

## STUDIES ON ALKALIGENESIS IN TISSUES

### II. AMMONIA PRODUCTION IN MUSCLE DURING CONTRACTION

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In the course of general wear and tear, all the tissues undergo a certain amount of protein decomposition, but how far functional activities of the body in general depend upon protein metabolism is a question not yet answered. In muscle, in spite of evidences brought out by Scaffadi (1), Burian (2), Tullio (3) and others (7) it is not yet conceded by all that its contraction involves a change in nitrogen metabolism. If there is any relationship between muscular activity and nitrogen metabolism one would expect to find some evidence of it in the urine. Failure to obtain such evidence, however, is not surprising, considering the many other factors of variability of urinary nitrogen.

The failure to obtain evidence of protein metabolism during muscular activity might be due to at least four difficulties. In the first place, the amount of metabolites might be exceedingly small. In the second place, the end products of metabolism of different proteins in the tissue might not necessarily be the same under all conditions. In the third place there might be mutual transformation among some of these possible metabolites. Finally, accurate methods of estimation of these different nitrogenous metabolites are not always available.

In the first study (4) on alkaligenesis of the tissues, we demonstrated that  $\text{NH}_3$  is one of the functional products of the nerve. Even if we assume that  $\text{NH}_3$  were a sole product of protein metabolism of muscle, the urinary or blood  $\text{NH}_3$  alone as determined by ordinary methods could not be used as a criterion in deciding whether or not similar metabolism occurs in contracting muscle. Errors of determination of  $\text{NH}_3$  in the body fluid, the transformation of ammonia into urea and vice versa, or slight decomposition of other nitrogenous constituents might easily mask a true functional variation. It is not surprising, therefore, to note that all the evidence so far recorded in the literature

of such nitrogenous metabolism was obtained not by examination of the urine but either in isolated muscle, or in blood which had been subjected to the least experimental disturbance and above all which had not been treated with such reagents as NaOH or  $\text{Ca}(\text{OH})_2$  to decompose ammonium salts.

We have undertaken an investigation to see whether or not  $\text{NH}_3$  is produced during muscular activity. We wished to know:

1. Whether muscle behaves similarly to nerve in increasing the output of  $\text{NH}_3$  during functional activity. A negative finding here would not rule out a possibility of protein metabolism during muscular contraction, but a positive result if proved to be due to a physiological process would be good evidence for it.

2. Whether a muscle contributed any basic substance to a salt solution in which it was immersed. Some investigators have studied the rate of  $\text{CO}_2$  production of different tissues by immersing them directly in an indicator solution. In order to test validity of this method, the actual basic substances contributed by muscle had to be measured.

3. How large the amount of  $\text{NH}_3$ , if any, given off by muscle is. For in order to know the fate of  $\text{NH}_3$  given off by nerves, and how far the body  $\text{NH}_3$  influences acid base balance in the body, it is desirable to know the actual amount of the base contributed by all the tissues.

In answering these questions we endeavored to measure the exact amount of volatile base produced in muscles rather than the total ammonium salts present in the muscle before, during and after contraction. The present communication records the amount of ammonia gas given off by a muscle under various physiological conditions.

**EXPERIMENTAL. Method and materials.** Gastrocnemius muscles of the frog, *Rana pipiens*, kept in laboratory aquarium from one day to many weeks were used exclusively. The muscle was quickly removed after the animal was decerebrated, and placed in Ringer's solution, avoiding as much as possible bruising or stimulating by contact with rough instruments. The adhering liquid is thoroughly removed with filter paper, the muscle weighed on a counterbalanced watch glass, and replaced in Ringer's solution for two or three minutes in order to lessen any stimulating effect, as well as to enable us to start the respiration experiment at fairly uniform intervals after the time of decerebration of the animal and isolation of the muscle from the body. The muscle was placed on electrodes fitted in paraffined corks, and placed in pyrex test tubes, containing each 1 cc. of indicator acid solution (MB-

MR-acid solution). The muscles were suspended over the liquid, which they did not touch. The respiration was allowed to continue usually fifteen minutes except on special occasions where different periods of respiration were desirable. The volatile base, if any, absorbed by the MB-MR-acid solution was analyzed in exactly the same way as described in the previous paper on nerve, by titration.<sup>1</sup>

Although all the quantitative data recorded in the following pages were obtained by the titration method alone, we ran extensive preliminary experiments with Graves' reagent. It will be recalled that a dilute  $\text{NH}_3$  solution, superimposed on Graves' reagent forms a white cloud ring at the junction of the two solutions.

RESULTS. *a. Resting and contracting muscle.* In order to eliminate as far as possible physiological variations of different animals, we have usually compared the two muscles of the same frog, one only being stimulated. Stimulation was applied directly to the muscle by means of a break induced shock just strong enough to give a vigorous contraction, the frequency being regulated to about 24 times per minute by a metronome. In this way ordinary fresh isolated muscles from a healthy frog could be kept contracting periodically for an hour or so. A detailed procedure of a typical experiment is exactly identical to that shown in table 3 in study I. In table 1 are given the results of quantitative estimations of  $\text{NH}_3$  production in the muscle during rest and contraction. Only those cases where the muscles of the same frog were used simultaneously for resting and stimulation experiments are included in the table.

As usual, the individual variation is considerable, no doubt due to the error of method as well as physiological variation. In the muscles of the same frog one very rarely fails to see the increased  $\text{NH}_3$  production during contraction. In the course of large numbers of experiments, occasionally we found that contracting muscle gave far less  $\text{NH}_3$  than the resting. The most of these cases we believe were due to unexpected injury, or the physiological condition of the muscle was such that they could not eliminate lactic acid quickly enough (see page 249). The results show that the average gastrocnemius muscle of the frog, ranging from 17 to 21 cm. in length, gives off  $3.8 \times 10^{-7}$  grams of  $\text{NH}_3$  without stimulation and  $7.56 \times 10^{-7}$  grams during contraction, calculated on the basis of 1 gram of the muscle and 15 minutes' respiration. Thus, during rhythmic muscular contraction,  $\text{NH}_3$  production is nearly doubled.

<sup>1</sup> For detailed method see page 532, lx, this JOURNAL.

Tullio (3) could not demonstrate an increased  $\text{NH}_3$  production in the blood of veins after vigorous contraction of muscles (man and animals), when he used milk of lime to displace  $\text{NH}_3$  from the blood. By utilizing blood alkalinity<sup>2</sup> instead of  $\text{Ca}(\text{OH})_2$ , he found 0.77 mgm. of  $\text{NH}_3$  in 100 grams of the blood of man during rest, and 0.84 mgm.

TABLE 1

Amount of  $\text{NH}_3$  given off by resting and contracting gastrocnemius muscle of frog, *Rana pipiens*

| EXPERIMENT                          | TEMPERATURE | WEIGHT OF MUSCLE | TIME ELAPSED FROM DECEREBRATION OF THE FROG TO THE BEGINNING OF RESPIRATION | DURATION OF RESPIRATION | CONTRACTION | TOTAL NH <sub>3</sub> GIVEN OFF | AMOUNTS NH <sub>3</sub> GIVEN OFF, CALCULATED ON BASIS OF 1 GRAM OF THE TISSUE AND 15 MINUTES RESPIRATION |
|-------------------------------------|-------------|------------------|---|-------------------------|-------------|---------------------------------|---|
|                                     | degrees C.  | mgm.             | minutes   | minutes                 |             | grams                           | grams   |
| 55                                  | 21.5        | 600              | 6½  | 15                      | +           | 7.5 × 10 <sup>-7</sup>          | 12.5 × 10 <sup>-7</sup>   |
|                                     |             | 635              | 6½  | 15                      | -           | 2.5 × 10 <sup>-7</sup>          | 3.93 × 10 <sup>-7</sup>   |
| 59                                  | 23.5        | 905              | 7   | 15                      | +           | 4.8 × 10 <sup>-7</sup>          | 5.3 × 10 <sup>-7</sup>  |
|                                     |             | 870              | 7   | 15                      | -           | 3.2 × 10 <sup>-7</sup>          | 3.67 × 10 <sup>-7</sup>   |
| 61                                  | 21.5        | 770              | 14  | 15                      | -           | 1.6 × 10 <sup>-7</sup>          | 2.07 × 10 <sup>-7</sup>   |
|                                     |             | 795              | 14  | 15                      | +           | 4.0 × 10 <sup>-7</sup>          | 5.03 × 10 <sup>-7</sup>   |
| 62                                  | 21.5        | 560              | 5   | 15                      | +           | 5.0 × 10 <sup>-7</sup>          | 8.92 × 10 <sup>-7</sup>   |
|                                     |             | 520              | 5   | 15                      | -           | 3.0 × 10 <sup>-7</sup>          | 5.76 × 10 <sup>-7</sup>   |
| 63                                  | 20.5        | 645              | 6   | 15                      | +           | 5.0 × 10 <sup>-7</sup>          | 7.75 × 10 <sup>-7</sup>   |
|                                     |             | 650              | 6   | 15                      | -           | 3.0 × 10 <sup>-7</sup>          | 4.61 × 10 <sup>-7</sup>   |
| 64                                  | 24.0        | 1010             | 10  | 15                      | -           | 3.0 × 10 <sup>-7</sup>          | 2.97 × 10 <sup>-7</sup>   |
|                                     |             | 1625             | 10  | 15                      | +           | 6.0 × 10 <sup>-7</sup>          | 5.91 × 10 <sup>-7</sup>   |
| Average for contracting muscle..... |             |                  |   |                         |             | 7.56 × 10 <sup>-7</sup> grams.  |   |
| Average for resting muscle.....     |             |                  |   |                         |             | 3.83 × 10 <sup>-7</sup> grams.  |   |

during muscular activity. In dogs' blood 0.27 mgm. in resting and 0.37 mgm. during muscular exertion. This is by far a smaller increase than our finding. Experimental conditions are, however, not comp-

<sup>2</sup> From his original Italian article we were unable to get an accurate notion of just how this was accomplished. We take it, however, that he probably passed an inert gas or air freed from  $\text{NH}_3$  and  $\text{CO}_2$  through the blood until all the  $\text{CO}_2$  was driven off and bicarbonate decomposed.

arable in the two cases. The important part of his conclusion is that the active muscle produces and eliminates more  $\text{NH}_3$  in a given time than muscle in repose.

The most striking point of our result is that the muscle gives off far less  $\text{NH}_3$  gas than does the nerve. As shown in table 2, the resting nerve produces thirteen times more  $\text{NH}_3$  per unit weight than the resting muscle. During functional activity the nerve gives off fourteen times more  $\text{NH}_3$  than the muscle. In other words, the amount of the base produced from the activated muscle is much smaller than the amount of acid produced under the same condition.  $\frac{\text{NH}_3}{\text{CO}_2}$  in case of the nerve was  $\frac{1}{17}$  by weight. In muscle, this ratio must be far less than  $\frac{1}{100}$ . Whether this means that the actual  $\text{NH}_3$  production in

TABLE 2

Showing comparative amounts of  $\text{NH}_3$  production in sciatic nerve and gastrocnemius muscle of frog, *Rana pipiens*

| CONDITIONS                        | TISSUE | TEMPERATURE | AMOUNT OF $\text{NH}_3$ GIVEN OFF, CALCULATED ON BASIS OF 1 GRAM DURING 15 MINUTES | NERVE $\text{NH}_3$ MUSCLE $\text{NH}_3$ |
|-----------------------------------|--------|-------------|--|--|
|                                   |        | °C.         | grams  |  |
| Resting.....                      | Nerve  | 20-24       | $51.2 \times 10^{-7}$  | 13.3                                     |
|                                   | Muscle | 20-24       | $3.8 \times 10^{-7}$   |  |
| During functional activities..... | Nerve  | 20-24       | $108.8 \times 10^{-7}$   | 14.2                                     |
|                                   | Muscle | 20-24       | $7.56 \times 10^{-7}$  |  |

muscle is really smaller than that in the nerve, or that the greater part of the base is held in the muscle by non-volatile acid formed during its activity might be determined by stimulating muscle at the beginning of the respiration period and collecting the gas during a longer period of respiration so that the lactic acid might be given an opportunity to be oxidized more completely. The result of such experiments show (see table 3, page 250), that the actual production of  $\text{NH}_3$  gas in the muscle is far less than that of the nerve.

b. *Injured muscle.* In these experiments, muscles were first "mashed," but not ground, by rough instruments, and then placed in the respiratory chamber. The results are exactly the same as were obtained with the nerve. In all cases, excepting two, no  $\text{NH}_3$  is produced from the severely injured muscle during first 15 minutes respiration. In two exceptions, muscle 74 gave  $6.2 \times 10^{-8}$  grams as muscle 76 gave 4.7

$\times 10^{-8}$ , both of which are less than one-tenth of that of the normal muscle.

*c. Tetanized muscle.* The muscle was stimulated with weak tetanizing current from induction coil, having the frequency of about 60 times per second. During the whole respiratory period the muscle is thus kept in complete tetanus. In every case muscle thus treated gives off no  $\text{NH}_3$ , as tested with our method. The results fail to show whether this muscle is producing less, or more  $\text{NH}_3$  than the resting nerve. Considering the well-known increase in acidity of the muscle during tetanus, this failure can be ascribed to the non-volatile acid holding the  $\text{NH}_3$  if any is produced in the muscle (see table 3). The important point is, however, that for experiment with contracting muscle the conditions tending to produce fatigue or tetanus must be avoided as much as possible. These results show beyond doubt that our method of titration actually measures a volatile base forming compound.

*d.  $\text{NH}_3$  production in muscles on standing under various conditions:*  
*1.  $\text{NH}_3$  production at successive intervals of survival.* In these experiments a series of 4 test tubes containing exactly the same amount of MB-MR-acid solution was prepared. The two muscles were prepared from the same frog, one placed in the first tube and the other in the next, the remaining two tubes of the group acting as controls. The muscle in the first tube was stimulated at definite intervals for 15 minutes. At the end of 15 minutes, after the usual procedure, both muscles were transferred into corresponding tubes of the second group, no more stimulation being applied to the stimulated muscle during subsequent respiration.

The results of titration of each of these tubes show that during the first 15 minutes the  $\text{NH}_3$  production is usually at the maximum in both resting and contracting muscle. During the next 15 minutes following contraction, the stimulated muscle gives a still greater amount than the unstimulated muscle. In the majority of cases, even at the end of the third 15 minutes, the muscle once activated produces a little more  $\text{NH}_3$  than the unstimulated. The result of these experiments are given in figure 1.

The slight rise during the fourth period is not significant. The fact is, the majority of muscles give less  $\text{NH}_3$  than that of the preceding period. The average value given here has been greatly influenced by the muscle of frog 57, which gave  $11.9 \times 10^{-7}$  grams for the resting and  $13.7 \times 10^{-7}$  grams for the one contracted. If we omit these two the

average will be much lower as indicated by the dotted lines. Just when the curve begins to rise is not the question we investigated carefully. For such an experiment one should control experimental condi-

TABLE 3  
*NH<sub>3</sub> production from the muscle determined during longer period of respiration*

| NUMBER OF EXPERIMENT | TEMPERATURE | FROG |      | WEIGHT OF MUSCLE | TIME ELAPSED FROM DEGENERATION OF PROTO BEGINNING OF RESPIRATION | RESPIRATION | TREATMENT | TOTAL NH <sub>3</sub> GIVEN OFF | NH <sub>3</sub> PRODUCED, CALCULATED ON THE BASIS OF 1 GRAM MUSCLE AND 15 MINUTES RESPIRATION |
|----------------------|-------------|------|------|------------------|--|-------------|-----------|---------------------------------|---|
|                      |             | Sex  | Size |                  |  |             |           |                                 |   |
|                      | degrees C.  |      | cm.  | mgm.             | minutes  | min-utes    |           | grams                           | grams   |
| 81                   | 28.5        | ♀    | 21   | 675              | 5½   | 129½        | S.        | $16.1 \times 10^{-7}$           | $1.83 \times 10^{-7}$   |
|                      |             |      |      | 690              | 5½   | 129½        | T.        | $4.9 \times 10^{-7}$            | $0.548 \times 10^{-7}$  |
| 83                   | 28.5        | ♂    | 20   | 580              | 10   | 127         | S.        | $7.7 \times 10^{-7}$            | $1.03 \times 10^{-7}$   |
|                      |             |      |      | 620              | 10   | 127         | T.        | $5.6 \times 10^{-7}$            | $0.713 \times 10^{-7}$  |
| 85                   | 29.5        | ♂    | 20   | 785              | 13   | 127         | E.        | $1.4 \times 10^{-7}$            | $0.14 \times 10^{-7}$   |
|                      |             |      |      | 780              | 13   | 127         | R.        | $9.1 \times 10^{-7}$            | $0.913 \times 10^{-7}$  |
| 86                   | 29.5        | ♀    | 20   | 796              | 7  | 127         | R.        | $4.9 \times 10^{-7}$            | $0.48 \times 10^{-7}$   |
|                      |             |      |      | 800              | 7  | 127         | E.        |                                 |   |
| 90                   | 28.0        | ♂    | 20   | 724              | 11   | 120         | S.        | $4.9 \times 10^{-7}$            | $0.558 \times 10^{-7}$  |
|                      |             |      |      | 756              | 11   | 120         | T.        | $2.1 \times 10^{-7}$            | $0.225 \times 10^{-7}$  |
| 91                   | 28.0        | ♀    | 20   | 655              | 5  | 108         | R.        | $8.4 \times 10^{-7}$            | $1.19 \times 10^{-7}$   |
|                      |             |      |      | 665              | 5  | 108         | E.        | $1.4 \times 10^{-7}$            | $0.194 \times 10^{-7}$  |

S. = Muscle is stimulated by break induced shock during first 15 minutes respiration.

T. = Muscle is tetanized by weak induced shocks during first 15 minutes respiration.

E. = Muscle is crushed before placing in the respiratory chamber.

R. = Unstimulated muscle.

Average S. =  $1.13 \times 10^{-7}$  grams.

T. =  $0.49 \times 10^{-7}$  grams.

E. =  $0.167 \times 10^{-7}$  grams.

R. =  $0.862 \times 10^{-7}$  grams.

tions more rigorously, as the temperature effect on the death point of the muscle and the NH<sub>3</sub> production is quite divergent. The point of importance is that NH<sub>3</sub> when measured as a gas, does not show a



steady increase during a short interval of survival as one would expect, if it is due to the post-mortem decomposition of urea, or other nitrogenous compounds.

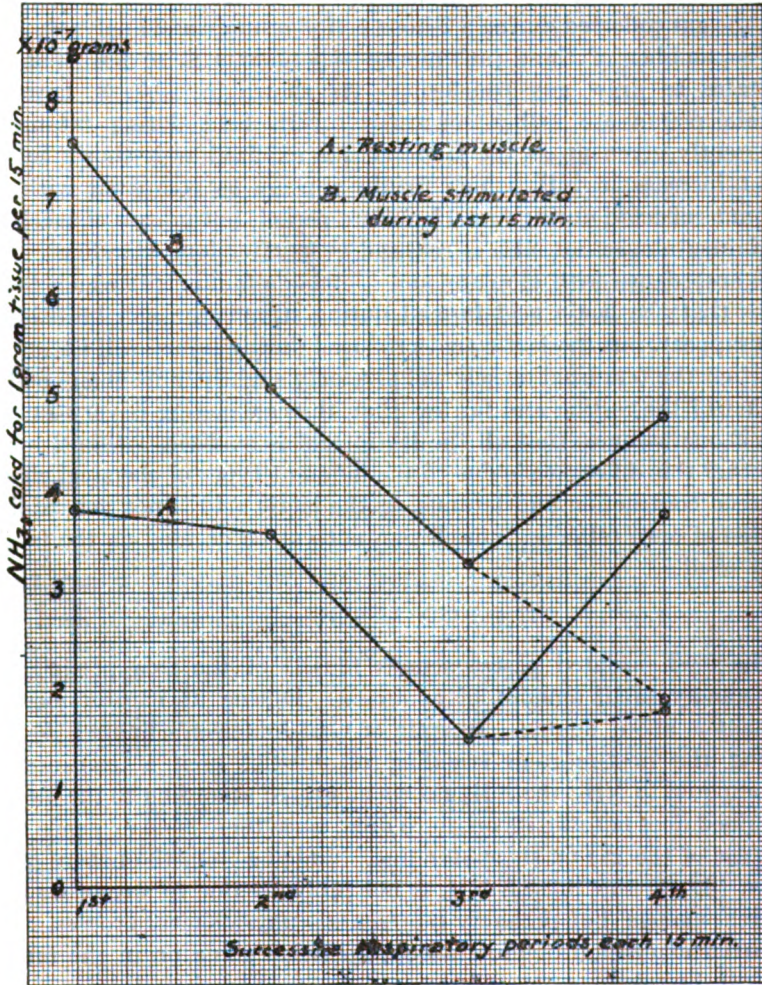


Fig. 1

2. *Experiments with longer intervals of respiration.* In order to see what is happening to NH<sub>3</sub> production during tetanus and injury where, even if the gas may be formed, the acid will be able to hold it, the muscles variously treated were allowed to respire for a longer period.

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In the case of contracting and tetanized muscles, the muscles were stimulated in the respiratory chamber during the first 15 minutes and let stand over 2 hours and the total  $\text{NH}_3$  given off was estimated. In the case of trauma, the muscle was injured before placing in the tube, and the gas collected during a 2-hour period or more and analyzed.

The number of these preliminary experiments is so few that one hesitates to draw any conclusion. As shown in table 3, however, certain points are rather suggestive.  $\text{NH}_3$  held during tetanus is apparently given up as the lactic acid is oxidized as is shown by the slight increase in  $\text{NH}_3$  which finally appears as compared with the amount collected during first 15-minute period, but in case of mechanical injury almost no  $\text{NH}_3$  is released even after long standing. This is in accordance with Hopkins and Fletcher's finding (6) which establishes that the lactic acid formed during stimulation can be oxidized or disappear, but the lactic acid produced during injury will not. The fact that the tetanized muscle will give only slightly more than one-half of that of the resting and far less than the contracting is very surprising, if one assumes that all the lactic acid is oxidized within 2 hours. Whether this difference is due to the different types of chemical change between contraction and tetanus, or the lactic acid is not yet oxidized in case of once tetanized muscle can not yet be decided on the face of these results.

#### CONCLUSION

The fresh isolated gastrocnemius muscle of the frog produces an exceedingly small amount of ammonia. During its contraction, the amount is about double. In spite of Gad-Andersen's finding (5) that during the post-mortem standing of muscle, urea is continuously decomposed to  $\text{NH}_3$ , the  $\text{NH}_3$  gas we measured according to the method described does not owe its origin to urea, since there is no evidence of an increase of the gas on short standing. Judging from the nature of the curve (fig. 1) this muscle  $\text{NH}_3$  is not probably due to any other post-mortem change, but probably represents a physiological process. It is a partial expression of protein decomposition going on in the muscle, and can be taken as an additive evidence to show that nitrogen metabolism is involved during its functional activity.

According to Burian, hypoxanthine at first increases on stimulation of muscle. If this base is produced from adenine, we expect a simultaneous production of  $\text{NH}_3$ .  $\text{NH}_3$  produced during functional activity in the muscle, however, is not likely due to the purine metabolism, considering the analogous case of the nerve fiber, where one would

expect the least possibility of such a metabolism. An analogy, however, does not prove any thing.

According to our results, the total muscles of a human being weighing<sup>3</sup> 150 pounds should give off 1.043 grams of  $\text{NH}_3$  per day if we calculate it on the basis of frog's gastrocnemius muscle and, at the maximum, the activity of all muscles in the body can never give beyond 2 grams of the gas. This calculation is given merely to show that muscle  $\text{NH}_3$  must be transformed to something else, probably urea. Under this condition, one would easily fail to detect an increase of N output in urine during severe muscular activity, no matter to which compound  $\text{NH}_3$  might be transformed.

As shown in table 2, muscle gives off the base-forming substance far less than does nerve. Since we have evidence to show that the various other tissues too give off an entirely different amount of this basic compound, it is an exceedingly unsafe procedure to depend on a method of estimation of  $\text{CO}_2$  by immersing the isolated tissues directly in an indicator solution and by measuring an amount of an increase of  $\text{H}^+$  concentration, or by determining rate at which the solution reaches a definite pH.

#### SUMMARY

1. A resting gastrocnemius muscle of the frog, *Rana pipiens*, gives off  $3.83 \times 10^{-7}$  grams  $\text{NH}_3$ , calculated on the basis of 1 gram, of the tissue and 15 minutes of respiration. During 360 contractions it gives off  $7.56 \times 10^{-7}$  grams  $\text{NH}_3$ . One gram of muscle, therefore, produces approximately  $1 \times 10^{-9}$  grams more  $\text{NH}_3$  during a single contraction.

2. Tetanized and injured muscles do not give off any  $\text{NH}_3$ , probably due to simultaneous production of a non-volatile acid.  $\text{NH}_3$  held by tetanized muscle will be released if we let it recover. Injured muscle will not.

3. For exactly the same unit, muscle gives  $\frac{1}{14}$  as much  $\text{NH}_3$  as nerve. This shows a danger of erroneous conclusion if for estimation of  $\text{CO}_2$  of the tissues one depends upon the direct indicator method alone, without eliminating the presence of the base-forming substance in the solution.

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<sup>3</sup> Forty-two per cent of the human body is considered to be made of muscles.