metabolism is interrupted may be able to reduce a dye but unable to grow. Thus, the KB system is different from those described previously.

Summary. A tissue culture bioautograph system has been described which allows identification of cytotoxic activities on paper chromatograms. Use of this procedure as a disc-plate assay was illustrated and its application in the screening and fractionation of beers with carcinolytic activities *in vitro* was discussed.

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Excretion of α-Ketoglutarate, Urea and Amino Acids by Normal and Muscular Dystrophic Mice.*† (25652)

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A preliminary chromatographic survey in this laboratory of urinary constituents of normal and muscular dystrophic strain 129 mice (1)‡ disclosed the presence of a substance which showed a reaction with semicarbazide reagent on chromatograms, and which appeared to be excreted in significantly larger quantities by dystrophic animals than by their littermate controls. The present report concerns identification of the unknown as α-ketoglutarate, further studies of excretion of this keto acid, and a simultaneous chromatographic investigation of amino acid and urea excretion. Amino acid excretion has been found altered or elevated in urines of other animals with various types of muscular dystrophy (2-7).

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‡ Obtained from Jackson Memorial Lab., Bar Harbor, Me.

Methods. Collection and preservation of urine. During urine collection periods, mice were housed in beakers with glass arms fused to the sides at a downward angle to serve as food containers. Access to food (ground Purina Chow) was given by means of holes in the sides of the beakers entering into the side arms. The mice were supported by wide mesh wire screens beneath which were placed at least 3 layers of glass beads pre-coated with thymol by immersion in a thymol-acetone solution followed by draining and drying. This arrangement tended to separate feces and scattered food from urine by allowing the latter to flow to the bottom of the beaker leaving the other materials trapped on top of the beads. Littermate pairs of mice, one dystrophic and one normal, were weighed and placed in the cages in the morning, and after 24 hours feces were removed and urine was collected with several washings. The combined washings were stirred, centrifuged, and supernatants were stored in glass vials at −10°C until analyzed. The urines of 5 pairs of mice were collected over various time intervals and analyzed for α-ketoglutarate, urea and amino

acids as described below. *Identification and analysis of compounds. α-Ketoglutarate.* The chromatographic behavior of the unknown material or its 2,4-dinitrophenylhydrazine (DNPH) derivative (which formed almost immediately at room temperature) was found to be identical with that of synthetic α-ketoglutarate or its DNPH derivative respectively in several different solvent systems(8,9,11). Semicarbazide(9) or DNPH‖ spray reagents were used to detect the free keto acid on chromatograms. Reduction of the DNPH derivative of the unknown by the method of Meister and Abendschein(10) yielded an amino acid, the identity of which was established as glutamic acid by demonstrating that the ninhydrin reacting chromatographic spot given by the unknown amino acid corresponded exactly with a radioautographic spot obtained from glutamic acid-2 C^{14} which had been added to the unknown amino acid as a marker. The amount of radioglutamic acid added was too small to give a detectable ninhydrin spot. Two-dimensional chromatography in 3 pairs of solvent systems and radioautography of chromatograms were carried out as previously described(11). To prepare the DNPH derivative, the reagent was added directly to urine and subsequent extractions of the derivative into ethyl acetate, sodium carbonate and, after acidification of the latter, again into ethyl acetate freed the derivative of contaminating urinary amino acids and excess reagent. Urines were analyzed quantitatively for α-ketoglutarate by a modified method of Bonting and Bonting(12). Reagents were employed at levels 50 times those of the published method(12) and either 0.05 ml or 0.1 ml of collected urine was used. It was shown that certain other unknown materials which appeared on chromatograms after DNPH spray were not visibly extractable from ethyl acetate by sodium carbonate and thus could not have contributed appreciably to the spectrophotometric values obtained for α-ketoglutarate dinitrophenylhydrazone. The absence of detectable levels of α-ketoglutarate in the food and its presence in easily detectable quantities in freshly voided urine were shown chromatographically. *Urea.* This substance was detected on chromatograms run in 80% acetone and sprayed with an acidified alcoholic solution of p-dimethylaminobenzaldehyde (Ehrlich's reagent)(13). If aliquots of the collected urine were diluted 1:10, 5 μl of the resulting solution were usually sufficient to provide a spot of satisfactory intensity for semiquantitative analysis by visual comparison with chromatographed standards. Urea was the only Ehrlich's reacting component detected with this amount of urine. Samples were run in triplicate and standards (1, 2, 3, 5, 7 and 10 μl of 0.01M urea) in duplicate. Each urine spot was evaluated by comparison with each set of standards and 6 resulting figures for each urine sample were averaged. *Amino acids.* These were detected and analyzed by 2 dimensional chromatography and ninhydrin spray as previously described(14). Levels of 100 μl per 8 ml of the collected urine sample were satisfactory for semi-quantitative analysis of the major ninhydrin reacting components. If scattered food was present in a significant amount, corrections were made for the levels of contaminating amino acids contained therein. Although 50-100% errors can sometimes occur for single amino acid measurements obtained by this chromatographic procedure, a large number of samples were run for each mouse, giving a fairly reliable estimate of average excretion over a long period of time. The daily variation in excretion far surpassed the analytical error which was also the case, to a still greater extent, for α-ketoglutarate and urea.

Results. α-Ketoglutarate and urea excretion. Graphs of urinary excretion of these substances per gram body weight together with weights of a representative littermate pair of dystrophic and control mice are shown in Fig. 1. Differences between dystrophics and controls in excretion of α-ketoglutarate and urea became more marked as the disease progressed, and in most cases it was found that even excretion of these compounds *per mouse* was greater by the dystrophic animals during the later stages of the disease.

Data concerning urinary excretion of α-ketoglutarate and urea for all 5 pairs of mice are shown in Table I. Only results obtained between 40 and 105 days were used since, dur-

‖ 0.05% 2,4-dinitrophenylhydrazine in 2 N HCl.

ing this interval, there appeared to be no marked changes in excretion as a function of age for any of the mice. A significantly larger amount of both compounds was excreted by dystrophic animals and the differences were often proportionately more marked for α-ketoglutarate than for urea, for which substance differences tended to remain fairly constant. Since a literature search failed to reveal reports of such high levels of α-ketoglutarate in the urines of any animals, urines of 2 other species and 2 other strains of mice were examined for approximate levels of the keto acid. These included Wistar rats, New Zealand white rabbits, CF_1 white mice, and a cross between C3H and strain A mice. Whereas 1-5 μl of undiluted urine from strain 129 mice usually showed a good semicarbazide or DNPH spot in the α-ketoglutarate position on chromatograms, urines from the other animals showed no such spot when chromatographed at levels exceeding those from the strain 129 mice by 4-10 fold. α-Ketoglutarate at high levels was detected chromatographically in the urines of dominant (DyDy) strain 129 mice, though no quantitative studies were done in an attempt to discriminate between this genotype and the heterozygote (Dydy).

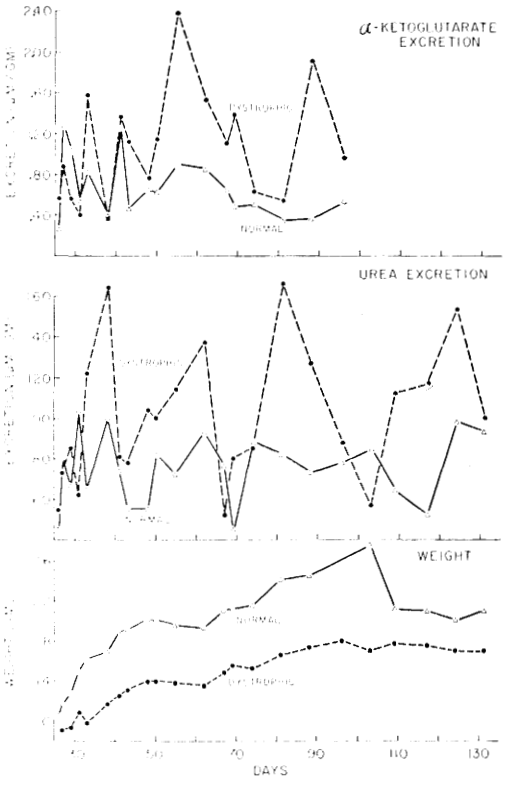

FIG. 1. Urinary excretion of α-ketoglutarate and urea by littermate dystrophic and normal mice.

TABLE I. Urinary Excretion of α-Ketoglutarate and Urea between 40 and 105 Days of Age for Littermate Dystrophic and Normal Mice.

Mouse	Sex	Net gain in wt (g)	α-Ketoglutarate Excretion (μM/g/day) Mean	Range	No. urines	Urea Excretion (μM/g/day) Mean	Range	No. urines
Dystrophic	♀	3.5	1.23	.38-2.4	11	90	70-170	12
Normal	♀	2.1	.82	.24-2.2	11	60	30-110	12
Dystr	♀	4.8	1.50	.99-2.3	11	120	90-150	12
Normal	♂	10.1	1.00	.49-1.5	11	80	40-120	12
Dystr	♀	2.4	3.00	2.4 -3.8	4	130	70-190	6
Normal	♀	4.0	1.31	.78-2.4	4	100	60-150	6
Dystr	♂	4.5	1.23	.54-2.4	12	100	50-170	13
Normal	♂	8.7	.63	.47-1.3	13	70	50- 90	13
Dystr	♂	10.0	1.40	.26-2.8	20	150	90-210	17
Normal	♂	10.4	.69	.21-2.1	20	110	60-230	17

Cumulative results

	Grand mean	S.D.*	Grand mean	S.D.*
Dystr	1.66	.75	118	24
Normals	.89	.28	86	18
	P† ~.003		P† ~.001	

* Stand. dev. † Probability that difference between means is not significant.

Amino acid excretion. The representatives of this class of compounds which were identified by their positions on chromatograms and comprised nearly the total amount of amino acid excretion were: taurine, glutamic acid, glycine, valine, alanine, aspartic acid, methionine sulfoxide, glutamine, phenylalanine, leucine, isoleucine and tyrosine. The listed order is approximately, from greatest to least, that of average daily excretion levels. Taurine was excreted at levels exceeding those of any other amino acid by several fold, the order of magnitude being around 1 μM/g/day. A few other unidentified ninhydrin reacting spots which appeared infrequently were not analyzed. There were no significant differences between dystrophic and normal mice in mean excretion per gram weight of any one or of total amino acids; however, daily excretion of all amino acids except taurine and methionine sulfoxide was consistently and markedly lower (around 50-75%) by 2 of the 5 dystrophic animals than by any of the controls. 1-Methylhistidine was never observed on chromatograms prepared from either normal or dystrophic mouse urines, although this amino acid was the predominant one excreted by rabbits with muscular dystrophy produced by a dietary Vit. E deficiency, whereas their littermate controls rarely excreted detectable levels(7).

Discussion. The consistency of differences in urea excretion between dystrophic and normal littermate mice as opposed to the large variability of differences in weight gain (Table I) tends to suggest that food consumption was fairly random and that variations in the latter probably had a relatively small influence on α-ketoglutarate and urea excretion as compared with the effect of the presence of muscular dystrophy. This is supported by a previous observation(15) that average food intake per unit weight by dystrophic animals was not markedly different from normals.

The increased urinary excretion of α-ketoglutarate by muscular dystrophic as compared with normal mice may have its basis in any of several physiological differences. Among possible reasons already suggested by other work can be included increased transaminase levels in blood and tissues of dystrophic mice (16), increased glutamic dehydrogenase levels in liver and muscle of the pathological animals(17), and increased protein catabolism by dystrophic muscle(18,19) furnishing more glutamate for transamination and deamination. Other metabolic derangements, changed kidney thresholds to α-ketoglutarate, or increased losses of glutamate or α-ketoglutarate from muscle because of increased permeability(20) cannot, however, at this stage be overlooked. The significance of the large excretion of α-ketoglutarate by strain 129 mice as compared with other species of animals and other strains of mice has not yet been assessed.

The elevated excretion of urea by the dystrophic mice could have its origin at least partly in an increased rate of protein catabolism by dystrophic mouse muscle(18,19) as well as an abnormal leakage of nitrogenous constituents from muscle(20) or other tissues. Increased nitrogen excretion has been observed in other forms of muscular dystrophy (21).

The failure in the present studies to observe an altered excretion of amino acids similar to that occurring in other dystrophies(2-7) could have resulted from the large individual variability under the conditions employed. The observation that 2 of the dystrophic animals consistently excreted less of most amino acids than any of the controls suggests an additional possibility that more extensive studies might disclose a deficient amino acid excretion by the pathological animals.

Summary. Unusually large amounts of α-ketoglutarate were excreted in the urines of strain 129 mice as compared with other species of animals and other strains of mice. A group of 5 muscular dystrophic strain 129 mice excreted significantly higher levels of α-ketoglutarate and urea/gram weight than did their 5 littermate controls. These differences became greater as the disease progressed from weaning through about 4 months of age. No significant differences were observed in urinary excretion of amino acids, either with regard to type or amount.

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Toxicity of Sphingosine and Other Long-Chain Amines. (25653)

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Rosenheim and Webster(1) found that feeding cerebrosides promoted conversion of cholesterol to coprosterol in the gastrointestinal tract. They fed various hydrolytic products of cerebrosides and noted that the basic component, sphingosine, could not be tested because the rats refused to eat diets containing either the free base or its sulfate salt. Recently we also observed that basic sphingosine-containing fractions from hydrolyzed cerebrosides were poorly accepted by rats even at very low levels in the diet(2). Further experiments suggested that toxicity was related to presence of free amine groups in these fractions and feeding tests were conducted with a series of simple aliphatic amines. These studies form the basis of the present paper.

Materials and methods. Methods used to prepare psychosine, ceramide, sphingosine and triacetylsphingosine from cerebrosides of beef brain or beef spinal cord have been described (2). Other amines used in present experiments were obtained from Matheson, Coleman and Bell, East Rutherford, N. J. Sulfate salts were prepared by mixing amines with a solution of sulfuric acid in ethanol as in preparation of sphingosine sulfate(3). Volume of ethanol was adjusted according to solubility of individual compounds. Male Sprague-Dawley rats weighing 80-100 g were used for most feeding experiments. Compounds to be tested were added to a synthetic diet consisting of: casein 21, glucose 58, corn oil 10, salt mixture 5 and celluflour 5 parts by weight, with an adequate supplement of water-soluble vitamins but no fat-soluble vitamins. In some experiments corn oil was omitted and glucose increased accordingly. In liquid diets fed by stomach tube, corn oil was retained but casein was replaced by a mixture of egg albumin and lactalbumin (1:3). The dry diet (150 g) was homogenized with water (100 ml) in Waring blendor.

Results. When young male rats were fed a synthetic diet containing 1 or 2% of free aminolipid prepared from beef spinal cord cerebrosides, they lost weight rapidly and

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