—
	Control	6	198	—	1.08	14,140	—	2.81

1,950 counts per gram and 4,730 to 8,270 for experimental groups. Ratios of experimental to corresponding control uptakes were 2.43 to 4.35. Net increases in uptake varied from 143 to 335% with a mean of 276%. Thus, average percent increase in uptake was 5½ times as great as average percent increase in weight.

Urinary excretion data are given in Table II. In the 39 hours of experiment nitrogen retention was manifest only in series II. The other series showed no change or an actual increased loss over controls. The average for the ratios of experimental to control nitrogen excretion for the 4 series was 1.01. Urinary excretion of the labelled compound was, however, decreased in all of the experiments in the 9 hours following its administration. Ratios of experimental to control excretions ranged from 0.83 to 0.35 or a decrease of 17 to 65% with an average of 51%. Thus, although there was no consistent decrease in nitrogen excretion as a result of administration of the steroids, there was a significant reduction in excretion of the AIB.

The changes in uptake of the other tissues analyzed for AIB were small in comparison to the levators. Serum, diaphragm, kidney and heart showed variable small changes, the experimental to control ratios being 1.03, 0.98, 0.95, and 1.06 respectively. Skeletal muscle (tongue was used) showed a consistent increase and liver and thymus a consistent decrease. The respective experimental to control ratios were 1.10, 0.88 and 0.86. These represent a 10 to 14% change which is only one-twentieth of that of the levator muscle.

The reproducibility of the method within the given groups is indicated by the standard errors in Table I. These are given in percent of the corresponding means to allow comparison between the two factors, weight gain and AIB uptake. The mean of the standard errors for the 15 groups is 6.3 (S.E. ± 0.73) for the levator weights and 4.6 (S.E. ± 0.29) for the uptakes. The latter is thus somewhat more accurate than the former. The reproducibility of the uptakes in the replicated groups (control and testosterone propionate) is indicated by the narrow range of the actual count values and the ratio values. Average counts in the control levator groups for the 4 series were 1,950, 1,780, 1,830 and 1,900 and for the 4 testosterone propionate groups, 7,590, 7,150, 7,040, and 7,780. The testosterone propionate to control ratios were 3.89, 4.01, 3.86 and 4.09 and the reciprocal ratios were 0.26, 0.25, 0.26 and 0.24 respectively.

Discussion. On the basis of the above results the levator ani-AIB test would appear to be as effective as the standard levator ani weight tests for determining the anabolic po-

tential of steroids. It has, in addition, a number of advantages over these tests, the Eisenberg-Gordan test and the modified test of Hershberger(2,3). The former requires 28 days for its performance and the latter 7 days, whereas the AIB test requires only 39 hours. The 30-hour interval from steroid injection to AIB administration was adopted to be sure of maximum effectiveness of the steroid, and the 9-hour interval from AIB administration to sacrifice to ensure an adequate circulating level of this compound; its previously determined half time in the circulation was 9 hours. Since anabolic steroids probably become effective soon after injection it may be possible to shorten the AIB test time. Studies are now being carried out to determine the shortest time after steroid administration in which statistically valid results are obtainable with the test. Admittedly, the test requires a little more working time (to process the levators for counting) than the simpler weighing test but it obviates the need for housing and feeding groups of rats for long periods of time and the need for daily injections of the test steroids. Another advantage of the AIB test, perhaps minor, is that precise excision of the levator ani is not critical to the test—the effect being measured not in total counts but in counts per gram of the muscle.

In this series of experiments the AIB test is somewhat more accurate than the weight test. The difference noted above between the means of the standard errors of the counts and those of the weights in the 15 experiments is statistically significant ($p < 0.05$). The reproducibility of the counts (per gram) in the 4 replicated control and testosterone propionate experiments is quite good. The coefficient of variation between the 4 values in the former was 4.7%, in the latter 2.7%, and between the respective ratios, 3.8%. The sensitivity of the AIB test is 5½ times that of the weight test on the basis of the ratio between percent increase in uptake to percent increase in weight. This sensitivity may actually be somewhat higher. If the first 2 weight ratios and the first uptake ratio are excluded from their respective columns (this is valid on statistical grounds) average net uptake increase would be 290% and average net weight increase would be 41% with a ratio of seven. Thus, AIB uptake is 5 to 7 times as sensitive as weight increase as an indicator of anabolic potential in the 39 hours of these experiments.

There was an appreciable reduction in urinary excretion of the AIB in the 9 hours after its administration but no consistent change in nitrogen excretion in the 39 hours after steroid administration. The explanation for this reduction in urinary AIB is not clear from these experiments. Although the relative kidney counts were 5 times as high as the average for the other soft tissues analyzed (20,000 *vs* 4,000 counts/g) the experimental animal kidney counts were the same as the controls. Also, the 10% increase in uptake by the skeletal muscle was not reflected by any change in the blood level which could have affected the excretion. This considerable reduction in AIB excretion, however, suggests the possibility of using this change as an added or alternate indicator of the activity of anabolic steroids. Prolonging the period of the test until nitrogen retention becomes established would allow quantitative correlation between AIB excretion and nitrogen retention to be determined and AIB itself could then serve as a monitor for nitrogen sparing.

At the high steroid dose level used there was less than a 2-fold spread, 2.43 to 4.35, in uptake ratios. Taking the mean, 3.96, of the 4 testosterone propionate ratios as only one value the mean of the ratios for the 8 compounds tested was 3.76 with an S.D. of $\pm$ 0.56 (15%). The 2 non-esterified compounds, methandrostenolone and norethandrolone, with known low androgenic potency had ratios below this mean (2.43, 3.23) and the parent compound, testosterone, with known high androgenic and anabolic activity had a ratio above this mean (4.35). The ratios of the 5 esterified compounds, on the other hand, were grouped very closely (3.96, 3.74, 3.88, 3.88, 4.02) with a mean of 3.90 and an S.D. of $\pm$ 0.13 (3.4%). It is interesting that the 2 nortestosterone esters, the phenpropionate and the dipropionate had identical values (3.88, 3.88), that the 17-

ethyl nortestosterone ester had a value very close to these (3.74), and that the 2 testosterone esters had practically identical values to each other (3.96, 4.02) and to the 3 nortestosterone values. Thus, deletion of the methyl group, presence of the 17-ethyl group, esterification at the 17 position, esterification at both the 3 and 17 positions and the nature of the esterifying groups did not seem to affect the common action of these compounds which is being expressed as an increase in uptake of the labelled amino acid. What proportion of uptake of the AIB is attributable to the anabolic effect and what proportion to the androgenic effect of these steroids cannot be deduced from these data. Nevertheless, the degree of agreement among the 5 esterified compounds suggests that the various compounds have the same effect on the uptake or concentrating mechanism or that their metabolic breakdown results in a common compound causing the effect noted.

Summary. 1. A test for determination of the anabolic potential of steroids using increase in uptake of a C^{14} labelled non-metabolizable amino acid by the levator ani muscle of the rat is described. 2. This test is considerably more rapid than the 2 standard levator ani weight increment tests. Reproducibility of the method within a given test is quite good as is reproducibility in serial tests with a given steroid. The sensitivity of this test is 5- to 7-fold that of the weight increment test. 3. The reduction in urinary AIB excretion observed even before any nitrogen retention becomes established suggests the possibility of using this reduction as an aditional or alternate indicator of the anabolic activity of steroids. 4. The close agreement in uptake ratios with 5 different steroid esters suggests that the metabolic product ultimately effective in causing the increase in uptake of the labelled compound is the same or that the various steroids have the same effect on the uptake mechanism.

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Effect of Size and Concentration of Latex Particles on Respiration of Human Blood Leucocytes.* (26743)

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Recent workers on physical and biochemical aspects of phagocytosis have used suspensions of polystyrene latex particles as the test substance(1,2). Using guinea pig exudate leucocytes and polystyrene latex particles 1.171 μ in diameter, Sbarra and Karnovsky concluded that phagocytosis requires energy, and that the oxygen consumed by the test cells is directly proportional to the number of particles ingested by them(2). Strauss and Stetson have noted the effect of 0.802 μ polystyrene latex particles and solutions of endotoxins of Gram negative bacteria on the respiratory activity of leucocytes of human peripheral blood(1).

The present paper describes effect of size and concentration of polystyrene latex particles upon oxygen consumption of leucocytes of heparinized human peripheral blood.

Materials and methods. Blood. Normal human peripheral blood was drawn and heparinized as described elsewhere(1). Blood

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