contrast to hydrocortisone acetate and cortisone acetate is also inactive by intra-tumoral injection in producing involution of the skin lesions in patients with Boeck's sarcoid and Hodgkin's disease.

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Excretion of Ether-Soluble Acids by Rats on Necrogenic Diet with and Without Supplements of Antibiotics.* (20577)

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Antibiotics such as aureomycin and penicillin when added to a necrogenic diet prolong the life of rats by delaying the onset of liver necrosis(1). Regardless of whether the antibiotics act by systemic action on the animal or by interaction with the intestinal flora, indirect effects should appear in the metabolism of the host. For this reason a comparison of the excretion products of rats on a necrogenic diet with those of animals receiving supplements of aureomycin or penicillin is of interest. The present study deals with the effect of the addition of these antibiotics to the basal necrogenic diet on the ether-soluble acid excretion of the rats.

Animals and methods. Male weanling rats (Sprague-Dawley or Carworth Farms) initially weighing approximately 45 g were kept in individual cages and were fed the necrogenic basal diet† with a daily supplement of vitamins(1). Aureomycin-HCl (Lederle) 25 mg, penicillin (N N'-Dibenzylethylene diamine-Wyeth) 5 mg, or vit. E 1 mg, were added to the daily rations of the treated animals. Control animals were offered 8.0 g of diet daily and the treated animals were pair-fed. For the *collection of urine* rats were placed in metabolic cages and kept on regular rations with water *ad lib.* Urine was collected under toluene. Funnels were washed down with hot water every day and the urine filtered and frozen. Analyses were run in duplicate on aliquots of combined 2- or 3-day samples of non-hydrolysed urine. *Nitrogen* was run by a semi-micro Kjeldahl method(2). Creatinine was run by the Jaffe reaction(3). Ammonia and urea after hydrolysis were determined by aeration and titration(4). Para aminobenzoic acid was determined by the usual diazotization method(5). Ether-soluble acids in urine were extracted by the method of Kanzaki(6) as modified by Bray *et al.*(7). Urine samples (10 ml) acidified with sulfuric acid (1 cc, 1 N) were continuously extracted with ether for 3 hours, the ether evaporated, the residue taken up in water and titrated with 0.1 N NaOH solution. Duplicate extracts of the same urine gave closely similar titration values. Hippuric acid (20 mg) added to a sample of urine was recovered 95-100% under the conditions of the experiments. Hippuric acid was determined after destruction of urea of the ether-soluble extract(8). Chromatograms of ether-soluble acids were run by descending technic with a butanol-acetic acid-water mixture (4:1:1) as solvent(9). Spots were developed with an alcoholic solution of brom cresol green.

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† Basal diet consists of: Corn starch 79; Baker's yeast 18 (British "D.C.L. Vit. B_1 yeast" from Distillers Corp., Ltd.); Salt Mix No. 2 USP 3; Peanut oil 0.5.

TABLE I. Effect of Aureomycin and of Penicillin on Daily Excretion of Ether-Soluble Acids in Rats on Necrogenic Diet.

Supplement to basal necrogenic diet	No. of days rats were on diet: 1–40 No. rats tested	1–40 ESA, μM × 10 / Creatinine, μM*	1–40 Diff. from control	40–200 No. rats tested	40–200 ESA, μM × 10 / Creatinine, μM	40–200 Diff. from control	Statistical significance (P) of diff.
0 (control)	5	88 ± 2.8		10	142 ± 12.4		—
Vit. E	8	100 ± 3.2	12	8	137 ± 7.5	5	0
Aureomycin	7	65 ± 10.4	23	14	77 ± 3.6	65	P 0.01
Penicillin	6	103 ± 4.0	15	12	67 ± 4.5	75	P 0.01

* ESA/N gives similar results: Daily quantities of creatinine and nitrogen were constant in each rat during duration of exp. There was no difference between the amounts excreted daily by rats from different groups.

Two of the ether-soluble acids found were crystallized. Two hundred ml of urine was extracted with ether for 20 hours. The ether fraction was then extracted with 200 ml 0.1 N $NaHCO_3$. The $NaHCO_3$ solution was acidified with H_2SO_4 to pH 1-2, decolorized with Norit A, and filtered. The filtrate was extracted with 200 ml ether, and the extract was evaporated. The residue was taken up in a small amount of ether, and the acid was precipitated out of solution by dropwise addition of petroleum ether in the cold. The two acids identified were crystallized directly from two different specimens of urine; no separation of the two acids was necessary.

Results. Urines of rats on a necrogenic diet were compared with that of treated animals of comparable weight, age, and food intake. The excretion of para-aminobenzoic acid, ammonia, urea, hippuric acid, nitrogen, and creatinine was similar in all the rats.

No differences in the quantity of ether soluble acids in the urine of treated and non-treated rats was observed during the first 40 days on the diet. Most rats kept on the necrogenic diet succumb to liver necrosis after 30 to 60 days, but a few survive. In these rats a statistically significantly higher excretion of ether soluble acids was found than in comparable animals protected with antibiotics (Table I). The animals given vit. E also showed an excretion rate comparable to the animals on the necrogenic diet. The pattern of spots in chromatograms of the ether soluble acids excreted by rats treated with antibiotics is different from that of non-supplemented animals or from animals given supplements of vit. E. These differences are shown most clearly in the chromatograms of the ether soluble acids of the older rats (Fig. 1). Ether soluble acids from the urine of non-treated or vit. E-treated rats showed spots at R_f 0.86 and 0.95. A few of these rats also showed spots at R_f 0.45. The rate at which these rats died was apparently unrelated to the presence or absence of the acid of R_f 0.45.

In contrast, almost all the rats treated with antibiotics have acids of R_f 0.45, R_f 0.86 and 0.95. In addition, all the rats treated with antibiotics have an additional acid of R_f 0.80. Hippuric acid gives a spot of R_f 0.93 and

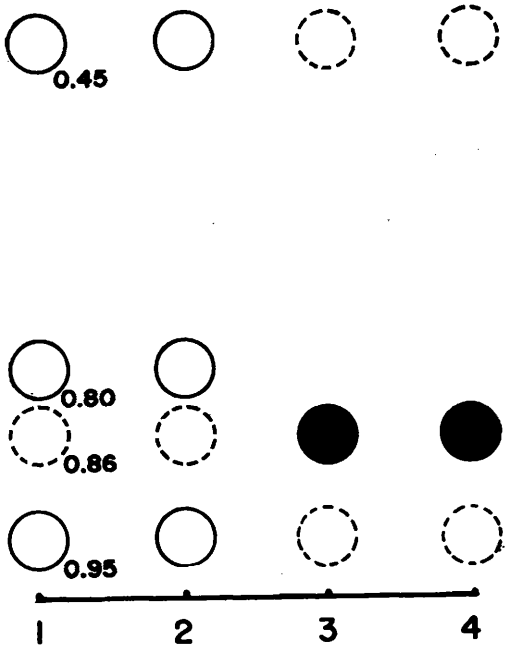

FIG. 1. Typical descending chromatogram of ether soluble acid excretion by rats on necrogenic diet with supplements 1. penicillin; 2. aureomycin; 3. necrogenic; 4. vitamin E.

PABA 0.92 under the conditions of our experiment. Five micromoles of total acid were found to give the clearest separation of spots; and this quantity was also used for test substances.

The acid of R_f 0.86 was crystallized from the urine of rats given vit. E by the method described above. The crystals were found to have a melting point of 129, an equivalent weight of 65, and elemental analysis C:46, H:8, O:64 (by difference), and were identified as methyl malonic acid. Mixed melting point, solubility in water, alcohol, ether, and petroleum ether, and R_f value were identical with that of pure methyl malonic acid.

The acid of R_f 0.95 was crystallized from the urine of rats given penicillin by the method described above. The crystals were found to have: m.p. 140, equiv. wt 75, and were identified as α,α-dimethyl succinic acid. Mixed melting point, solubility in water, alcohol, ether, and petroleum ether, and R_f value were identical with that of pure α,α-dimethyl succinic acid.

The ether-soluble acids of R_f 0.45 and 0.80 have not yet been identified.

Titration of the chromatographic spots (Fig. 1) after cutting and eluting showed the following distribution:

R_f	No supplement, %	Vit. E, %	Aureomycin, %	Penicillin, %
.45	*	*	10	10
.80	None	None	25–30	25–30
.86	85–90	85–90	15–40	15–40
.95	10–15	10–15	30–60	20–50

* Too small to measure.

Discussion. Aureomycin, penicillin and vit. E, when added to the necrogenic experimental diet, caused no quantitative alteration in the ammonia, urea, nitrogen, creatinine, hippuric acid, or para-aminobenzoic acid excretion in the urine.

The significance of the ether-soluble acid excretion in the urine of rats is unknown. Presumably, these acids represent end products of the metabolism of the animals.

Both the surviving non-supplemented rats and those receiving vit. E excrete high levels of ether-soluble acids. Either the surviving animal on the necrogenic diet has undetermined vit. E reserves or has developed a pathway similar to that of vit. E-treated animals.

No high levels of ether-soluble acids were found in the urines of rats receiving antibiotics. Since antibiotics prolong the lives of the rats but do not offer the permanent protection given by vit. E supplements, a different metabolic pathway is suggested for the acid excretion of the 2 groups of rats.

Methyl malonic acid forms the largest part of the total ether-soluble acid in the untreated or vit. E-treated animal, whereas α,α-dimethyl succinic acid and acid of R_f 0.80 forms the largest part of the acid excretion of the animals treated with antibiotics. No significant quantities of homogentisic acid were found in any of these urines(10). Methyl malonic acid has been described in the urines of rats fed anthracene(11), but was apparently unrelated to death in these animals. α,α-dimethyl succinic acid has not previously been described in the urine of rats.

Summary. 1. Addition of aureomycin or penicillin to the necrogenic diet showed no effect on the quantity of nitrogen, urea, ammonia, creatinine, hippuric acid or para-aminobenzoic acid excreted in the urine by rats. 2. Most rats on necrogenic diet die of liver necrosis between 30 and 60 days; a few survive. In these rats a statistically significantly higher urinary excretion of ether soluble acids was found than in rats treated with aureomycin or penicillin. Chromatograms of these ether-soluble acids indicated that addition of aureomycin or penicillin to the diet but not vitamin E altered the components of these acids in the urine. 3. The ether-soluble acids in the non-hydrolysed urine consist of para-aminobenzoic acid, hippuric acid, methyl malonic acid, α,α-dimethyl succinic acid and at least 2 unidentified acids. Methyl malonic acid forms a large part of the total ether-soluble acids in the non-treated and vit. E-treated animal, and forms a smaller fraction of the acid excretion of the animals receiving penicillin or aureomycin.

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Leptospiral Antigen Demonstrated by the Fluorescent Antibody Technic in Human Muscle Lesions of *Leptospirosis icterohemorrhagiae*.* (20578)

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Leptospiral antigen was demonstrated with the fluorescent antibody technic in the muscle lesions of a patient with *L. icterohemorrhagiae* infection.

Materials and methods. A 1 cm square piece of the left gastrocnemius muscle was excised from a 42-year-old Negro construction worker on the 9th day of an acute febrile illness, associated with icterus, azotemia, muscle pain which was particularly marked in the calf, headache and malaise. Half the specimen was immediately quick frozen in dry ice and alcohol and stored at —20°C. The remaining tissue was divided and fixed in Zenker's fluid with 5% glacial acetic acid and in 10% formalin. Routine histologic preparations revealed the typical lesions of acute Weil's disease (Fig. 1). These lesions consisted of focal areas of necrosis of muscle fibers with slight proliferation of sarcolemmal nuclei and minimal inflammatory cell infiltration(1). Sections stained by the Whartin-Starry method failed to reveal any organisms. Agglutination tests were positive for *L. icterohemorrhagiae* with titres of 1:6400 on the 16th day of illness and 1:12800 on the 31st day. Both serum samples were also positive for *L. mitis* at a titre of 1:640 and for *L. canicola* at a titre of 1:200. During convalescence, 31 days after the onset of the illness, a biopsy of the right gastrocnemius muscle was taken. This tissue was prepared as described above. Routine histologic preparations of this specimen revealed advanced healing by regeneration. Silver stains for leptospira again showed no organisms. Frozen sections of unfixed frozen muscle were prepared following the technic of Linderstrøm-Lang as modified by Coons *et al.*(2). Preliminary to staining, the mounted sections were fixed either by dipping briefly in 70% ethanol and rinsing with buffered saline or by covering with acetone for 15 minutes with subsequent evaporation of the acetone in an incubator at 37°C. These 2 types of fixation had been arrived at while adapting the fluorescent antibody technic to the study of experimental leptospiroses in Syrian hamsters.

The sections were stained for 30 minutes with the specific conjugate prepared according to the method of Coons and his associates(3). Conjugates with fluorescein isocyanate were prepared from immune sera obtained from rabbits injected with either *L. icterohemorrhagiae* or *L. canicola*.† The agglutination

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