TABLE I. Survival of 8-Day Eggs, Cooled 24 Hours, Inoculated with 0.1 cc Suspensions of *Coccidioides immitis*.

No. of 8-day eggs	Dosage	Days of survival in total age of embryo							
		9	10	11	12	13	14	15	16
46	1×10^6 spores	45	12	5	1	0	0	0	0
47	1×10^5	46	27	15	10	8	7	4	4
47	1×10^4	46	41	37	33	27	24	23	23
47	1×10^3	45	41	38	37	33	29	26	23
45	0.1 cc 5% glucose in saline	44	42	41	40	40	38	38	38

length of time and temperature of cooling can be chosen so that various degrees of susceptibility might possibly be attained if other invading hosts were to be used. 2. However, a word of caution is needed if chick embryos are to be used in a pseudo-quantitative manner to evaluate virulence or invasive capacity of an infective agent. Considerable variation on the hatchability or vigor of the embryos may occur from one region or one strain of chickens to another(4). Also nutritional factors of the mother hen, her age, and the phase of the egg laying season(5-8), all enter into the percentage of eggs that will hatch; hence, reflect the possibility of inherited variation of the susceptibility of the host.

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Proteolytic Activity and Protein Catabolism of Rat Diaphragm in Hemorrhagic Shock.* (20360)

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This work forms part of a study concerned with the biologic role of certain intracellular proteolytic enzymes. These peptidases, intensively and extensively studied by Bergmann and associates(1), exhibit a high degree of specificity so that it has been possible to measure accurately enzyme activity of highly inhomogenous preparations as a function of the rate at which, under standard conditions, a specific synthetic peptide substrate is hydrolyzed. Thus, glycylglycine‡ (GG) is hydrolyzed by a dipeptidase (GGase), present in many tissues, which has been shown by Smith(2,3) to be quite specific in its cleavage of this particular dipeptide.

That a state of shock serves as a potent stimulus to protein catabolic processes appears to be well documented. A review of these and other metabolic changes has been presented recently by Engel(4). The present investigation was undertaken to determine whether shock-induced acceleration of protein

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breakdown would be demonstrable in isolated rat diaphragm.

Materials and methods. After an 18-hour fast, adult male rats (Vanderbilt strain) were lightly anesthetized with Nembutal, 4 mg per 100 g body weight, and then bled from the cut tail. The bleeding was timed so that 3 to 3.5% of body weight was withdrawn in the first hour, a procedure which rather uniformly induces shock(5). The state of shock was maintained for the subsequent 2 to 6 hours. Terminal blood samples were obtained from the abdominal aorta followed by removal of the diaphragm. Plasma amino acid concentration was determined by a modification of the photometric ninhydrin procedure of Moore and Stein(6) with leucine as a standard. Throughout this investigation, control determinations on unbled animals were made concurrently with those on rats in hemorrhagic shock.

Experimental. GGase activity was measured in both extracted and surviving diaphragm. Aqueous extracts of diaphragm were prepared by grinding the isolated diaphragm in a mortar with a known volume (2.2 to 3 ml) of a medium(7) containing 103 mM of sodium chloride, 25 mM of sodium bicarbonate, 2.5 mM of calcium chloride, and 20 mM of sodium pyruvate. After centrifugation, the GGase activity of the clear supernatant solution was determined by a photometric ninhydrin technic(8). Dipeptidase activity of surviving diaphragm was measured by a procedure(9) in which the increment of free glycine is determined following incubation of washed isolated diaphragm with an excess of GG in the incubating medium. GGase activity, defined as μM of GG hydrolyzed per minute per g of diaphragm, was determined using a medium containing 42.8 μM/ml of GG and an incubation period of 15 minutes.

TABLE I. Effect of Hemorrhagic Shock on GGase Activity of Extracted and Surviving Rat Diaphragm.

	Extracted diaphragm		Surviving diaphragm	
	Control	Shock	Control	Shock
GGase* activity	2.25	2.00	.12	.12
S. E.	.184	.231	.004	.011
No. of animals	3	3	22	3

* GGase activity expressed as μM GG/ml of hydrolysis mixture, hydrolyzed/min./g of diaphragm.

As indicated in Table I, when compared with control values, there is no appreciable change in the GGase activity of either extracted or surviving diaphragm from rats in shock.

Results reported below support the conclusion that the metabolic effects of shock are secondary to tissue hypoxia. Since in the procedure for measuring GGase activity of surviving diaphragm(9), the incubated hemidiaphragm is repeatedly washed in media maintained in an *oxygen* atmosphere for one hour *prior* to incubation with substrate, it was conceivable that the incubated tissue "recovered" from shock during the preliminary exposure to oxygen, and, consequently, dipeptidase activity was found to be normal. Therefore, the experiment was repeated with only a 15-minute preliminary washing period. Again, the difference between GGase activity of diaphragms of 6 normal and 6 shocked animals was insignificant; the values obtained being 0.13 ± 0.021 and 0.09 ± 0.041, respectively.

In an effort to determine whether the protein catabolic effect of hemorrhagic shock was, in fact, demonstrable *in vitro,* net protein catabolism of normal and shock rats was studied. An amplification of a technic described by Kline(10) was used. Since, with this method, no exogenous substrate was added to the *in vitro* system, no specific proteolytic enzyme activity could be determined. Instead, net amino acid accumulation, which, in muscle, is taken to be a measure of net protein catabolism was measured. The freshly removed diaphragm was wiped free of blood on saline saturated gauze, and bisected. The control hemidiaphragm, after being weighed on a torsion balance, was immediately placed in 1% picric acid solution. The remaining hemidiaphragm was weighed and then placed in 2.7 ml of the medium described above, contained in a 20 ml beaker which had already been equilibrated with the 95% oxygen-5% carbon dioxide atmosphere in a Dubnoff metabolic incubator. The incubation was continued for a known interval of time at 37°C with shaking maintained at 90 cycles per minute. The hemidiaphragm was then removed, blotted on saline moistened gauze,

TABLE II. Effect of Hemorrhagic Shock on Net Protein Catabolism of Rat Diaphragm Incubated Aerobically and Anaerobically for 2 Hr.

Incubation atmosphere	Condition of animals	Diaphragm: Control	Diaphragm: Incubated	Incubated medium	Net protein catabolism (NPC)	D*/M	Terminal plasma amino N, mg %
		μM α-amino N per g diaphragm					
Oxygen	Normal	36.3 ± .6† (8)‡	17.6 ± .5 (10)	34.0 ± 1.2 (10)	16.4 ± .9 (8)	.53 ± .03 (8)	5.38 ± .17 (10)
	Shocked	35.0 (2)	17.5 (2)	31.6 (2)	14.0 (2)	.56 (2)	13.09 (2)
Nitrogen	Normal	37.4 ± .4 (3)	11.9 ± .8 (3)	48.2 ± 2.3 (3)	23.0 ±1.1 (3)	.25 ± .03 (3)	4.53 ± .23 (3)
	Shocked	35.4 ±1.7 (3)	10.4 ± .2 (3)	49.3 ± 2.2 (3)	24.3 ± .7 (3)	.21 ± .01 (3)	9.95 ±1.43 (3)
Oxygen	Normal	36.2 ± .5 (7)	14.8 ± .6 (7)	38.3 ± .6 (7)	16.9 ± .8 (7)	.38 ± .02 (7)	5.38 ± .29 (6)
Serial transfers to fresh media	Shocked	36.8 ± .9 (4)	11.4 ± .8 (4)	40.6 ± 1.0 (4)	15.2 ±2.3 (4)	.28 ± .02 (4)	13.9 ±1.29 (4)

Data above double line were obtained following uninterrupted incubation. The same procedure was followed to obtain the data placed below the double line except that incubated diaphragms were transferred to fresh media every 15 min. of the first hr of incubation.

* $\frac{D}{M} = \frac{\text{Incubated diaphragm } \alpha\text{-amino N}}{\text{Incubated medium } \alpha\text{-amino N}}$.

† Mean ± stand. error. ‡ No. of animals.

and placed in 7 ml of 1% picric acid. Two ml of incubated medium were diluted to 10 ml with picric acid solution. The hemidiaphragms were finely ground and the resultant suspension sedimented by centrifugation. Non-protein α-amino nitrogen (AN) content was determined in the picric acid supernates of control hemidiaphragm, incubated hemidiaphragm and incubated medium by a photometric method(6). Leucine was selected for the preparation of standard curves and the values reported below represent, therefore, AN relative to the color yield of pure leucine. The sum of AN concentrations of incubated diaphragm and medium minus the AN concentration of the control hemidiaphragm (all expressed as μM AN per g diaphragm) is the AN concentration which accumulated during the time of incubation. This difference is referred to as net protein catabolism (NPC). Factors affecting NPC have subsequently been evaluated in detail and will be the subject of another report(11).

NPC was estimated first during a 2-hour period of incubation. Results are presented in Table II. The state of shock is reflected in the increased plasma AN concentration in the experimental group and this was a constant finding throughout the present study. Using the *two-hour* incubation period, it was apparent that NPC values of diaphragms from shocked animals were not appreciably different from those of unbled controls. This was true regardless of whether incubation took place in an oxygen or nitrogen atmosphere.

However, in both the normal and shocked state, the removal of oxygen from the incubation atmosphere resulted in striking changes. Anoxia was accompanied by a highly significant increase in NPC. In addition, the AN content of the incubated diaphragm fell and that of the medium rose so that the ratio of the two determinations fell from 0.53 in oxygen atmosphere to 0.25 under nitrogen.

Since it is held that all the metabolic concomitants of shock are the sequelae of tissue hypoxia(4), the increased "leakage" of amino acids from the anaerobically incubated diaphragm suggested that a similar change might occur aerobically in the shocked state. Accordingly, the comparison between NPC values of normal and shocked diaphragms main-

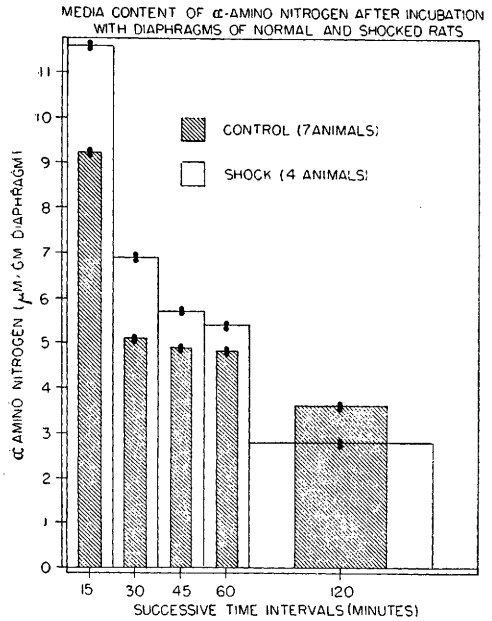

FIG. 1. See text for discussion. Black dots represent stand. errors.

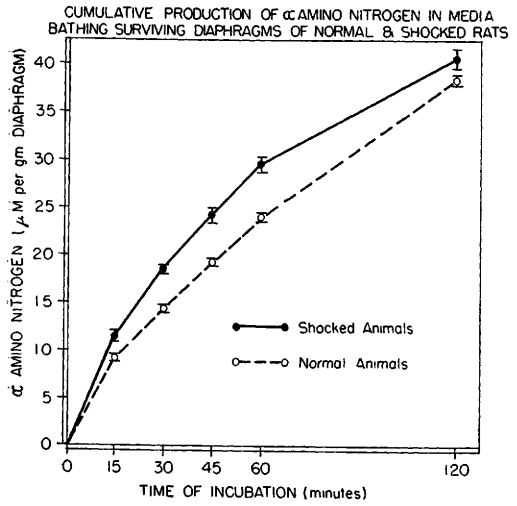

FIG. 2. See text for discussion. Vertical lines represent stand. errors.

tained in oxygen was repeated. In this instance, however, the incubated diaphragms were transferred to fresh media every 15 minutes for one hour followed by further uninterrupted incubation for the subsequent hour. Again, as recorded in Table II, there are only inconclusive differences between NPC values of normal and shocked tissue.

An examination of the serial media, however, proved to be fruitful as shown in Fig. 1. During the first 4 transfer periods, significantly more AN was released from the shocked rat diaphragms than from the normal controls. During the second hour of incubation, the reverse was observed so that the two effects cancelled each other over the total 2-hour period, as indicated in Fig. 2. Here, increments in AN content in the medium are plotted in a cumulative fashion. The increase in AN released from the shocked rat diaphragm remained consistently and significantly greater than that from the normal for the first hour. This increase fell off during the second hour so that, at the end of the 2-hour period, the differences became insignificant.

These findings led to the surmise that the determination of NPC after only *one hour* incubation might be profitable. The results, presented in Fig. 3, show clearly that under

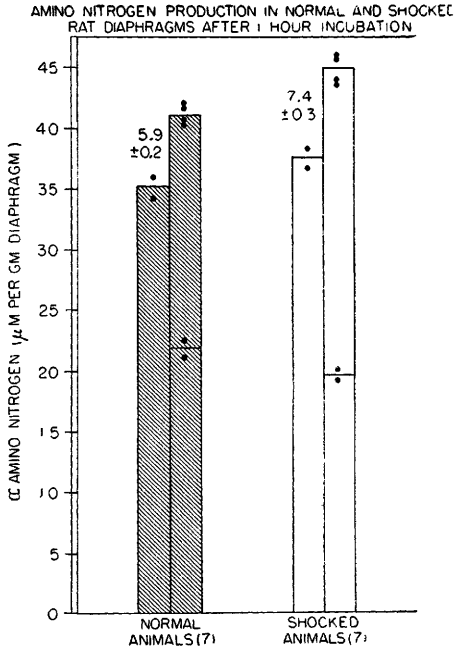

FIG. 3. Hatched column on far left represents AN content of control diaphragms from normal animals. Lower portion of second hatched column defines AN content of incubated diaphragm. Added to this is the AN content (per g diaphragm) of incubated medium. Difference between the heights of these 2 columns is 5.9 ± 0.02 μM/g diaphragm, the AN formed during incubation. Clear columns on the right are similarly charted. Black dots represent stand. errors.

these conditions, there is a relatively small but highly significant ($p < 0.01$) increase in NPC in diaphragms from shocked rats as compared with normal controls.

Discussion. There is convincing indirect evidence that increased protein breakdown in peripheral tissues is an integral part of the metabolic pattern found in hemorrhagic shock. Using the eviscerated rat, a preparation in which deamination of amino acids is negligible, Russell, Long, and Engel(12) showed that the rate of accumulation of AN in the blood was considerably greater in bled than in unbled animals. In dogs subjected to massive hemorrhage, Kline(13) found that the plasma AN arteriovenous difference became negative across the leg. The present demonstration that, after one hour incubation, the net protein breakdown from diaphragms of shocked rats is significantly increased above normal provides direct *in vitro* confirmation of the *in vivo* observations.

The report of Russell and Long(14) that the AN content of rat gastrocnemius was significantly increased following severe hemorrhage was not corroborated with the procedures employed in the present investigation, with rat diaphragm (Table II). The reason for this discrepancy has not been elucidated, but it should be recalled that the level of intracellular AN in muscle is dependent on membrane integrity(7) as well as intracellular protein metabolism. The present study provides clear-cut evidence of increased "leakage" of AN from diaphragms of shocked rats as well as increased protein breakdown in such tissues so that the "normal" AN content found may be the resultant of the 2 processes operating concurrently.

After the induction of hemorrhagic shock in the rat, its isolated diaphragm, in an oxygen atmosphere, behaves in a biphasic fashion with reference to release of AN; a period of relatively increased amino acid release, presumably the result of abnormal permeability, is followed by a period of decreased amino acid transfer. Further, during exposure to oxygen, the increased net protein breakdown in shocked rat diaphragm found after *one hour* incubation is no longer demonstrable if the time of incubation is doubled. Both of these observations may be interpreted as evidence of some degree of *in vitro* "recovery" from the shocked state. In view of the parallel metabolic effects of shock and anoxia, it does not seem unreasonable to attribute this "recovery" to the presence of high concentrations of oxygen in the metabolic incubator. Again, somewhat analogous observations have been made *in vivo*. Kline(13) found that the outpouring of AN from the leg of the dog, in hemorrhagic shock, could be reversed by replacing the blood previously removed.

Since, under properly selected circumstances, net protein catabolism of shocked rat diaphragm is enhanced, why are changes not discernible in specific peptidase activity? Various possibilities come to mind. First, it may be that shifts in the dynamic state which maintains protein mass are produced by changes in the rate of synthesis alone, without significant alteration in the rate of protein breakdown. Second, the highly specific dipeptidase chosen for study may not be representative of the many proteolytic enzymes which undoubtedly are involved in the catabolic process. Third, the negative nitrogen balance which occurs in shock could be provoked by relatively small increases in peptidase activity. Perhaps the presently available technics are simply too insensitive to reveal changes of this magnitude, although significant alterations have been produced(15) in GGase activity of surviving diaphragm following parenteral administration of an activator (cobalt) and inhibitor (cysteine) to rats.

Summary. The induction of hemorrhagic shock in the rat is accompanied by 1) no change in dipeptidase activity of extracted or surviving diaphragm; and 2) a significant increase in net protein catabolism of surviving diaphragm. Evidence is presented indicating that isolated diaphragms from shocked animals undergo some degree of "recovery" when exposed to an oxygen atmosphere.

The assistance of Mrs. Catherine C. Towbin is gratefully acknowledged.

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Oxidation of L-Glutamic Acid by Rat Heart.* (20361)

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Glutamic acid is known to be oxidized in tissues other than the liver and kidney and the enzymes responsible for its oxidation are known in a number of instances. Weil-Malherbe(1) for example observed that brain was able to oxidize glutamic acid. Dewan(2) isolated the enzyme and established its requirements. Independently from Dewan, von Euler and associates(3-5) made similar observations and reported an exhaustive study on glutamic dehydrogenase. In a large number of tissues, however, glutamic dehydrogenase exists in very low concentrations and could not account for the rapid utilization of glutamic acid. In heart muscle the glutamic dehydrogenase activity is very low or nonexistent(6), but glutamic acid is well utilized by homogenates of this organ, when fortified with ATP and coenzyme I. The results reported here clearly indicate that glutamic acid is oxidized only after transaminating with oxalacetic acid; the resulting α-ketoglutaric acid is then oxidized by way of the Krebs' cycle.

Methods. Male rats of the Sprague-Dawley strain and weighing approximately 250 g were used in all the experiments. The animals were sacrificed by decapitation, blood was drained off as much as possible, the heart removed and placed on a Petri dish resting over cracked ice. The muscle was freed of connective tissue and blood, then weighed and homogenized in a Potter homogenizer with ice cold isotonic KCl solution. The final concentration of the homogenate was 10%. In all experiments, 0.5 ml of the 1:10 homogenate was used in a final volume of 3 ml. The composition of the medium was identical to that given by Potter, Pardee, and Lyle(7) for the oxalacetic acid system, except that 500 γ of DPN were added in our experiments. The substrate concentration was 20 μM per flask unless stated otherwise. Oxygen uptake was measured by conventional Warburg methods at 38°C with air as the gas phase. *Ammonia* was determined in the deproteinized medium by distillation and nesslerization of the distillate. Aspartic acid and glutamic acid were determined by quanti-

TABLE I. Oxidations in Heart Homogenates.

Substrate (20/flask)	O_2 uptake 15m	30m	60m
No substrate	42	42	43
Glutamate	100	208	400
Succinate	60	120	219
α-Ketoglutarate	91	195	369
Oxalacetate	150	296	395
Citrate	42	46	54
Fumarate	80	160	273

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