directions, it is not surprising that phloridzin not only blocks the tubular reabsorption of D-glucose, but also the secretion of L-glucose.

3MG, being an analogue of D-glucose, is considered in many respects to behave similarly to D-glucose. It is transported by the intestinal mucose actively against a concentration gradient(3), and it augments the sodium influx and short circuit current across the intestinal mucosa(12,13). However, as shown in the structural model constructed in Fig. 4, 3MG possesses a configuration similar to that of L-glucose. It is therefore reasonable to predict that 3MG be secreted by the renal tubules in a manner similar to L-glucose and that this secretion also be abolished by the infusion of phloridzin.

*Summary.* Studies with close arterial injection technique have demonstrated that the renal excretion of 3MG, L-glucose, L-galactose, L-fucose and L-mannose was greater than that of the simultaneously injected inulin, suggesting a renal tubular secretion of these compounds. Steady-state experiments also showed that a positive T value was obtained with L-glucose and 3MG. Phloridzin abolished the tubular secretion of these compounds. It is therefore postulated that a carrier system for sugar transport is located in the brush border of renal tubules. This carrier has a complementary structure resembling that of hexoses. Its luminal site reacts complementarily with D-hexoses and the cytoplasmic facing site of the carrier reacts with the 3MG and L-hexose.

1. Pitts, R. F., Physiology of the kidney and body fluids, Year Book Med. Publisher, Chicago, 1963, p71.
2. Wilson, T. H., Landau, B. R., Am. J. Physiol., 1960, v198, 99.
3. Csaky, T. Z., Fernald, G. W., ibid., 1960, v198, 445.
4. LeFevre, P. G., Marshall, J. K., ibid., 1958, v194, 333.
5. Chinard, F. P., ibid., 1955, v180, 617.
6. Huang, K. C., JPET, 1961, v134, 257.
7. Nelson, N. J., Biol. Chem., 1944, v153, 375.
8. Roe, J. H., Epstein, J. H., Goldstein, N. P., ibid., 1949, v178, 839.
9. Bray, G. A., Anal. Biochem., 1960, v1, 279.
10. Stahl, E., Thin-Layer Chromatography, Acad. Press Inc., New York, 1965, p461.
11. LeFevre, P. G., Marshall, J. K., J. Biol. Chem., 1959, v234, 3022.
12. Current, R. F., J. Gen. Physiol., 1959, v43, 1137.
13. Schultz, S. G., Zalusk, R., ibid., 1964, v47, 1043.

Received June 30, 1966. P.S.E.B.M., 1967, v124.

## Further Studies on Effects of Anabolic Steroids on the Course of Murine Muscular Dystrophy.* (31658)

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The availability of a mutant strain of mice afflicted with an hereditary disease bearing some resemblance to human muscular dystrophy has made possible studies of drug effects. The administration of the anabolic steroid, 1-methyl-$\Delta^1$-androsten-17$\beta$-ol-3-one and some other androgenic-anabolic steroids, but not testosterone propionate, has been reported to result in increased longevity accompanied by a slowing in the rate of loss of muscle strength(1-3).

Residual muscle from dystrophic mice, like muscle from patients with the Duchenne form of muscular dystrophy, contains less potassium, more sodium and more chloride than muscle from normal mice(3,4). Dystrophic muscle appears to be "leaky" to potassium and to soluble cytoplasmic enzymes such as

* This research was supported by grants AM-08096 and AM-09715 from Nat. Inst. Health and by a grant from National Foundation for Neuromuscular Diseases.

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aldolase and creatine kinase as evidenced by abnormally high efflux rates(5,6). Probably as a result of the increased rate of efflux from dystrophic skeletal muscle, the intracellular levels of aldolase and other soluble enzymes are decreased while the serum levels of these enzymes are increased(7,8). The administration of a longevity increasing anabolic steroid to dystrophic mice resulted in a significant, but not dramatic, lowering of the muscle sodium and chloride toward normal(3). This steroid also produced a relatively greater lowering of the abnormally high $K^{42}$ efflux rate characteristic of dystrophic muscle(3).

In this communication, results are reported of further studies undertaken to establish structure-activity relationships and to define better the effects of steroid treatment on the electrolyte abnormality of dystrophic muscle.

*Materials and methods.* Mean attained ages were determined using dystrophic mutants of strain 129/Re mice obtained as weanlings from the Jackson Memorial Laboratory, Bar Harbor, Maine. The details of handling and feeding have been described(3). The compounds tested and dosage used are listed in Table I. Drug administration was begun when the animals were 5 weeks of age. For some drugs, parallel but separate groups were exercised and muscle strength was assessed by measuring the length of time animals could hang on a vertical screen.

Groups of 5-week-old dystrophic mice were given 0.25 $\mu$g/g body weight of 7$\alpha$-methyl-19-nortestosterone, 0.02 $\mu$g/g body weight of digitoxin, or a combination of these drugs in 60% propylene glycol by subcutaneous injection. Control dystrophic animals and control parent strain (normal) animals received solvent alone. After 2 weeks of treatment, the animals were sacrificed, exsanguinated and a dry, fat-free muscle powder (DFFM) prepared(3) from the rump muscles. Sodium and potassium were determined in a 0.6 N perchloric acid extract and DFFM using a Baird-Atomic KY-2 flame photometer. Chloride was determined in a 0.75 N nitric acid extract of DFFM by the method of Cotlove *et al*(9). Non-collagenous nitrogen was determined in DFFM by the method of Lilienthal *et al*(10).

Potassium efflux from excised *peroneus longus* muscle was measured using $K^{42}$ by a modification(3) of Zierler's procedure(6). Serum creatine kinase was determined by the procedure of Hughes(11) and the distribution of serum lactate dehydrogenase isozymes was determined by acrylamide gel electrophoresis substantially as described by Dewey and Conklin(12). Samples of muscle were fixed in neutral formalin and stained with hematoxylin-eosin for microscopic examination.‡

*Results and discussion.* The effect of treatment with anabolic steroids on the mean attained age ($ST_{50}$) of dystrophic mice is summarized in Table I. Control female dystrophic mice had a slightly greater $ST_{50}$ than males and exercised dystrophic animals lived somewhat longer than animals not actively exercised. Treatment with all steroids resulted in longer $ST_{50}$'s; the greatest lengthening of life span occurred with 7$\alpha$-methyl-19-nortestosterone and 2$\alpha$-methyl-5$\alpha$-androstan-17$\beta$-ol-3-one. The increase in $ST_{50}$ with drug administration was slightly less in male than in female dystrophic mice. The augmentation of life span compared to control dystrophic mice sometimes decreased as larger doses of drug were administered. The prolongation of survival of dystrophic mice by treatment with anabolic steroids was accompanied by an increase in the length of time mice could hang on a vertical screen (Fig. 1). In groups treated with a supra-optimal dose of steroid, the hanging time first increased rapidly, reaching a peak value after a few weeks, and then fell rather rapidly. These data indicate a complex action of these steroids, the salutary effect being offset by a toxic effect at higher doses. A similar diphasic action of anabolic steroids on heart actomyosin content has been observed(13). Administration of bovine growth hormone did not prolong survival.

Microscopic examination of sections of muscle from dystrophic controls showed changes which have been described previously(14), hyaline degeneration with loss of cross striations, some variation in fiber size

‡ The authors are indebted to Dr. Charlotte Maddock for preparing and reviewing the microscopic sections.

TABLE I. Effect of Steroids on the Mean Attained Age of Dystrophic Mice.

| Drug | Daily dose ($\mu$g/g) | Mean attained age (days) ± S.E.M. Female | Male |
|---|---|---|---|
| Control (solvent only) | — | 196 ± 11 (12)* | 174 ± 17 (17)* |
| " , exercised | — | 216 ± 11 (6) | 199 ± 20 (6) |
| 1-methyl-$\Delta^1$-androstenolone acetate | .5 | 294 ± 14 (8)† | |
| Digitoxin | .04 | 264 ± 23 (6)† | |
| 7$\alpha$-methyl-19-nortestosterone | .25 | 385 ± 22 (5)† | |
| *Idem* | 1.0 | 326 ± 26 (6)† | 271 ± 29 (6)† |
| " | 2.5 | 245 ± 17 (6) | |
| " , exercised | .5 | 317 ± 19 (6)† | |
| 7$\alpha$-methyl-19-nortestosterone + Digitoxin | .5 + .4 | 297 ± 12 (6)† | |
| Same as above, exercised | | 342 ± 14 (6)† | |
| 2$\alpha$, 7$\alpha$-dimethyltestosterone | 2.5 | 279 ± 27 (6)† | |
| 7$\alpha$-methyl-17$\beta$-hydroxy-1,4-androstandiene-3-one acetate | 2.5 | 325 ± 13 (6)† | 248 ± 18 (6)† |
| 7$\alpha$-methyltestosterone | 2.5 | 221 ± 18 (6) | |
| 1$\alpha$,17$\alpha$-dimethylandrostan-17$\beta$-ol-3-one | 2.5 | | 198 ± 25 (6) |
| 17$\alpha$-ethyl-19-nortestosterone | 5.0 | | 234 ± 17 (6) |
| 2$\alpha$-methyl-5$\alpha$-androstan-17$\beta$-ol-3-one | .5 | 351 ± 26 (5)† | |
| 2$\alpha$,3$\alpha$-episulfido-17$\beta$-(3-cyclopentanyl)propionoxy-5$\alpha$-androstane | .5 | 371 ± 20 (5)† | |
| *Idem* | 1.0 | 290 ± 24 (5)† | |
| Testosterone trimethylsilyl ether | .5 | 339 ± 41 (5)† | |
| Bovine growth hormone | 25 | | 178 ± 17 (6) |

* Numbers in parentheses are No. of animals in group.
† Significant difference ($p < .05$) compared to controls.

with proliferation, particularly of small, intensely hyperchromatic nuclei, a few being centrally located. Also present were areas of necrosis, usually limited to a segment of a single muscle fiber and often accompanied by active phagocytosis. No differences were observed in them icroscopic picture of muscle from treated dystrophic mice.

The serum creatine kinase levels were markedly elevated in the dystrophic mice compared to normals; pretreatment with 7$\alpha$-methyl-19 nortestosterone resulted in a slight lowering of this level. Patients with muscular dystrophy show a marked decrease in the $LDH_5$ lactate dehydrogenase isoenzyme. Normal mice have a low $LDH_5$ level in serum, and while dystrophic mice have an elevation in the absolute level of serum lactate dehydrogenase, the isoenzyme distribution in dystrophic mouse serum resembles that of normal mice(15). Administration of anabolic steroids produced no significant change in lactate dehydrogenase isoenzyme distribution.

The electrolyte data from control and treated dystrophic mouse muscle are summarized in Table II. DFFM from dystrophic animals showed a lower potassium and higher sodium and chloride content than muscle from

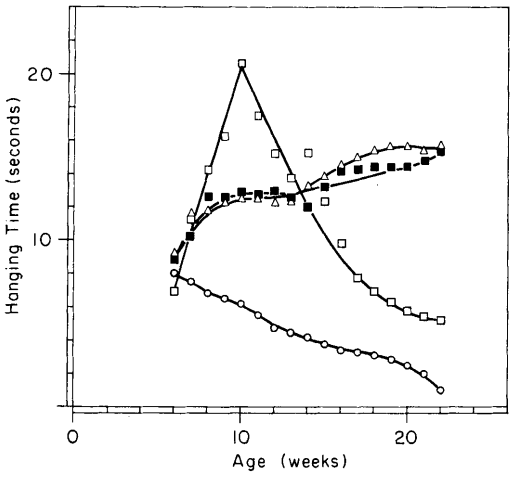


FIG. 1. Hanging time of dystrophic mice on a vertical screen: ○—○ control dystrophic, □—□ 7$\alpha$-methyl-19-nortestosterone 0.5 $\mu$g/g/day, △—△ 2$\alpha$,3$\alpha$-episulfido-17$\beta$-(3-cyclopentanyl)propionoxy-5$\alpha$-androstane 0.5 $\mu$g/g/day, and ■—■ 2$\alpha$-methyl-5$\alpha$-androstan-17$\beta$-ol-3-one 0.5 $\mu$g/g/day. Each point is the mean of 6 animals.

TABLE II. Skeletal Muscle Electrolytes, $K^{42}$ Efflux Rates and Serum Creatine Kinase in Dystrophic Mice.

| Group | | I | II | III | IV |
|---|---|---|---|---|---|
| No. animals | | 11 | 17 | 6 | 6 |
| Drug dose | | Normal control | Dystrophic control | U-11828, 0.25 μg/g | U-11828, 0.25 μg/g + Dig, 0.02 μg/g |
| Skeletal muscle DFFM: (mean ± S.E.M.) | Na (meq/kg) | 85 ± 4 | 188 ± 12 | 171 ± 22 | 167 ± 20 |
| | K " | 435 ± 7 | 381 ± 18 | 391 ± 19 | 386 ± 18 |
| | Cl " | 78 ± 2 | 130 ± 12 | 114 ± 8 | 113 ± 17 |
| | NCN (g/kg) | 126 ± 8 | 126 ± 11 | 121 ± 16 | 123 ± 15 |
| | Wet wt/DFFM | 4.90 ± .08 | 6.04 ± .55 | 5.38 ± .46 | 5.02 ± 1.05 |
| | K/Na | 5.12 | 2.03 | 2.28 | 2.32 |
| $K^{42}$ efflux from excised peroneus longus muscle ($hr^{-1}$ ± S.E.M.) | | .372 ± .014 | .680 ± .027 | .624 ± .022 | .621 ± .028 |
| Serum creatine kinase (μmoles /ml/hr) | | 2.47 ± .46 | 11.74 ± .68 | 9.84 ± .55 | |

U-11828 is 7α-methyl-19-nortestosterone.
Dig=digitoxin.

normal (parent strain) animals, which was not altered significantly by treatment. The non-collagenous nitrogen (NCN) content of DFFM muscle was approximately the same for normals, dystrophic controls, or treated dystrophic animals, indicating that either NCN or DFFM are equally good reference bases for composition data. Measurement of potassium efflux from excised *peroneus longus* muscle from parent strain and dystrophic mice showed a highly significant difference. Efflux rates from muscle of dystrophic rates treated with 7α-methyl-19-nortestosterone or a combination of this drug with digitoxin showed differences which gave $0.1 > p > 0.05$ when compared with efflux rates of dystrophic controls. Administration of anabolic steroids resulted in a return of the high wet wt/DFFM ratio of dystrophic muscle toward normal.

Although the return toward normal of the abnormal potassium efflux, electrolyte content and wet wt/DFFM ratio of dystrophic muscle were not dramatic, they may be sufficient to explain the prolonged survival following administration of anabolic steroids. It is also possible, however, that the increased longevity may result through another mechanism. Protein turnover in dystrophic muscle is much greater than in normal muscle(16); the anabolic steroids may act to facilitate protein synthesis at the high rates which are required or to diminish the abnormally high rates of turnover. Experiments are in progress to assess these possibilities.

*Summary.* Administration of 7α-methyl-19-nortestosterone, 2α-methyl-5α-androstan-17β-ol-3-one, 2α,3α-episulfido-17β-(3-cyclopentanyd)propionoxy-5α-androstane and related anabolic steroids to dystrophic mice resulted in prolonged survival and increased ability to hang on a vertical screen. A lowering of the abnormally high potassium efflux and wet wt/dry fat-free muscle ratio characteristic of dystrophic muscle was found after treatment with these steroids. Slight but not statistically significant changes in the steady state levels of muscle potassium, sodium and chloride toward normal were observed. Treatment with these steroids did not alter the microscopic changes of dystrophic muscle.

1. Dowben, R. M., Nature, 1959, v184, 1966.
2. Borgman, R. F., ibid., 1963, v197, 1304.
3. Dowben, R. M., Zuckerman, L., Gordon, P., Sniderman, S. P., Am. J. Physiol., 1964, v206, 1049.
4. Young, H. L. Young, W., Edelman, I. S., ibid., 1959, v197, 487.
5. Zierler, K. L., Bull. Johns Hopkins Hosp., 1958, v102, 17.
6. ———, ibid., 1961, v108, 208.
7. Schapira, F., Schapira, G., Dreyfus, J. C., Compt. rend., 1957, v245, 753.
8. Nichol, C., McGeer, P. L., Miller, J. R., Canad. J. Biochem. Physiol., 1962, v40, 443.
9. Cotlove, E., Trantham, H. V., Bowman, R. L., J. Lab. Clin. Med., 1958, v51, 461.
10. Lilienthal, J. L., Zierler, K. M., Folk, B. P., Buka, R., Riley, M. J., J. Biol. Chem., 1950, v182, 501.

11. Hughes, B. P., Clin. Chim. Acta, 1962, v7, 597.
12. Dewey, M. M., Conklin, J. L., Proc. Soc. Exp. Biol. and Med., 1960, v105, 492.
13. Nowy, H., Frings, H. D., Hohenbleicher, R. E., Arzn. Forsch., 1965, v15, 1253.
14. West, W. T., Murphy, E. D., Anat. Rec., 1960, v137, 279.
15. Lauryssens, M. G., Lauryssens, M. J., Zondag, H. A., Clin. Chim. Acta, 1964, v9, 276.
16. Kruh, J., Dreyfus, J. C., Schapira, G., Gey, G. O., J. Clin. Invest., 1960, v39, 1180.

Received July 5, 1966. P.S.E.B.M., 1967, v124.

## Comparative Immunology: Hemolytic Complement in Elasmobranchs.* (31659)

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Considerable interest has been displayed recently in the phylogeny of the immune response, particularly with regard to antibody synthesis in lower vertebrates(1-6). The present report deals with the related area of serum complement as studied in 3 species of elasmobranchs.

It was previously observed that amphibians possess measurable hemolytic complement (C′) levels comparable to those of mammalian sera(7). Titers were much lower than those found in guinea pig sera, however. Sera from each of 3 species of *Amphibia* were capable of reaction in a rabbit antibodysheep erythrocyte hemolytic system; although certain mammalian complements lack this ability(8). Whole complement has also been demonstrated in snakes and certain fish(9-11).

Many species of fish have been shown to elicit a specific immune response(2-6). By using such parameters as response to antigenic stimulation, homograft rejection, and the presence of a thymus and organized lymphopoietic tissues, a progressively decreasing capacity for immune response was suggested as the phylogenetic scale was descended. Minimal responses were noted in certain elasmobranchs and the lamprey, with immunologic activity reaching a virtual null state in the hagfish(12,13). The purpose of the present investigation was to determine whether complement levels decreased in similar fashion in the lower vertebrates.

*Materials and methods.* Specimens of lemon shark, *Negaparion brevirostris*, and nurse shark, *Ginglymostoma cirratum*, weighing 1000-3000 g, were captured in the waters surrounding Bimini, Bahamas and maintained in outdoor seawater pens in natural environmental conditions at the Lerner Marine Laboratory. The animals were bled from the hemal arch, anterior to the caudal fin, under tricaine methane sulfonate (MS-222) anesthesia. Blood was allowed to clot for one hour at room temperature followed by 2 hours at 10°C. The sera were centrifuged at 3 hours post-bleeding to minimize loss of activity and maintained in an ice bath. Quantitative complement titrations were initiated within 4 hours after bleeding, and all other procedures were begun within 24 hours. Blood samples from the sting ray, *Dasyatis americana*, were obtained by cardiac puncture from mature specimens captured at Bimini. Certain of the sting ray sera, otherwise treated as described above, were maintained in an ice bath for periods up to 24 hours before use.

The natural hemolytic activity of pooled sera from each species of elasmobranch was evaluated by combining serum dilutions with constant amounts of erythrocyte suspensions from several foreign species. A standard pH 7.4 barbital buffer, containing optimal calcium and magnesium ions, was used for all dilutions(14). Each tube contained a final volume of 0.4 ml which included 0.1 ml

* Supported by USPHS Grant AI-02693.
† Conducted during tenure of a traineeship on Grant DE-7 from Nat. Inst. of Dental Research.