Effects of Certain Compounds on Experimental Muscular Dystrophy

By NATHAN WATZMAN†, WILLIAM J. KINNARD, MARIO D. ACETO, and JOSEPH P. BUCKLEY

Several compounds which represent classifications of drugs known to have effects on muscular efficiency were administered to dystrophic animals in which atrophy was achieved utilizing a vitamin E-deficient diet. A myopathic mutation of the house mouse (dystrophia muscularis) was also utilized in certain portions of the study. Motor coordination, weight, muscle strength, and urinary creatine and creatinine were measured. The effects of some of the compounds on the isolated diaphragm muscle of normal and dystrophic mice were also studied. Norethandrolone, methimazole, and digoxin increased the ability of vitamin E-deficient rats to perform forced motor activity. The same compounds increased the offspring survival rate of vitamin E-deficient female rats. Potassium chloride (10 mg./Kg./day) temporarily increased the mean rotarod performance time and arrested loss of weight of dystrophic mice. Although there was no statistical difference between the life span of the control and drug-treated groups of strain-129 mice, the group receiving digoxin, 0.05 mg./Kg. subcutaneously, twice a week survived the longest.

MUSCULAR DYSTROPHY is a chronic disease manifested by a gradual weakening of the voluntary muscles. As the condition progresses, there occurs loss of function, mild contractions, and finally contractures in which the muscle is entirely inert and no longer capable of response to stimuli.

The etiology of muscular dystrophy has eluded investigators for many years, but it appears to be due to some defect in metabolism. The following metabolic alterations and abnormalities invariably appear in dystrophic muscle tissue: decreased concentration of creatine and increased concentration of creatinine in striated muscle (1); creatinuria (2); decreased concentration of extracellular potassium and magnesium (3); diminished glycogenolysis (4); increased collagen nitrogen (5); and decreased concentrations of the enzymes aldolase and phosphorylase (5).

In the light of these consistent findings, it seemed plausible to administer certain therapeutic agents which might metabolically or pharmacologically reverse the specific patterns elicited by the dystrophic conditions.

Methimazole (1-methyl-2-mercaptoimidazole),[1] one of the most active of the antithyroid agents (6), was selected as one of the test compounds. Increasing evidence exists that muscle weakness, tremors, and reduced muscle efficiency occur in hyperthyroidism. Du Toit (7) reported that the oxidative efficiency of phosphorylation is reduced in the presence of thyroxin, suggesting that muscle efficiency might be reduced in hyperthyroidism. Thomson and his associates (8) reported that hyperthyroidism was accompanied by tremors, atrophy, myasthenia, and muscle lesions. In their studies, denervation atrophy was accelerated by thyroxin and retarded by thiouracil, an antithyroid agent. In addition, Boldrini (9) using liver slices of hyperthyroid rats, showed that the creatine content of liver and muscle of hyperthyroid animals was less than that of normal animals. Creatine synthesis was also depressed and this was reversed by methylthiouracil, an antithyroid agent.

The wasting of muscle in muscular dystrophy suggested the use of norethandrolone (17 alpha-ethyl - 17 - hydroxy - 19 - nor - 4 - androsten - 3-one),[2] an androgenic hormone, which enhances protein anabolism by reversing negative nitrogen balance. Saunders and Drill (10), using the levator ani muscle of the rat reported that norethandrolone had marked myotrophic activity. The most familiar example of the anabolic effects of the androgens is the rapid growth and muscular development of the adolescent male. Methandrostenolone,[3] another androgenic steroid with anabolic properties, was also selected as a test compound.

Digoxin[4] is widely used in the treatment of congestive heart failure. It acts directly on the myocardium to increase muscle tone and contractile force, thereby increasing the mechanical efficiency of the failing heart (11). This compound is deposited in skeletal muscle as well as the myocardium and might possibly produce an effect in skeletal muscle similar to that produced in the failing heart.

The finding of increased collagen nitrogen (12)

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[1] Marketed as Tapazole by Eli Lilly & Co.
[2] Marketed as Nilevar by G. D. Searle & Co.
[3] Marketed as Dianabol by Ciba Pharmaceutical Co.
[4] Marketed as Lanoxin by Burroughs Wellcome & Co.

in the dystrophic muscle suggests an increase in fibrous tissue resulting from an inflammatory condition. This fact, coupled with evidence of diminished glycogenolysis (4) led to the consideration of an anti-inflammatory corticosteroid as one of the experimental compounds. Methylprednisolone acetate,[5] the 6-methyl derivative of prednisolone (delta-1-hydrocortisone), was selected because it has been reported to be more potent than prednisolone and less prone to produce early side effects (13).

Evidence of electrolytic disturbances in atrophic muscle such as decreased concentrations of extracellular potassium and magnesium ions (14) and increased sodium and calcium ions (14) led to the selection of the electrolytic compound, potassium chloride, as another test substance. Dystrophic muscles have been reported to have a slightly lower potassium content than normal muscle (15). Overholt and his associates (16) have reported the prevention of periodic paralysis associated with hyperthyroidism by the use of a high potassium diet.

Finally, three other pharmacologically active agents were utilized on modified *in vitro* diaphragm preparations. Ephedrine was employed because of evidence that this substance reversed the effects of fatigue, a condition not unlike that of muscular dystrophy. Burns (17) demonstrated in dogs that ephedrine causes a marked and prolonged rise in tension of skeletal muscle fatigued by repeated stimulation of the sciatic nerve. The second compound selected for *in vitro* study was ouabain (g-strophanthin), a cardiac glycoside having a very quick onset of action. Cattell (18) has shown that this compound produces an increase in force of striated muscle contraction. Caffeine is known to increase the amplitude of muscle contractions (19). Contractions of isolated nerve-muscle preparations are enhanced and muscle made less susceptible to fatigue if low concentrations of xanthines are present in the bath solution (20); therefore, theophylline (21) was selected as the third compound.

Several experimental methods for the development of dystrophic symptoms in laboratory animals are available to investigators in this field. Muscular weakness and wasting similar to human muscular dystrophy have been accomplished by vitamin E-deficient (22) or choline-deficient (23) diets in many species of animals. Also, through the efforts of the staff at Jackson Memorial Laboratories, Bar Harbor, Maine, a myopathic mutation appeared among a colony of inbred strain-129 mice (dystrophia muscularis) in 1951. The clinical manifestations of the disease in this species are first recognizable at $3^1/_2$ weeks when ataxia and paresis of the hind limbs are observed.

The objectives of this study were: (*a*) to determine if the test compounds delay or arrest, to any degree, the onset of experimental muscular dystrophy and (*b*) to determine if the test compounds affect the contraction of normal and fatigued muscle.

EXPERIMENTAL

Development of Experimental Muscular Dystrophy.—Muscular weakness and ataxia were observed in albino Wistar rats after 5 months feeding on the vitamin E-deficient diet utilized successfully by Olcott (22).

Dose Regimen.—Thirty albino Wistar rats, weighing approximately 100 Gm., were divided into five groups of six animals each (two males and four females). Group 1 received a normal rat diet (Purina Rat Chow); group 2 received a vitamin E-deficient diet; group 3 received the vitamin E-deficient diet and norethandrolone, 2 mg./Kg. orally; group 4 received the vitamin E-deficient diet and digoxin, 0.2 mg./Kg. initially and then 0.1 mg./Kg. daily by oral route; group 5 received the vitamin E-deficient diet and methimazole, 4 mg./Kg. orally. Drugs were administered daily, 6 days a week.

One hundred and sixty-four pairs of dystrophic mice (dystrophia muscularis, Jackson Memorial Laboratories, Bar Harbor, Maine) and their littermate controls were divided into 14 groups of not less than nine pairs each. Twenty pairs comprised the control group. Group 1 (controls) received no drugs; group 2 received norethandrolone, 2 mg./Kg. orally; group 3 received norethandrolone, 10 mg./Kg. orally; group 4 received methimazole, 4 mg./Kg. orally; group 5 received methimazole, 20 mg./Kg. orally; group 6 received digoxin, 0.2 mg./Kg. for 1 week and then 0.05 mg./Kg. daily by the subcutaneous route; group 7 received digoxin, 0.2 mg./Kg. initially for one dose and then 0.1 mg./Kg. daily by the subcutaneous route; group 8 received digoxin, 0.2 mg./Kg. daily by the subcutaneous route; group 9 received potassium chloride, 10 mg./Kg. orally; group 10 received potassium chloride, 50 mg./Kg. orally; group 11 received potassium chloride, 100 mg./Kg. orally; group 12 received methylprednisolone acetate, 10 mg./Kg. intramuscularly; group 13 received methylprednisolone acetate, 100 mg./Kg. intramuscularly; and group 14 received methandostrenolone, 10 mg./Kg. orally. Drugs were administered daily, 7 days per week except methylprednisolone acetate which was given twice weekly. The animals were weighed every fourth day and careful records maintained on their life spans.

Measurement of Motor Coordination.—Rats were placed on an electrically driven rotarod (24), $1^1/_4$ inches in diameter, which was set to turn at 18 r.p.m. The animals were forced to walk the turning rod without side excursions until they fell off. The rats were trained for a period of 1 week and then the relative forced motor activity and muscle coordination were determined by the length of time

[5] Marketed as Depo-Medrol by The Upjohn Co.

the animals could maintain their position on the rod, allowing a maximum of 2 minutes per animal. Coordination in strain-129 mice (dystrophia muscularis) was measured in the same manner as the rats, using a rod of smaller diameter (1 inch), set to rotate 11 r.p.m.

Measurement of Muscle Strength.—This factor was measured in rat offspring and dystrophic mice by placing them on a vertical screen (90 degrees) and recording the length of time they could remain on it. The maximum experimental time was established at 60 seconds and the average of each group recorded in seconds.

Measurement of Urinary Creatine-Creatinine Ratio.—Twenty-four hour urine samples were obtained from rats placed in individual metabolism cages every 2 weeks. A urine analysis of creatine and creatinine was determined according to a modification of Folin's method (25).

Survival of Offspring of Pretreated Vitamin E-Deficient Female Rats.—Female rats of all groups were mated with normal males 90 days following the initiation of the vitamin E-deficient diet. On the first day of mating, a single oral dose of 0.5 Gm. of wheat germ oil (not less than 2 i.u. vitamin E per Gm.) was administered to each female to assure proper reproductive ability. Size of litters and survival rates as well as motor coordination and muscle force of the offspring were recorded.

In Vitro Studies.—The effect of the test compounds on the amplitude of contractions of the diaphragm muscle of normal and dystrophic mice (dystrophia muscularis) was studied utilizing a modified method of Bülbring (26). The test compounds were added to the muscle bath to produce the following concentrations: potassium chloride (1:1500); ephedrine sulfate (1:1000); ouabain (1:35,000,000); theophylline ethylenediamine (1:50,000). A similar study was done, utilizing the fatigued diaphragm of normal and dystrophic mice. A submaximal threshold was established for each preparation and then the muscle was fatigued by stimulating every 10 seconds until a 50% decrease in amplitude of contraction was achieved. The test compounds were then administered directly into the bath and effects on the amplitude of contractions and recovery recorded.

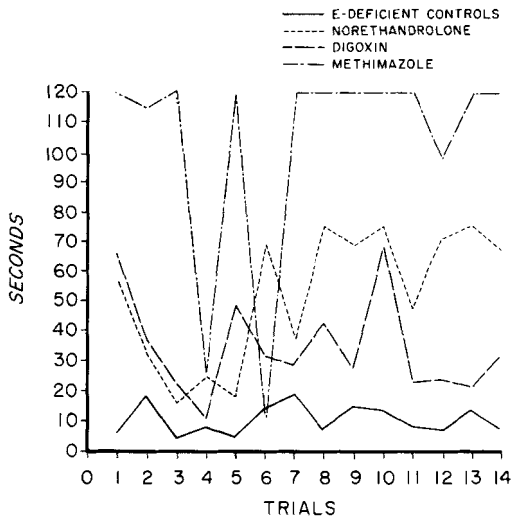

Fig. 1.—Effects of norethandrolone, digoxin, and methimazole on he rotarod performance times of vitamin E-deficient male rats. Doses: norethandrolone, 2 mg./Kg., daily; digoxin, 0.1 mg./Kg., daily; and methimazole, 4 mg./Kg., daily.

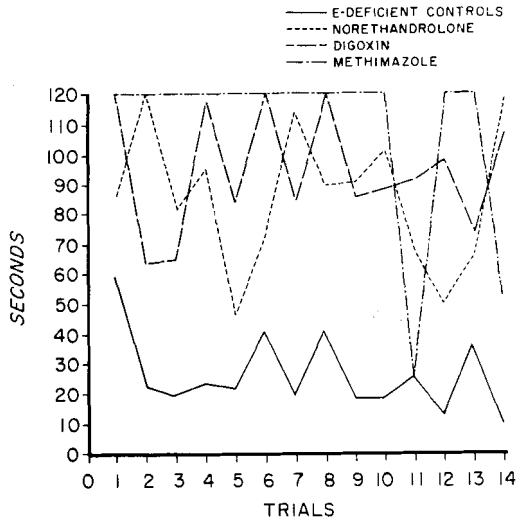

Fig. 2.—Effects of norethandrolone, digoxin, and methimazole on the rotarod performance times of vitamin E-deficient female rats. Doses: norethandrolone, 2 mg./Kg., daily; digoxin, 0.1 mg./Kg., daily; and methimazole, 4 mg./Kg., daily.

RESULTS AND DISCUSSION

The initial pilot study of this investigation in experimental muscular dystrophy utilized albino rats of both sexes. After 5 months on a vitamin E-deficient diet it was quite apparent that there was a significant difference in the abilities of the treated groups to perform on the rotarod apparatus (Figs. 1 and 2). Methimazole-treated rats demonstrated the greatest ability to perform forced motor activity. The data further indicated that the methimazole, digoxin, and norethandrolone-treated rats demonstrated greater ability to perform forced motor activity than the untreated vitamin E-deficient rats. This was true of both sexes (Figs. 1 and 2), and it appears that the compounds in some way alleviated the paralysis-producing phenomenon of a vitamin E-deficient diet.

The consistently lower weights of the methimazole-treated animals from the fifth week on (Fig. 3) are unexplainable in view of the fact that since methimazole is an antithyroid compound, it would be expected that animals treated with this compound might be heavier than the nontreated rats.

The data on the urinary creatine-creatinine ratios were inconclusive. There appeared to be no correlation between loss of motor ability and creatine-creatinine values. The vitamin E-deficient control ratios were moderately higher than the other groups but were generally within the normal 0.1–1.0 range.

The moderate success of the experimental compounds in the pilot study on vitamin E-deficient rats gave impetus to the investigation of the offspring of vitamin E-deficient females. As observed by Olcott (12), offspring of vitamin E-

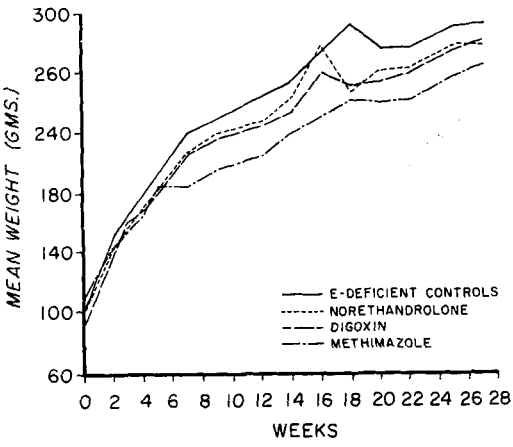

Fig. 3.—Effects of norethandrolone, digoxin, and methimazole on the weights of vitamin E-deficient female rats. Doses: norethandrolone, 2 mg./Kg., daily; digoxin, 0.1 mg./Kg., daily; and methimazole, 4 mg./Kg., daily.

deficient female rats developed a temporary muscular weakness on about the twentieth day of life. The phenomenon of muscular involvement in vitamin E-deficient animals is not understood. It would appear that the characteristic symptoms are genetically transferred to offspring. However, the temporary nature of these abnormalities at a specific age is not consistent with the fixed, permanent nature of genetics. This investigation corroborated Olcott's results and, in addition, elaborated two significant findings. A total of 74 offspring were produced by all groups (Table I). The normal control group

TABLE I.—EFFECTS OF NORETHANDROLONE, METHIMAZOLE, AND DIGOXIN ON OFFSPRING SURVIVAL OF VITAMIN E-DEFICIENT ALBINO RATS

	Group[a]				
	1	2	3	4	5
Offspring, No.	27.0	14.0	15.0	12.0	6.0
Offspring litter	9.0	5.0	5.0	4.0	3.0
Survival, %	70.3	0.0	26.6	25.0	50.0

[a] Group 1, normal control; group 2, E-deficient control; group 3, norethandrolone; group 4, digoxin; and group 5, methimazole.

had a mean of nine offspring per litter whereas the other groups ranged between three and five animals per litter. Nineteen offspring from normal control females survived, three from methimazole-treated females, four from norethandrolone-treated females, and three from digoxin-treated females. Per cent survival was fairly good for all groups with the exception of the vitamin E-deficient control group of which all offspring died within 18 days. The normal control group had a significantly higher per cent survival, 70.3%. The evidence thus indicated that all of the test compounds administered to the females increased the ability of the offspring of vitamin E-deficient rats to survive. However, in the light of the role of vitamin E as an antioxidant, the mechanisms by which the test compounds were able to increase offspring survival rate could not be resolved.

The second notable finding was that the offspring of norethandrolone-treated females were able to perform forced motor activity consistently better than the offspring of normal females (Fig. 4) and they experienced only slight difficulty in remaining on the vertical screen. In general, there appeared to be no correlation between motor coordination (as measured on the rotarod) and muscle strength (as measured by the vertical screen). This was evidenced by the poor performance of the digoxin offspring on the rotarod (Fig. 4), and their excellent agility on the vertical screen. Although norethandrolone produced highly beneficial effects in the vitamin E-deficient dystrophic rats, the effects of this compound in human muscular dystrophy has been discouraging. Dowben and Perlstein (27) have reported that the administration of 0.5 mg./Kg. of norethandrolone daily failed to produce an improvement in muscle function and strength.

The effects of the experimental compounds on the life span, motor coordination, and muscle strength of the strain-129 dystrophic mouse were observed. Rotarod performance graphs, life-span charts, cage score and weight graphs were carefully scrutinized to determine if any of the test compounds were successful in simultaneously (*a*) prolonging the life-span, (*b*) increasing motor performance times, (*c*) increasing the vertical cage scores, (*d*) increasing weight or at least arresting loss of weight. The data indicate that potassium chloride in the dose 10 mg./Kg. (group 9) was able to produce these responses temporarily (Figs. 5 and 6). From the 16th–64th day of therapy, mean rotarod performance times consistently ranged from 10.0–39.6 seconds. Thereafter the scores steadily decreased to zero. In comparison, the control dystrophics (group 1) never achieved a mean score higher than 5.4 seconds. Secondly, the mean weights of the potassium chloride-treated (10 mg./Kg.) animals ranged consistently between 13–17 Gm. with no apparent pattern of steady weight loss through the 120th day of drug administration. The mean cage

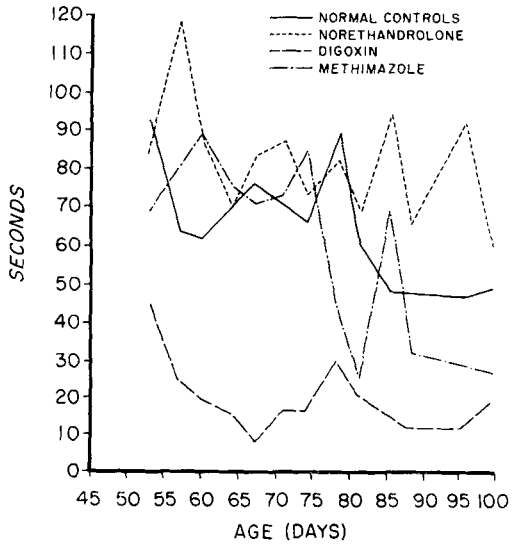

Fig. 4.—Effects of norethandrolone, digoxin, and methimazole on the rotarod performance times of the offspring of vitamin E-deficient female rats.

scores ranged steadily from 25.0–53.0 seconds through the 104th day of therapy whereas the control scores ranged from 7.0–28.0 seconds. Furthermore, the mean life-span of the potassium chloride-treated group was prolonged 16 days over that of the controls (Table II). However, statistical analysis showed this to be an insignificant response. The temporary beneficial results of potassium chloride in this study were understandable in the light of potassium evoked muscular improvements noted in the literature and previously discussed in the statement of the problem. Although norethandrolone has been reported to increase the survival time of strain-129 dystrophic mice (28), daily administration of this compound failed to increase the life-span of this strain of mice in this present study.

Although there was no statistical difference between the life spans of the untreated and drug-treated groups of strain-129 dystrophic mice, the group receiving digoxin 0.05 mg./Kg. subcutaneously twice a week (group 6) survived the longest. There appeared to be no correlation between mean rotarod performance times, mean cage scores, and life spans.

The results of the *in vitro* studies indicated that ouabain (1:35,000,000) and potassium chloride (1:1500) increased the amplitude of the contractions of the diaphragm muscle obtained from normal mice, 20.4 and 11.5%, respectively. None of the test compounds was able to evoke a change in the amplitude of diaphragm contractions of the dystrophic mouse. However, when the diaphragm muscle was partially fatigued, potassium chloride (1:1500) and ephedrine sulfate (1:1000) produced significant mean per cent recoveries, 33.5 and 20.3, respectively. Potassium chloride also produced a mean per cent recovery of 20.0 in the fatigued diaphragm of the normal mouse. The

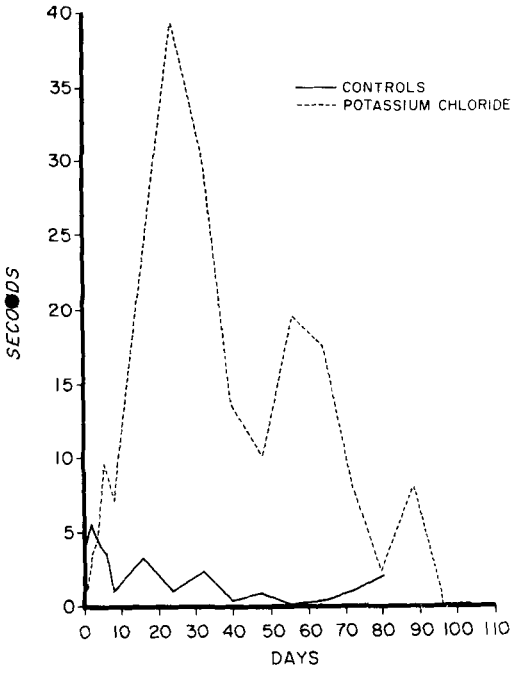

Fig. 5.—Effects of 10 mg./Kg. daily of potassium chloride on the rotarod performance times of dystrophic mice (dystrophia muscularis).

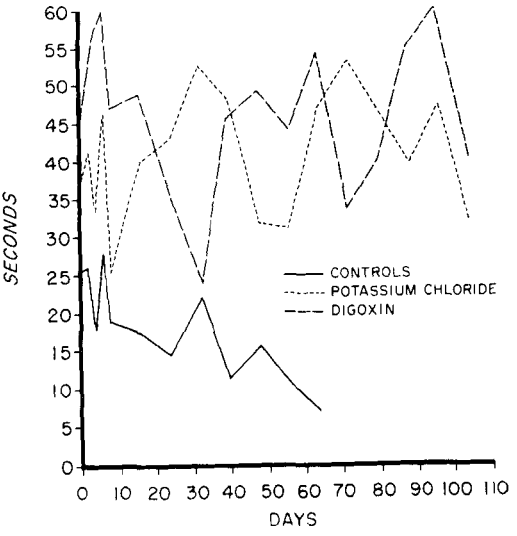

Fig. 6.—The effects of 10 mg./Kg. daily of potassium chloride and 0.05 mg./Kg. daily of digoxin on the vertical cage scores of dystrophic mice (dystrophia muscularis).

TABLE II.—EFFECTS OF EXPERIMENTAL COMPOUNDS ON LIFE SPAN OF STRAIN-129 DYSTROPHIC MOUSE (DYSTROPHIA MUSCULARIS)

Drug	Dose, mg./Kg.	Route of Administration	No. Animals	Mean Life Span ± S.D., Days
Controls	...	...	20	123 ± 20
Digoxin[a]	0.05	subcu.	9	151 ± 49
Digoxin[a]	0.10	subcu.	10	132 ± 50
Digoxin	0.20	subcu.	13	122 ± 31
Methimazole	4.00	oral	9	109 ± 34
Methimazole	20.00	oral	11	95 ± 17
Potassium chloride	10.00	oral	10	139 ± 48
Potassium chloride	50.00	oral	9	127 ± 41
Potassium chloride	100.00	oral	10	104 ± 26
Norethandrolone	2.00	oral	9	99 ± 23
Norethandrolone	10.00	oral	9	100 ± 14
Methandrostenolone	10.00	oral	16	100 ± 15
Methylprednisolone[b]	10.00	i.m.	10	130 ± 45
Methylprednisolone[b]	100.00	i.m.	18	92 ± 6

[a] 0.2 mg./Kg. initially (subcutaneously). [b] Administered twice weekly.

moderate success of potassium chloride in both *in vitro* and *in vivo* experiments on this special strain of mouse indicates that further investigation into the role of potassium as well as other ions should be carried out.

CONCLUSIONS

1. Methimazole-, digoxin-, and norethandrolone-treated rats of both sexes fed on a vitamin E-deficient diet demonstrated greater ability to perform forced motor activity than the untreated control groups.

2. Norethandrolone, digoxin, and methimazole increased the offspring survival rate of vitamin E-deficient female rats.

3. There appeared to be no correlation between motor coordination and muscle strength in the offspring of vitamin E-deficient female rats.

4. Oral dose of potassium chloride, 10 mg./Kg., appears to temporarily improve motor coordination in the strain-129 dystrophic mouse (dystrophia muscularis).

5. There appears to be no correlation between weights, motor coordination, and muscle strength in the strain-129 dystrophic mouse (dystrophia muscularis).

6. There was no statistical difference between the life spans of the control group and the drug-treated groups of strain-129 dystrophic mice (dystrophia muscularis).

7. Potassium chloride (1:1500) and ephedrine sulfate (1:1000) increased the amplitude of contractions in the partially fatigued diaphragm muscle of the strain-129 dystrophic mouse (dystrophia muscularis).

8. None of the test compounds appears to significantly delay or arrest the onset of experimental muscular dystrophy.

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Gas Chromatography of Alkaloids, Alkaloidal Salts, and Derivatives

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A number of alkaloids and alkaloidal derivatives have been gas chromatographed on columns of silicone rubber SE-30. The method has also been applied to alkaloidal extracts of crude drugs. Phenolic alkaloids are readily gas chromatographed as the trimethylsilyl ethers. Alkaloidal salts have been gas chromatographed directly. The salt dissociates in the flash heater and the alkaloid is eluted as the base. Under certain conditions, many alkaloids decompose when subjected to gas chromatography. These decompositions often appear to be catalyzed by the glass wool used on top of the column packing.

In 1958 Quin used gas-liquid chromatography to study the alkaloidal composition of tobacco smoke (1–3). Two years later, Lloyd, *et al.*, demonstrated that a large number of high molecular weight alkaloids could be successfully gas chromatographed (4). This furnished a new and useful tool for research in an area in which pharmaceutical chemists have been interested since the days of Sertürner, more than 150 years ago.

This paper reports the gas chromatography of several alkaloids and alkaloidal derivatives, many of which have not been analyzed previously by this method. The gas chromatographic technique has also been applied to ex-

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