Barrera-Oro, J. G., and Levy, B. M., J. Immunol. 92, 596 (1964).

13. Vogt, P. K., in "Advances in Virus Research," Smith, K. M. and Lauffer, M., eds., Vol. 11, p. 293. Academic Press, New York, 1965.

14. Epstein, M. A. and Achong, B. G., J. Natl. Cancer Inst. 34, 491 (1967).

15. Darfman, R. F., J. Natl. Cancer Inst. 38, 491 (1967).

Received July 28, 1967. P.S.E.B.M., 1968, Vol. 127.

Cathepsin Activity of Liver and Muscle Fractions of Adrenalectomized Rats* (32651)

JOHN W. C. BIRD, TROND BERG, AND JAMES H. LEATHEM[1]

Department of Physiology and Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey 08903

The release of acid hydrolases from lysosomes has been associated with several physiological and pathological states. De Duve *et al.* (1) postulated that if lysosomal enzymes were to function in the normal economy of cell catabolism or renewal, mechanisms must exist whereby the release of these potentially injurious enzymes could be facilitated or retarded. *In vitro* and *in vivo* experiments have indicated that various physiological compounds may stabilize or labilize the lysosomal membrane.

Under several experimental conditions cortisone and cortisol have been shown to stabilize the lysosomal membrane. De Duve *et al.* (1) observed that these corticoids stabilized liver lysosomes in incubation at acid pH. Weissmann and Thomas (2) found that cortisone administered *in vivo* protects rabbits against the cartilage lesions of hypervitaminosis A; and lysosomes isolated from the tissues of animals treated with cortisone appear to be more resistant to incubation with labilizers.

If one of the effects of corticoids is to stabilize lysosomal membranes, it appeared of interest to study the results of a drastic reduction of corticoids in the organism, and thus our experiments were designed to measure lysosomal enzyme activity in liver and muscle tissues of adrenalectomized rats. In that adrenalectomized animals have a reduced food intake, other experiments were designed to study the influence of starvation on lysosomal enzyme activity.

Materials and Methods. Weanling male Long-Evans rats were maintained on Purina Fox Chow until they attained a body weight of 60–65 gm, at which time they were placed on experiment. A semipurified 20% casein diet (3) was fed *ad libitum* to all adrenalectomized rats and their intact controls. Other groups were pair-fed to the adrenalectomized rats.

Adrenal glands were removed under ether anesthesia through bilateral incisions in the lumbar region. Care was taken to remove the glands encapsulated with as much adherent fat as possible. In one series of experiments untreated adrenalectomized animals and their controls were sacrificed 5 days after operation. In another series the adrenalectomized animals were given 1% saline to drink, and sacrificed with their controls 10 days after operation. In a final series, intact animals were pair-fed 25% of the food intake of their intact controls.

Animals were sacrificed by decapitation. Liver and gastrocnemius muscles were rapidly excised and chilled in a sucrose solution. After rinsing the tissues in additional sucrose solution, the tissues were cut into small pieces with scissors, and prepared into a 10% (w/v) homogenate with 0.25 *M* sucrose containing 1 m*M* EDTA. The homogenization was done in chilled glass, conical, motor driven homogenizers with the pestle rotating about 1000

* This study was aided in part by USPHS grants AM-06625, A462, and a Career Development Award to J.W.C.B.

[1] The authors wish to express their appreciation to Miss Sandra Goodstone for her technical assistance.

rpm. For liver, the grinding consisted of two complete up and down runs of the pestle, a 10-min rest in an ice slurry, and then the procedure repeated. The muscle was homogenized by 10 up and down runs of the pestle in one min, 10 min in the ice slurry, and the procedure repeated.

Cathepsin activity was determined by a modification of the Anson hemoglobin method (4). Bovine hemoglobin type II (Sigma) was used as a substrate. Liver samples were incubated for 10 min and muscle samples for 30 min with the substrate. Gianetto and De Duve (5) found that the relationship between activity and duration of incubation or enzyme concentration, while linear, did not go through the origin of the axes. In their method, the blanks were incubated for 2 min with enzyme before adding TCA. We found that by incubating substrate in the blanks for the same period of time as the experimental samples, and then adding TCA followed by enzyme, that the relationship between activity and enzyme concentration was linear through the origin of the axis.

Protein was determined by the method of Lowry *et al* (6) with Bovine albumen as the standard.

Tissues, homogenates and tissue fractions were kept below 4°C at all times, except when incubated at 38°C for enzyme or protein assays.

Distribution of muscle enzyme activity was determined by differential centrifugation of the homogenate. A nuclear fraction was separated at 1000 *g* for 10 min and the remaining cytoplasmic extract was divided into a heavy mitochondrial fraction (10,000 *g* for 15 min), a light mitochondrial fraction (20,000 *g* for 15 min), a microsomal fraction (78,000 *g* for 45 min) and a soluble fraction (final supernatant). All fractions except the final supernatant were treated with Triton X-100, at a final concentration of 0.2%, before the cathepsin assay. Preliminary experiments indicated that Triton X-100 had no effect on activity in the final supernatant.

Total enzyme activity, expressed as μgrams of tyrosine per mg protein, was obtained by adding Triton X-100 to the whole homogenate in the case of muscle. For liver, total activity was calculated as the sum of the activities from the nuclear fraction and the cytoplasmic extract, after treating with Triton X-100. *Free activity* is here defined as the enzyme activity in the final supernatant and was expressed either as percentage of total activity or as specific activity.

In vitro studies on the stabilizing influence of cortisone acetate and the labilizing influence of vitamin A alcohol were performed using the lysosome-rich total mitochondrial fraction (i.e., light and heavy mitochondrial fractions) from muscle. Cortisone acetate was suspended in aqueous solution by sonification and added to whole homogenate in a final concentration of 10^{-4} *M*. After incubation at 38°C for 30 min, the total mitochondrial fraction was separated, washed in 0.25 *M* sucrose, and resuspended in 0.25 *M* sucrose with 0.1 *M* phosphate buffer, pH 7.0. To some of the preparations vitamin A alcohol in 95% ethanol was added to a final concentration of 0.2 mg/ml, and all the suspensions and their controls were incubated at 38°C under nitrogen. At time intervals of 0, 30, and 60 min, 5 ml aliquots were removed from the incubator, centrifuged for 20 min at 15,000 *g*, and enzyme activity assayed in the supernatant.

For studies of kinetics and the effect of pH on enzyme activity, total muscle homogenates were treated with Triton X-100, and the nonsedimentable activity of 78,000 *g* for 45 min was assayed. Acetic acid–sodium acetate buffer was used over the range of its buffering capacity. A combination of glycine hydrochloride and citric acid–sodium phosphate buffers were tested to extend the range of pH's from 2.3 to 5.6. The pH's were checked at the middle of the incubation period. Acetic acid–sodium acetate buffer at pH 3.9 was used for the kinetic studies.

Students small sample *t* test was used for the statistical analyses with a probability of ≤ 0.01 being accepted as revealing a significant difference.

Results. The combined glycine-HCl and citric acid–sodium phosphate buffers, through the pH range of 2.3–5.6 indicate pH optimums at 3.2 and 4.9 for rat skeletal muscle cathepsin activity (Fig. 1A). Preliminary experi-

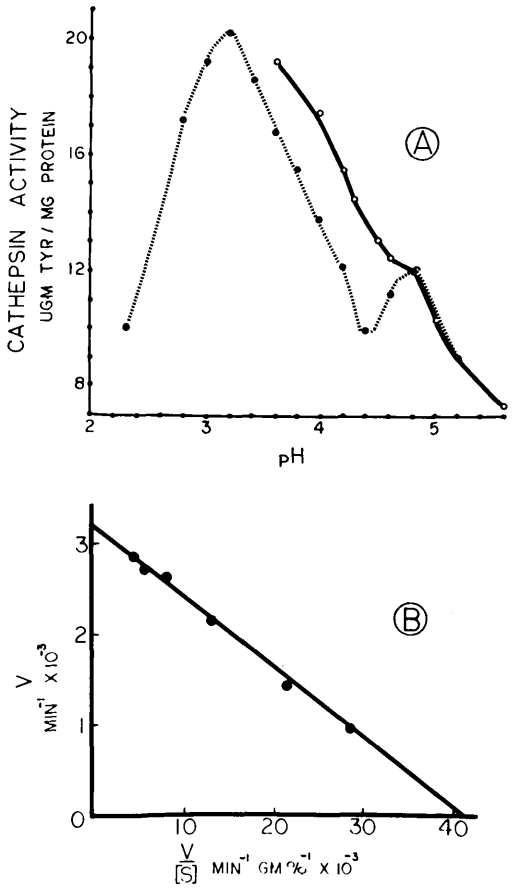

FIG. 1A. Dependence of muscle cathepsin activity on pH. The experimental conditions were the same as the cathepsin assay except that total muscle homogenates were treated with Triton X-100, and the nonsedimentable activity at 78,000*g* for 45 min was assayed. Activity is expressed as μg tyrosine per mg protein per 30 min. ..... combination of glycine hydrochloride and citric acid–sodium phosphate buffer and solid line, acetic acid–sodium acetate buffer. B. Hofstee plot of kinetic data, using acid-denatured hemoglobin as substrate, acetic acid–sodium acetate buffer, pH 3.9 38°C. K_m = 0.08 gm/100ml; V_{max} = 3.2 × 10^{-3} min^{-1}.

ments suggest that the maximum activity at pH 3.2 was due to cathepsin D. The second curve (Fig. 1A), representing the specific activity of cathepsins with acetic acid buffer, also demonstrates that the activity is optimum in the acid pH range. Acetic acid has been the buffer of choice in studies of proteolytic activity, and the one used in our experiments.

The Hofstee plot (Fig. 1B) of our kinetic data is linear and indicates that hemoglobin is forming a complex with cathepsin, and the complex remains at a relatively constant concentration throughout the period of observation. The V_{max} is 3.2 × 10^{-3} min^{-1} and K_m is 0.08% gm. Plotting the data by the modified Lineweaver-Burk method ([*S*]/v vs [*S*]) gave the same V_{max} and K_m values.

In addition to the demonstration of an acid pH optimum, the distribution of enzyme activity among the various subcellular fractions shows a pattern typical for lysosomal enzymes, i.e., highest relative specific activity in the light mitochondrial fraction (Fig. 2A). The large nuclear fraction is attributable to the homogenization procedure. This can be reduced by more severe homogenization; how-

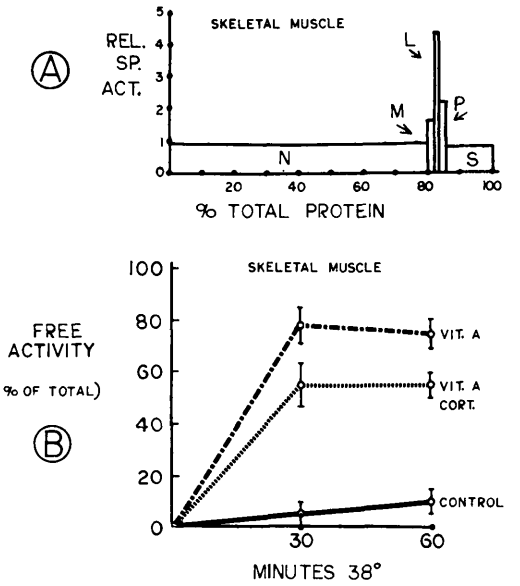

FIG. 2A. Distribution pattern of muscle cathepsin. Ordinate: mean relative specific activity of fractions. Abscissa: fractions are represented by their relative protein content, in the order in which they were isolated, i.e., from left to right: N, nuclear; M, heavy mitochondrial; L, light mitochondrial; P, microsomal; and S, final supernatant fraction. B. Labilizing and stabilizing effects of vitamin A alcohol and cortisone acetate, respectively, on total mitochondrial fractions. Particles are suspended in 0.25 *M* sucrose with 0.1 *M* phosphate buffer, pH 7.0 incubated at 38°, and at intervals the nonsedimentable activity was measured. The results at each time are expressed as a percentage of the total activity of the suspension ..... preparations pretreated with cortisone acetate, then vitamin A alcohol; —.— preparations pretreated with vitamin A alcohol; —— control preparations, with no additions. Each point represents the mean ± S.E. of seven experiments.

TABLE I. Cathepsin Activity of Rat Muscle and Liver Following Adrenalectomy and Food Restriction.[a]

Animal group	No. rats	Av. daily food intake (gm)	Change in body wt (gm)	Cathepsin activity: Total (μg Tyr/gm prot) Muscle	Total (μg Tyr/gm prot) Liver	Free (% of total) Muscle	Free (% of total) Liver
Adx × 5 days	7	5.8 ± 0.5	0.5 ± 1.0	7.2 ± 0.4[c]	15.1 ± 1.0	29.5 ± 1.9[c,d]	23.5 ± 2.4[b]
Normal, P.F.	7	5.8 ± 0.5	5.4 ± 2.1	6.3 ± 0.5	13.4 ± 1.0	23.5 ± 1.7	12.3 ± 1.8
Normal, *Ad lib.*	7	7.6 ± 0.8	18.4 ± 2.3	5.7 ± 0.4	13.8 ± 0.8	20.7 ± 1.0	8.3 ± 1.3
Adx + 1% NaCl × 10 days	7	6.8 ± 0.5	26.0 ± 4.1	6.9 ± 0.7	13.5 ± 1.0	26.0 ± 1.1	18.7 ± 1.6[b]
Normal, P.F.	7	6.8 ± 0.5	29.6 ± 3.0	5.5 ± 0.4	13.4 ± 0.9	21.3 ± 2.8	8.1 ± 2.7
Normal, *Ad lib.*	7	7.3 ± 0.7	40.1 ± 1.9	5.3 ± 0.4	13.1 ± 0.8	20.7 ± 1.0	8.0 ± 1.0
Partially starved × 5 days	9	1.9 ± 0.1	−6.7 ± 2.4	6.6 ± 0.3[b]	16.5 ± 1.0[b]	19.7 ± 2.4	5.7 ± 1.0
Normal, *Ad lib.*	9	7.6 ± 0.4	18.4 ± 4.1	5.4 ± 0.3	12.5 ± 0.8	20.9 ± 2.4	8.1 ± 1.0

[a] Adx, adrenalectomized; P.F., pair fed to adrenalectomized animals; *Ad lib.*, *ad libitum*-fed animals. Values represent means ± SE. Total cathepsin activity is represented by μg tyrosine per mg protein per 30 min for muscle and 10 min for liver. Free cathepsin activity is the percentage of total activity non-sedimentable at 78,000*g* for 45 min.

[b] Adx > *Ad lib.* and P.F. ($p < .01$); [c] Adx > *Ad lib.* ($p < .01$); [d] Adx > P.F. ($p = .03$). Details in text.

ever an undesirable increase in soluble activity in the final supernatant occurs. Another possibility for the nuclear activity is that some of the lysosomes, or solubilized enzyme, have agglutinated to the nuclei. Since Triton X-100 will not rupture nuclei, this should eliminate a nuclear origin of the activity found in the nuclear fraction.

Structure-linked latency of cathepsin was demonstrated in our preparations from gastrocnemius muscle (Fig. 2B). The labilizing effect of vitamin A alcohol is significantly inhibited by pretreating the homogenate with an aqueous suspension of cortisone acetate prior to fractionating the tissue. The final concentration of cortisone acetate in the total homogenate was 10^{-4} *M*. Due to the very limited water solubility of cortisone acetate, it is difficult to predict how much was actually available to the lysosomes, but its stabilizing effect is quite evident. Other experiments were performed in which the lysosome-rich suspensions were preincubated with only cortisone acetate, and no measurable release of cathepsin was evident in the 60-min thermal activation (not shown in Fig. 2B).

The adrenalectomized animals and their pair-fed controls had a food intake which was 75% of the *ad libitum*-fed animals, resulting in a much smaller gain in weight (Table I). Animals fed 25% of the food intake of the *ad libitum*-fed animals lost weight in the 5-day period. Adrenalectomized animals treated with saline had the same food intake as the *ad libitum* animals, and the change in weight was not significantly different from their pair-fed controls.

There was a significant increase in cathepsin free activity in both liver and muscle when adrenalectomized animals are compared with their *ad libitum* and pair-fed controls (Table I). The differences were significant whether the results were expressed as specific activity or as percentage of total activity. If one examines the pair-fed values for 5-day muscle-total, muscle-free, and liver-free activities, one notes that these data are intermediate between the values for adrenalectomized and *ad libitum*-fed animals, suggesting a possible effect of reduced food intake. The only significant change in total activity of adrenalectomized animals is the increase in muscle activity, as compared to normal animals.

When intact normal animals were fed 25% of the food intake of *ad libitum*-fed normals, both muscle and liver from pair-fed animals

had significant increases in total cathepsin activity, as compared to the *ad libitum*-fed animals, with no significant differences in free cathepsin activity for either tissue.

Discussion. Biochemical criteria of preparations. The lysosomal particle, as demonstrated by the electron microscope in several tissues, has never been shown in normal skeletal muscle. For this reason several experiments were performed to insure that cathepsins of this tissue conformed to the biochemical criteria defined by De Duve (7) for inclusion within this group of cytoplasmic particles. Our preliminary data indicated that the enzyme cathepsin D is associated at least partly with particles possessing the typical properties of lysosomes. Press *et al.* (8) were able to attribute two-thirds of their proteolytic activity, using a mince of spleen tissue, to cathepsin D. Iodice *et al.* (9) studying proteinase isolated from skeletal muscle of chickens concluded from their evidence that cathepsin D is the predominant, if not the only, proteinase present in the lysosome of muscle.

Valid assay conditions for cathepsin in muscle were established by a series of kinetic investigations, exploring such variables as enzyme concentration, substrate concentration, incubation time, and pH. Our results indicate that the enzyme has its optimum activity at pH 3.2 in our preparations, and is linear with respect to enzyme concentration and duration of incubation. Press *et al.* (8) found that purified cathepsin D from spleen had a pH optimum of 3.0, using hemoglobin as substrate. Iodice *et al.* (9), studying chicken skeletal muscle, and also using hemoglobin as substrate, showed an optimal activity occurring at pH 2.8 for purified cathepsin D. The latter authors found that when using total mitochondrial fractions the pH activity curve was biphasic, as we found in the present experiments, and that as much activity was found at pH 3.8 as 2.8. They felt that the measurement of activity in "crude extract" at pH 3.8 was as reliable an index of activity as that at pH 2.8 with purified enzyme.

The presence of two peaks in the pH activity curve indicates the presence of two enzymes in our system. However, Hofstee plots of our kinetic data at pH 3.9 were linear, suggesting that only one enzyme is acting on the substrate during the course of the experiment. If more than one enzyme were acting on the substrate at the same time, there would have been a sag in the line, which is believed to be due to a composite of separate straight lines for each enzyme involved (10). Another possibility, although unlikely, would be that each of the enzymes has the same K_m. We have not identified the enzyme giving rise to the peak at pH 4.8 in our preparation but Iodice *et al.* (9) have published convincing evidence, using chicken muscle, that the second enzyme is cathepsin A. Their results suggest that peptides produced by cathepsin D were being utilized by cathepsin A as substrate.

Distribution studies showed that less than 1% of the total protein (light mitochondrial fraction) contained approximately 44% of the total relative specific activity. The structure-linked latency of our particles was demonstrated by the increased activity after adding the nonionic detergent Triton X-100, or by treating the fraction with a physiological solubilizer, vitamin A alcohol.

Adrenalectomy experiments. The experiments presented here show that adrenalectomy of young male rats results in a significant increase in the free activity of cathepsin in muscle and liver preparations, and is true whether the enzyme activity is expressed as specific activity or percentage of total activity. This percentage is considered to be a measure of the stability of the particles to homogenization. The term "free" as used in this connection does not necessarily imply that the enzyme is free in the cell cytoplasm, but can be an indication of the lability of the particle to mechanical or chemical stress. There was also shown to be an increase in total activity in muscle from adrenalectomized animals.

Several physiological changes are taking place in the untreated adrenalectomized animals, and a change in the stability of the lysosomal membrane may be the result of factors other than the withdrawal of the glucocorticoids per se. The negligible gain in weight of the adrenalectomized animals, as compared to their pair-fed controls, suggests a reduction in absorption from the gut (11). Our results

with pair-fed intact animals, having 75% of the food intake of the *ad libitum* fed animals, indicated a small increase in free activity in muscle and liver. Experiments were performed to test this observation further by reducing the food intake of intact animals to 25% of normal, thereby giving a more pronounced starvation effect. These experiments showed that the greater starvation stress resulted in a tendency for less free activity in liver and muscle tissue than the normals, and a significant increase in specific total activity for both tissues. Swift and Hruban (12) have shown by electron microscopy that membrane-bound structures are formed in the liver following starvation, and propose that these structures are secondary lysosomes (autophages) which enable their digested material to enter the metabolic pool for reutilization. These secondary lysosomes would explain our increases in total activity in starvation.

The stress of starvation should increase the output of ACTH, which would result in a greater secretion of corticoids by the adrenal cortex. The increased titers of corticoids should enhance the stability of the lysosomal particles, which would explain the low free cathepsin activity in our starved animals. The inhibition of the labilizing effect of vitamin A alcohol by cortisone acetate in our *in vitro* muscle, and liver preparation (13) would further support the above argument. Beaufay *et al.* (14) have injected normal rats with hydrocortisone for 3 days prior to sacrificing the animals, and found significant decreases in total activity of liver cathepsin. Unsedimentable activities of several lysosomal hydrolases were either unchanged or lower than normal. We have confirmed these findings in preliminary studies on liver and muscle with corticosterone.

In an earlier study on cathepsin activity in liver tissue from guinea pigs fasted for 18 hours (15) we reported an increased rate of solubilization of cathepsins when the preparations were thermally activated or treated with vitamin A alcohol. Dingle *et al.* (16) have found that starving young rats (comparable to those used in the present experiment) for 24 hours caused on increase in free proteolytic activity of the liver, whereas there was no change in activity when adult rats were used. Beaufay *et al.* (14) fasted adult rats for 6 days and tested several acid hydrolases of the liver. They reported a slightly increased unsedimentable activity after starvation (significant only for cathepsin) which decreased during refeeding, and a significant increase in total activity. Zalkin *et al.* (17) reported no change in the activity of several acid hydrolases in the skeletal muscle of rabbits "deprived of food."

With the present data it would be difficult to state how much, if any, of the increased free activity in our preparations from adrenalectomized animals is due to starvation effects, as the animals have a reduced food adsorption from the intestines and may therefore be in a more severe state of starvation than indicated by food intake. Levin *et al.* (18) have recently demonstrated with *in vivo* and *in vitro* experiments on intestine a reduction in absorption of sugars, glycine, and glycyl-glycine in adrenalectomized rats, and a reduction in transfer of glucose and glycine in adrenalectomized and starved rats. Feeding 1% NaCl to adrenalectomized rats restored intestinal absorption of sugars, glycine, and glycylglycine. However, adrenalectomized animals maintained with saline in our experiments had significantly greater free cathepsin activity in liver than their pair-fed and *ad libitum*-fed controls. In that the pair-fed and *ad libitum*-fed groups were not significantly different from each other, these data would suggest that the increased free activity in the tissues from maintained animals was not due to electrolyte imbalance or starvation.

Summary. Cathepsin activity was determined in muscle and liver tissue fractions of adrenalectomized rats, adrenalectomized rats supplemented with saline, pair-fed intact controls, and *ad libitum*-fed controls. Cathepsin activity of skeletal muscle tissue conformed to the biochemical criteria necessary to be associated with lysosomes. Adrenalectomized rats had a significant increase in free cathepsin activity in muscle and liver, and a significant increase in total activity in muscle tissue. Intact animals fed 25% of the food intake of *ad libitum*-fed controls had significant in-

creases in total activity, with no significant differences in free cathepsin activity. Adrenalectomized animals maintained with saline had greater free cathepsin activity in liver and muscle than their pair-fed and *ad libitum*-fed controls, suggesting that the increased free activity was not due to electrolyte imbalance or starvation.

1. De Duve, C., Wattiaux, R., and Wibo, M., Biochem. Pharmacol. 9, 97 (1962).
2. Weissmann, G. and Thomas, L., Recent Progr. Hormone Res. 20, 215 (1964).
3. Leathem, J. H. and Wolf, R. C., Proc. Soc. Exptl. Biol. Med. 86, 724 (1954).
4. Anson, M. L., J. Gen. Physiol. 20, 565 (1936).
5. Gianetto, R., and De Duve, C., Biochem. J., v59, 433 (1955).
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
7. De Duve, C., Ciba Found. Symp. Lysosomes, p. 1-31. Little, Brown, Boston, 1963.
8. Press, E. M., Porter, R. R., and Cebra, J., Biochem. J. 74, 501 (1960).
9. Iodice, A. A., Leong, V., and Weinstock, I. M., Arch. Biochem. Biophys. 117, 477 (1966).
10. Patton, A. R., "Biochemical Energetics and Kinetics," p. 92. Saunders, Philadelphia, Pennsylvania 1965.
11. Noble, R. L., "The Hormones," Vol. 2, p. 69. New York, 1950.
12. Swift, H. and Hruban, Z., Federation Proc. 23, 1026 (1964).
13. Weissmann, G., and Thomas, L., J. Clin. Invest. 42, 661 (1963).
14. Beaufay, H., Van Campenhout, E., and De Duve, C., Biochem. J. 73, 617 (1959).
15. Bond, J. and Bird, J. W. C., Federation Proc. 25, 242 (1966).
16. Dingle, J. T., Sharman, I. M., and Moore, T., Biochem. J. 98, 476 (1966).
17. Zalkin, H., Tappel, A. L., Caldwell, K. A., Shibko, S., Desai, I. D., and Holliday, T. A., J. Biol. Chem. 237, 2678 (1962).
18. Levin, R. J., Newey, H., and Smyth, D. H., J. Physiol., (London) 177, 58 (1965).

Received July 28, 1967. P.S.E.B.M., 1968, Vol. 127.

Aflatoxin Toxicity in Beef Cattle (32652)

W. N. GARRETT,[1] H. HEITMAN, JR.,[1] AND A. N. BOOTH[2]
University of California,[1] Davis, California 95616, and Western Region Research Laboratory,[2] Albany, California 94710

Loosmore and Markson in 1961 first reported the adverse effects of aflatoxin in cattle (1). Subsequently, Clegg and Bryson described an outbreak of poisoning in cattle which was attributed to Brazilian groundnut (peanut) meal (2). In 1963 Allcroft and Lewis reported their findings on the effects of toxic Brazilian peanut meal for calves, cows, and heifers (3). In calves the first symptomatic effect of continuous ingestion of toxic peanut meal was a reduced growth rate followed by severe tenesmus a few days before death. Postmortem examination revealed fibrosis of the liver, ascites, and visceral edema.

Since aflatoxin is toxic for cattle, this raises the question as to whether a "no effect" or harmless level of intake of aflatoxin for cattle can be established. The primary purpose of this work was to relate the level of intake of aflatoxin B_1 to the degree of toxic effects produced in a beef cattle feeding study under practical conditions.

Methods and Materials. Twenty tons of prime quality California grown cottonseed meal, containing not less than 41% protein and not more than 0.04% free gossypol, was purchased locally for the feeding trial. Three tons of this meal was shipped to the Southern Regional Research Laboratory for fortifying with rice which had been cultured with mold at the Northern Regional Research Labora-

[1] Department of Animal Husbandry.
[2] Western Utilization Research & Development Div., Agricultural Research Service, U. S. Dept. of Agriculture.