STUDIES ON ALKALIGENESIS IN TISSUES

I. Ammonia Production in the Nerve Fiber during Excitation[1]

SHIRO TASHIRO

From the Department of Biochemistry, University of Cincinnati

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Immediately after the publication of an article (1) in which the author reported that the resting nerve respires and that this respiration increases during the passage of the impulse, he made some attempts to devise a quick and easy method practicable for ordinary class experiments to demonstrate this increased metabolism during the stimulation. On account of the necessity of an elaborate device to make air free from CO_2, the principle used in his original apparatus could not be used. Since we know today more about the proper use of indicators than in early days, when physiologists used them for detection of CO_2 in metabolism experiments on nerves without any degree of success, and since we know the exact amounts of CO_2 produced in the nerve under various conditions, the indicator method was next tried. We immersed the nerve in Ringer's solution, saturated with phenolphthalein and containing enough alkali to give a slight pink color but not enough to affect appreciably nervous activity. Although this method was good enough to show that the resting nerve gives off an acid (CO_2), yet it would not show a decided difference in rates of decolorization in tubes containing resting and stimulated nerves.

This failure of detection of an increased carbon dioxide by the stimulated nerve meant one of two things. Either the $Ba(OH)_2$ method the author previously used was wrong and the stimulated nerve does not give any more CO_2 than the resting, or the increased CO_2 production is masked when an indicator method was used. Because of several hundred repetitions with the same results of his original method, demonstrated to many and confirmed by all of his students who learned the proper use of his apparatus, this apparent failure with the indicator

[1] Preliminary report of this work was given before the Chicago Meeting of the American Physiological Society, 1920, and its abstract appeared in this Journal, 1921, lv, 282.

method was not taken by him as conclusive evidence against his original contention but was considered to be due to some difficulty with the indicator method.

With an assumption, therefore, that his original method of estimating CO_2 is accurate and that CO_2 production does increase in the nerve during excitation, we proceeded to inquire what could be the interfering factors if the indicator method was used.

There are two obvious conditions which might mask the increase of acidity due to production of more CO_2—one is the simultaneous production of any base-forming compounds; the other is the presence of buffers in the tissue itself or its production in or diffusion out of the tissue.

During preliminary experiments, the author discovered that when two nerves of equal weight were placed in chamber A and B of his CO_2 apparatus and hemispheres of Nessler's solution were introduced instead of $Ba(OH)_2$, the Nessler drop in the chamber in which the stimulated nerve is placed gave a brownish precipitate much quicker than that of the resting nerve. This suggested the possibility of more NH_3 formation in the stimulated nerve, but it was not necessarily NH_3 since CO_2 is known to give a precipitate with Nessler. To scrutinize this difficulty, drops of NaOH solution were placed in both chambers for the purpose of eliminating any CO_2 which we knew to be produced. The same result could be obtained as before, showing that the precipitate was not due to CO_2, but it was impossible to use this method for estimating quantities. The first difficulty was in producing a perfectly clear Nessler drop which is the essential requirement for quantitative determinations in the biometer. The second difficulty was that the stopcock caused a great deal of trouble on account of its strong alkali. The substitution of a pinchcock for the stopcock caused a further difficulty in that it was impossible to place a stationary hemispherical drop of Nessler's reagent on the top of the tube.

In spite of these difficulties and the lack of quantitative data, however, the conclusion was warranted that there is at least one other volatile compound produced besides CO_2. Consequently the method of immersing an isolated nerve directly into a solution to estimate CO_2 by measuring the rate of change of the hydrogen ion concentration might cause a very serious error, unles we can ascertain that not only the other gas has no influence upon H^+ produced by CO_2, but also that there are no other compounds diffusing in or out of the tissue in the solution which effects the reaction of the medium.

In view of this experience and considering the easy availability of standard indicator tubes now on the market for determination of the H^+ concentration, the author expected some one to report similar results, namely, that the increased metabolism accompanying stimulation cannot be detected by a direct indicator method. It is not surprising, therefore, to see an article (2) by A. R. Moore of Rutgers College in which such a negative result is reported; and to him all the credit for this rediscovery of the negative result obtained by very early workers should be given. It is, however, surprising indeed to see him dismiss this problem by saying that "*The nerve impulse does not depend upon processes leading to the production of carbon dioxide.*"

The present communication is, however, not to show whether or not an indicator method should be used for estimation of CO_2 in the nerve fiber, nor to consider all factors which are sufficient to explain why contradictory results were obtained with the indicator and our original CO_2 methods (3). It is, first, to prove that the nerve fiber gives off a basic substance simultaneously with an increased production of carbon dioxide and that this substance is most probably ammonia; and second, to consider the relationship between irritability and ammonia formation in the nerve under various conditions.

EXPERIMENTAL: *Part 1. Preliminary consideration.* If the sciatic nerve of a frog is placed in Ringer's solution, made slightly alkaline and colored with any weak acid indicator like phenolphthalein, sooner or later the fluid gives an acid reaction. If, therefore, we assume that the failure to detect the increase of CO_2 during the stimulation is due entirely to ammonia alone, we should expect that the maximum amount of ammonia production cannot be greater than that necessary to neutralize 8.7×10^{-7} grams of CO_2 for 10 mgm. of the nerve during 10 minutes of respiration using phenolphthalein as an indicator, since for the same units, a resting nerve gives off 5.5×10^{-7} grams and an activated nerve 14.2×10^{-7} grams CO_2 at ordinary room temperture, provided of course our previous estimation of CO_2 is correct.

Theoretically, 34 grams of NH_3 should neutralize 44 grams of CO_2, and 8.7×10^{-7} grams $\times \frac{34}{44} = 6.7 \times 10^{-7}$ grams of NH_3 should be required to completely neutralize just the amount of increased CO_2 produced during excitation. However, since NH_4OH is more highly ionized than H_2CO_3, the probability is that far less an amount of NH_3 than 6.7×10^{-7} grams will be able to maintain a definite level of H^+ concentration in presence of 8.7×10^{-7} grams of CO_2, which is the

amount increased in 10 minutes by 10 mgm. of the stimulated sciatic nerve of a frog, under ordinary conditions.

How far this hydrolysis of $(NH_4)_2CO_3$ will affect the turning point of the indicator will depend not only on the concentration of $(NH_4)_2CO_3$, and kind of indicators and the amount of free CO_2 present, but also upon the presence of other salts as well as on temperature. Since available data on these points are almost inapplicable to the condition under which we are working, the exact relationship between NH_3 and CO_2 in respect to maintenance of certain H^+ concentration in dilution approximately that of our problem is under separate investigation, the results of which will be published elsewhere in conjunction with Mr. L. S. Friedman.

The point of the foregoing consideration is, however, to show that the amount of NH_3 produced under the conditions stated might be far less than 6.7×10^{-7} grams and that any method for detection of the gas must necessarily be exceedingly delicate.

Part 2. Qualitative experiments. In the following experiments we shall see whether or not the living nerve gives off something else besides CO_2, and if so, we shall attempt to identify the nature of this compound. In these cases, we used more than 100 mgm. of fresh sciatic nerves of the frog, and let them respire more than 15 minutes.

a. Does the resting nerve give off something else than CO_2 when immersed in Ringer's solution?

Experiment 1. Tube i: 3 cc. Ringer + nerves; tube ii: 3 cc. Ringer.

When tubes i and ii are Nesslerized after a definite time of respiration, tube i will show a more intense yellowish color than ii. This suggests a possibility of NH_3 formation but not necessarily, since it is known that CO_2 and other compounds by virtues of forming precipitates or similar colors, might produce different colorations.

The following experiments may eliminate CO_2 factor.

Experiment 2. Tube i: 3 cc. Ringer + nerve; ii: 3 cc. Ringer.

At the end of respiration, 1 cc. of N/800 H_2SO_4 is added to each tube and placed in boiling water for 5 minutes. The obvious reason of this treatment is of course to boil off CO_2 without losing NH_3 if present.

Since under this condition tube i is still more intensely colored after Nesslerization than the control, it is certain that the living nerve when immersed in Ringer's solution, gives off something besides CO_2.

b. Is it ammonia? The experiments cited above do not rule out the possibility that the slight amount of neutral ammonium salt might have diffused out of the nerve or that some compound other than NH_3 might be diffused or produced from it.

1. If it is ammonia, it should be volatile; we ought to be able to absorb it out of the air by acid.

Experiment 3. Tube i: 1 cc. of N/800 H_2SO_4 + nerve (hanging without touching the solution); tube ii: 1 cc. of N/800 H_2SO_4.

At the end of respiration, the tubes are shaken without moistening the nerve and the tissue removed, and both of the tubes are immersed in boiling water for 6 minutes. To the bottom of cooled solutions, 1 cc. of Graves' reagent[2] is carefully introduced. Graves' reagent gives a white precipitate with ammonia, Tube i gives a faint white cloud at the junction of the 2 fluids. Tube ii gives no precipitate. The ring test thus performed shows conclusively that the compound is volatile and gives an insoluble complex salt with $NaHgCl_3$ in alkaline solution, exactly in the same manner as do NH_4 salts.

2. If this is ammonia, it should not only be volatile, but also should form a base. The base-forming property of this compound can easliy be demonstrated by the following experiments.

Experiment 4. Tube i: 1 cc. Indicator[3] + unstimulated nerve; ii: 1 cc. Indicator.

The tubes are corked and allowed to stand.

At the end of respiration these tubes are shaken and the tissue is removed. The tubes are then immersed open in boiling water for 6 minutes. If at the end of the boiling there is not a detectable difference in the color, then to each tube add alternately drop by drop of distilled H_2O kept in *ordinary glass* to each tube. In the course of adding this exceedingly weak alkaline solution, it will be noted that tube *i* which has had the nerve will decolorize first, then *ii*. Since this indicator will lose its pink color when H^+ concentration reaches pH = 5 to 6, it is evident that as less alkali needs to be added to tube i this compound given off by the nerve has a base forming property.

c. Does the activated nerve give off this compound more than the resting? In similar experiments as described above but by substituting for the control the tube containing a stimulated nerve, it can be demonstrated that the nerve when stimulated gives off more of this compound than the resting.

d. Is this an amine or ammonia? 1. The fact that this compound is volatile and forms a yellow complex salt with Nessler's reagent and a white precipitate with $NaHgCl_3$ (Graves' reagent), strongly suggests that it is NH_3 gas, but does not absolutely rule out the possibility of

[2] See page 525.
[3] See page 528.

its being one of the volatile amines. There are theoretically many ways by which we may be able to differentiate amines from NH_3, yet we have not succeeded in applying them satisfactorily to such a small concentration as that with which we are dealing. If, however, the quantitative data obtained by two methods based on entirely different chemical properties of the substance, agree within experimental errors, then the identity of this compound can easily be ascertained.

Thus by estimating basicity produced by this compound, we may calculate it on the basis of NH_3, and compare the results with those obtained by Nessler or Graves' methods using standard ammonium salt. If such results do not agree with each other, we may calculate the basicity on the basis of all volatile amines, and compare the results obtained from the other method using standard solution of various alkyl ammonium salts.

Considering the difficulty and large sources of error obtained by turbidity experiments with exceedingly minute amount of this substance, the results obtained by these two methods are satisfactorily concordant to show that it is ammonia. (See page 523.)

On the basis of this evidence, we shall from now on call this compound ammonia until we shall have evidence to the contrary.

Part 3. Quantitative methods.[4] The two well-known properties of NH_3, used in ordinary methods, can be applied for measurement of exceedingly minute quantities. With slight modification and a great deal of care, one can detect an amount of NH_3 gas as small as 0.000,000,1 gram either by means of converting it to a complex salt with Hg. (Nessler or Graves'), or by measuring the amount of base formed by the gas. Since, however, the presence of CO_2 will interfere with all the methods based on these properties (the basicity in the greatest degree), it is absolutely necessary to have a device to eliminate CO_2. It is equally essential to avoid a direct contact between the tissue and liquid in which NH_3 is to be estimated, on account of possible diffusion of neutral NH_4 salts or other nitrogenous extractives that would react with direct Nesslerization or Graves' reagents, and on account of the possible presence of buffers either diffused out of the tissue or present in the tissue itself that would mask the true basicity attributed to ammonia alone. The amount of NH_3 gas actually given off by the nerve can only be estimated by suspending the tissue over a very small amount of dilute acid, the concentration of which should be kept con-

[4] For checking up the method as well as certain experiments, the author is greatly indebted to Miss Olive Pearl Lee and Dr. H. Sugata.

stant not only for all the nerves and controls, but also for the standard in each set of experiments.

A. Ring test with Graves' reagent. A new precipitant for ammonia recommended by Graves[5] is just as sensitive as that of Nessler. When used as a ring test, moreover, we found that as small as 1×10^{-7} grams N in form of NH_3 can be easily detected. This formation of a ring depends, however, upon two factors, i.e., concentration of the acid and of NH_4. For example in N/400 H_2SO_4, 2.5×10^{-7} grams N per cc. is barely detectable, while in N/800 H_2SO_4, 1×10^{-7} grams N will form a white ring within 3 to 4 minutes.

By varying acidity, therefore, over which the nerve is to respire, and by determining how much NH_4 is necessary to form a ring in the acidity in which a positive ring was formed, and over which nerve was suspended, we can estimate how much NH_3 is produced from the given nerve during a given period of respiration.

There are two main difficulties with this method. First, it is exceedingly difficult to determine the absolute point at which ring formation occurs, the speed of which depends not only on the concentration of NH_3, acidity, but also temperature. Second, it involves maintenance of a series of standard acids whose blank ammonium contents must be kept constant. If this varies, there is no way of correcting an error without performing a series of elaborate quantitative estimations with the different ammonium standards in different concentrations of the acids. Although this latter difficulty can be eliminated to a great degree by making up for each experiment a series of standards of the ammonia in the same acidities which are used for absorption of NH_3 from the nerve, yet the amount of acidity reduced by the NH_3 has not been reckoned with the standard.

In addition to this, any quantitative method based on such a formation of a barely visible ring is impossible to make free from a personal factor. But for a quick qualitative-quantitative test to determine which gives more NH_3, this ring test with Graves' reagent is very convenient and reliable.

B. Nephelometer method. This is essentially Graves' method. Since, however, the amount of NH_3 is so small a minor modification is necessary. In this, the use of starch is omitted on account of the

[5] Graves' reagent is made up as follows: To 80 grams of NaCl, are added 130 cc. H_2O and 100 cc. of cold sat. $HgCl_2$ solution with shaking. When the salt is practically all dissolved, 70 cc. of sat. Li_2CO_3 are added slowly while shaking, so that no mercury oxide forms on the side of the flask.

fact that the precipitate is so little as to maintain almost permanent turbidity without the use of any protective colloid. In order to keep the turbidity at the possible maximum, the dilution with NH_3-free H_2O is omitted. Ordinarily, for each cubic centimeter of N/800 H_2SO_4 over which the nerve is suspended, 10 cc. of Graves' reagent are added and the resulting turbidity is matched against the standard which contains 10 cc. of the reagent and 1 cc. of the standard $(NH_4)_2SO_4$ made up in N/800 H_2SO_4.

Although this method should be theoretically far superior to that of the ring test, the accurate matching of this exceedingly small turbidity is exceedingly difficult with the best nephelometer on the market even to the best and most experienced person. Any effort to increase the amount of turbidity by using a larger number of the isolated nerves is apt to introduce very serious physiological errors.

In addition to these, the method is so tedious on account of necessity of having perfectly clean and dry tubes ready, that it is impossible to run the quantitative estimations more than two dozen a day without having two or three dozens of nephelometer tubes that are uniform in all regards. Expense has precluded obtaining any such number. Unless for the purpose of identifying NH_3 by checking the result with the titration method, we have discarded the use of this method for a routine determination of NH_3.

C. Titration method: 1. Principle. By far the most satisfactory method, we found, is a titration method. The method itself has no claim of originality. The nerve is suspended over a definite quantity of an acid which contains a proper indicator. At the end of the respiration, CO_2 is driven off by immersing in boiling water 6 minutes, and the remaining acidity is titrated drop by drop with an alkali.

In spite of simplicity of the principle, however, there are many necessary details and precautions, without which the method is not dependable.

2. Apparatus. No special apparatus is necessary except that all, unless otherwise stated, should be made of Pyrex glass. Respiration and titration are performed in ordinary 6 inch Pyrex test tubes. One of the greatest sources of error and difficulty will be with these test tubes. For a successful experiment, it is convenient to have over 100 of the Pyrex test tubes and have them ready according to the following procedures:

These tubes are boiled in about 10 per cent H_2SO_4 for one hour and washed thoroughly with ordinary distilled water several times. Finally

these are again rinsed at least twice in ordinary distilled water, filling the tube to the top in each rinsing. These are then boiled in ordinary distilled water for one hour twice, and finally boiled in NH_3-silicate free[6] H_2O for one hour.

After being heated in a hot oven for 2 hours, the tubes are directly transferred in a con. H_2SO_4 desiccator. The tubes thus prepared should be tested for their cleanness in the following way.

When a solution of phenolphthalein made alkaline so that it is just perceptibly pink is added to the tube, it should not be decolorized in the cold. The faint pink color of methylene blue-methyl red (see p. 528) should stay permanent in the test tube without heating. When 1 cc. of the same indicator-acid solution is heated in this tube for 6 minutes in boiling water, the pink color should not be intensified. On the other hand, if this color changes to deep yellow the tube should be discarded.

All the tubes which stood a successful test were rinsed with redistilled H_2O and boiled again in redistilled H_2O once, and finally boiled in water free from ammonia and silicate for one hour. After being heated in a hot oven for 2 hours, they are directly transferred in a con. H_2SO_4 desiccator, ready for the experiment.

The tubes once used successfully for ordinary respiratory experiment should be subjected to similar cleaning as described in the last paragraph, i.e., they need not be boiled again in the strong acid.

In spite of all these precautions, one often finds a tube which is contaminated with alkali or acid. In the course of the experiment, one may also desire to add an excess of alkali or acid for various reasons to a tube. Such tubes should be set aside, separate from the regular tubes, and be subjected to more careful washings than the tubes which are used for routine quantitative experiments.

3. Solutions: a. Water free from NH_3 and silicate. Ordinary method of preparation of NH_3-free-H_2O is employed in preparing this water, care being taken, however, to use Pyrex glass in every part of the distillation apparatus where water or its vapor comes in contact with it. Thus it is recommended to use Pyrex distilling flask, a condenser whose inside tube is made of Pyrex, and receptor and adapter, all made of the same material. This NH_3-free-H_2O freed as much as possible from silicate is kept in a Pyrex bottle or flask tightly stoppered with paraffined cork.

[6] See last paragraph, this page.

b. Indicators: 1. Methylene blue solution, 0.5 gram of methylene blue, special,[7] is dissolved in 200 cc. NH_3-silicate-free H_2O. A 0.025 per cent solution is made from this stock solution by diluting 10 cc. to 100 cc. with NH_3-free H_2O.

2. Methyl red. Methyl red, special, recrystallized from alcohol, is saturated in 50 per cent of redistilled alcohol at room temperature.

3. To make methylene blue-methyl red (MB-MR) indicator. For 1 cc. of 0.025 per cent of methylene blue, add 10 cc. of methyl red solution.

4. Ordinary alcoholic solution of phenolphthalein.

c. Standard solutions: 1. N/20 H_2SO_4 solution, made up from a standard acid by diluting with NH_3-free H_2O, is kept in several small bottles well protected from the air.

2. Standard alkaline solution. The error of the experiment will depend a great deal upon the concentration of the standard alkali solution. If it is too low, the end point will not be sharp. If it is too high, the NH_3 can not be detected. Any alkaline solution which contains available alkalinity of N/10,000 or about, is satisfactory, that is when 0.1 cc. N/20 H_2SO_4 + 1.2 cc. MR-MB is made up by this solution to 100 cc., the pink color should disappear when immersed in boiling water for 6 minutes. If such alkali solution is completely ionized, H^+ should correspond to between pH 8–9, just the turning point of phenolphthalein, and of course way up on the alkali side of MB-MR. When, however, such a solution contains a trace of silicates, even if it is decidedly acid to phenolphthalein, the solution will often be too strongly alkaline for our purpose. Therefore as long as we are not sure of absence of silicate in the water, the ideal method of preparation of this solution on the basis of free H^+ concentration alone as determined by indicators will not be safe unless it is checked by a titration in a manner described below.

The method by which one can prepare the satisfactory concentration of alkaline water is as follows. Several liters of NH_3-silicate-free H_2O is poured into a large Pyrex flask and a few drops of phenolphthalein solution added. After addition of each drop of N/20 NaOH, the

[7] Presence of proper amount of methylene blue is very important for detecting the end point. Since we all know that no two brands of methylene blue on the market are the same, we have specified the amount on the basis of methylene blue, special, prepared by Coleman and Bell, Norwood, Ohio. If one prefers to use other brands, it will be necessary for him to determine proper concentration of the indicator to use.

bottle is tightly stoppered with paraffined cork and shaken until a faint but distinct pink color persists. Then enough N/20 H_2SO_4 is added to barely decolorize the pink color. The flask is filled with a Pyrex syphon; the inlet of air is protected with 15 per cent NaOH and concentrated H_2SO_4. The tip of the syphon is also provided with cork to which a Pyrex test tube can be inserted to protect against diffusion of the gases through the tip.

This solution should be tested out for its proper alkalinity in the following way.

To a Pyrex flask marked at 100 cc., 0.1 cc. of N/20 H_2SO_4 and 1.2 cc. of MB-MR are added using Pyrex pipettes. The volume is made up to 100 cc. mark with the alkaline solution just described. If the resulting solution is greenish yellow, it is apt to be too strongly alkaline. It should be colored faintly pink in cold, but should become a pale greenish yellow when a few cubic centimeter of this are placed in the Pyrex test tubes, previously tested, and immersed in boiling water for 6 minutes.

When 0.2 cc. of N/20 H_2SO_4 is taken and tested in the same way, the color should be faintly pink after immersion in the water for the same period of time. In other words the concentration of the alkali should be somewhere around N/10,000.

3. Standardization of the standard alkali. This approximately right alkali solution is standardized as follows.

To each of four Pyrex flasks marked at 100 cc. various fractions of cubic centimeter from 0.5–0.2 cc. of N/20 H_2SO_4 accurately measured with Pyrex pipette and 1.2 cc. of MB-MR, measured also with Pyrex pipette, are added, and made up to the volume with the alkali solution. One cubic centimeter of each is measured off by Pyrex pipette from each flask into a clean Pyrex test tube, previously tested, and immersed in boiling H_2O. At the end of 6 minutes, each tube is titrated drop by drop with the same alkaline solution. The typical result of titration of a particular standard solution we made up as shown in table 1.

The average of these determinations, then, shows that 7.2 drops of alkali solution were necessary to neutralize 1 cc. of N/20,000 H_2SO_4, since each flask contains increment increase of 0.1 cc. of N/20 per 100 cc. and since we took for titration 1 cc. only. On the basis of this titration, each drop of the alkali is equivalent to 1/7.2 cc. of N/20,000 alkalinity, which in terms of NH_3 corresponds to 0.000,000,12 gram.

It should be noted that in the above calculation, we have not ignored the effect of the indicator itself, but have eliminated it on account of the

fact that each cubic centimeter of the acid we took contained exactly the same amount of indicator, and by subtracting number of drops from the one above, the acidity due to indicator itself is canceled.

TABLE 1

TUBE	CUBIC CENTIMETERS OF N/20 N_2SO_4	CUBIC CENTIMETERS OF MB-MR	MADE UP WITH THE ALKALINE SOLUTION	NUMBER OF DROPS REQUIRED TO NEUTRALIZE 1 CC. OF THE SOLUTION	DIFFERENCE FOR EACH TUBE
					drops
A	0.5	1.2	100	22	7.0
B	0.4	1.2	100	15	7.5
C	0.3	1.2	100	7	7.0
D	0.2	1.2	100	0	
Average drops.....					7.2

From the same data, however, we may calculate the acidity contributed by the indicator, as shown in the following table.

TABLE 2

TUBE	CUBIC CENTIMETERS OF N/20 H_2SO_4	CUBIC CENTIMETERS OF MB-MR USED	MADE UP WITH THE ALKALINE SOLUTION	NUMBER OF DROPS OF THE ALKALINE SOLUTION NECESSARY TO NEUTRALIZE 1 CC. OF THE SOLUTION	NUMBER OF DROPS CONTAINED IN 1 CC. PIPETTE USED TO MEASURE THE ACID-MB-MR	SUM OF DROPS	NUMBER OF DROPS REQUIRED TO NEUTRALIZE 1 CC. N/20000
A	0.5	1.2	100	22	18	40	40/5 = 8.0
B	0.4	1.2	100	15	18	32	32/4 = 8.0
C	0.3	1.2	100	7	18	35	25/3 = 8.3
D	0.2	1.2	100	0	18	18	18/2 = 9.0

Average for first three = 8.1 drop. Average for all, including the last which was already decolorized before titration = 8.3. Average of these two = 8.2; 8.2 − 7.2 = 1 drop = the amount of alkali required to neutralize 1.2/100 cc. of MB-MR indicator, the amount of indicator contained in 1 cc. of MB-MR-acid.

If we can ignore the acidity contributed by the indicator, the standardization of the alkali solution can be done by a simple method. For instance, take 0.5 cc. of N/20 H_2SO_4 and 1.2 cc. of MB-MR, and dilute it to 100 cc. with solution to be standardized. Titrate 1 cc. of this solution according to the method described above. Add the number of drops contained in 1 cc. of the pipette used for titration, to the

number of drops of the alkali required to neutralize the acid-MB-MR. The sum of the drops divided by 5 will be equivalent to 1 cc. of N/20,000. The general formula for this calculation is as follows:

$$\text{One drop} = \frac{1}{\frac{(a+b)}{c}} \times 0.000{,}000{,}85 \text{ gram } NH_3$$

where a = number of drops required to neutralize 1 cc. of the solution.

b = number of drops contained in each cubic centimeter of the pipette used for titration.

c = number of cubic centimeters of 1/200 cc. H_2SO_4 (number of tenths of cubic centimeter of N/20 H_2SO_4) used to make the solution and 0.000,000,85 gram is amount of NH_3 contained in 1 cc. of N/20,000 NH_4OH solution.

If one uses always the same fresh indicator, and is sure of the amount of acid it contributes, then the following formula can be used, since 1.2/100 cc. of MB-MR does neutralize 0.000,000,02 gram of NH_3.

$$\text{One drop} = \frac{1}{\frac{(a+b)}{c}} \times 0.000{,}000{,}85 \text{ gram } NH_3 + 0.000{,}000{,}02 \text{ gram.}$$

Under strictly ideal experimental conditions, there are 3 factors which will determine the error of determination: a, correct standardization of the alkali; b, any factors which influence size of the drop, and c, end point. The first two factors concern the accurate estimation of the amount of alkali taken. If one wishes, therefore, to be exceedingly accurate, instead of measuring it by drops, use of a very small Pyrex pipette, accurately calibrated to hundredths of a cubic centimeter will narrow the limit of error. The effect of temperature on the number of the drop should, of course, be always remembered.

Although the end point is sharp within $\frac{1}{2}$ drop of the alkali we ordinarily use, the error will be enormously great if the number of total drops of the alkali used is very small. It is highly desirable for one to decide what point is the end and use that criterion for all the experiments, both standardizations of the alkali, and titration of the remaining acid after the respiration. The point of change from pink to faint greenish yellow is the most sharp. Many unnecessary errors will be eliminated if one uses a control tube in which the same amount of the indicator is placed and which has the greenish yellow color to be compared.

During titration, care should be taken not to let the alkali drop touch the side of the tube, but to let it drop directly into the solution.

4. Preparation of MB-MR-acid solution. If we know the exact concentration of the alkali, theoretically any strength of the acid should be satisfactory, provided we run a control tube containing exactly the same amount of the acid. Like the Kjeldahl titration, however, it is best to have such an acidity that each cubic centimeter of the control should not require more than 1 cc. of the standard alkali solution. That is, if one used the alkali solution to dilute the N/20 H_2SO_4 to 100 cc. with MR-MB, the original acidity should be slightly more than twice as strong as the standard alkali. If too strong acid is used, the indicator is so diluted during titration, that the end point may be a little obscure. If too weak acid is used, the acid may be already neutralized by the ammonia before titration. Thus according to the type of the tissue, length of the respiratory period and weight of the nerve, the acidity may have to be altered after a preliminary experiment. For ordinary 15 minutes' respiration with 2 sciatic nerves, a MB-MR-acid solution prepared in the following ratio is found to be satisfactory: 0.4 cc. N/20 H_2SO_4 + 1.2 cc. MB-MR are made up to 100 cc. with the standardized alkali.

D. Detailed method of estimation of the NH_3 given off by the nerve. A typical experiment to estimate simultaneously the amount of NH_3 given off by resting and stimulated nerve is as follows: Four clean test tubes are placed in the rack, each tube being provided with a paraffined cork, having 2 electrode attachments. Prepare the indicator-acid solution, by taking 0.4 cc. N/20 H_2SO_4 + 1.2 cc. MR-MB and making it up to 100 cc. with the standard alkali solution, using a flask and both pipettes made of Pyrex. By means of another Pyrex pipette, 1 cc. of each of the indicator-acid solution is placed in the above tubes, and tightly stoppered with the cork. Two pairs of the sciatic nerves are quickly isolated from two frogs, and immersed in each of two dishes containing Ringer's solution. One out of each pair is taken together, blotted and weighed. The remaining two are also likewise weighed. After the adhering liquid is carefully removed by means of filter paper, each set of nerves is placed in tubes *S* and *R*, respectively, the corks are replaced and stimulation by a weak induction shock is applied to the nerves in tube *S*, recording the time at which the respiration begins. The stimulus should be so weak as to be barely perceptible to the tongue. Tubes c_1 and c_2 are controls. All the tubes are shaken gently once or twice during experiment, care being taken not to let the liquid touch the nerves. The best way is to shake the rack gently without touching each tube with finger, in order to avoid temporary rise of temperature of the tube.

At the end of 15 minutes' respiration, each tube is shaken before the tissue is removed. After the stopper is removed, the upper third of the tube is wiped with filter paper so as to remove any liquid of the tissue which might have been left on the side of the tube.

The four tubes are placed in a basket which is hung in boiling H_2O, and which is placed at least one inch above the bottom of the beaker in which the water is boiling. At the end of 6 minutes, the tubes are removed and titrated while still hot with the standard alkali. The standard alkali can be introduced either from Pyrex buret, syphon or pipette, provided we have standardized value of each drop of our alkali with the same apparatus.

The different amounts of drops of alkali required to neutralize control tubes and tubes containing the nerves when multiplied by number of grams of NH_3 equivalent to one drop of the alkali will indicate the amount of NH_3 given off by the nerves during that period of respiration.

The record given in table 3 will give an idea of the details of the method used in above experiments.[8]

TABLE 3

Exper. 8. 12/24/1920. Two frogs, Rana pipiens (♂, 22 cm.; ♂, 20 cm.) of lot VI (arrived at the laboratory on 11/12/1920) decerebrated simultaneously at 10:57 a.m. Respiration at 24°C.

TISSUE			NUMBER OF TUBES*	STIMULATION	RESPIRATION (15 MINUTES)		DROPS OF ALKALI NECESSARY TO NEUTRALIZE THE REMAINING ACID	AMOUNT OF ACID NEUTRALIZED BY NH_3	TOTAL NH_3 GIVEN OFF	NH_3 GIVEN OFF BY 10 MGM. OF THE TISSUE DURING 10 MINUTES
KIND	Isolated at	Weight of			From	To				
	a. m.	*mgm.*			*a. m.*	*a. m.*		*drops*†	*gram*	*gram*
2 sciatics	10:58	80	S	Yes	11:09	11:24	7	7	0.98×10^{-6}	0.81×10^{-7}
2 sciatics	10:59	80	R	No	11:09	11:24	11	3	0.42×10^{-6}	0.35×10^{-7}
		0	C_1		11:10	11:25	14			
		0	C_2		11:10	11:25	14			

* Each tube contains 1 cc. of MB-MR-acid solution.

† Each drop of the alkali is equivalent to 0.14×10^{-6} gram NH_3.

[8] Immediately after smoking, the breath contains appreciable amounts of base-forming substances. It is always safer, therefore, for a smoker to wash his mouth thoroughly before he uses pipettes.

RESULTS: *1. Resting nerve.* The results obtained by the last method show that the resting nerve gives off exceedingly small, but quite definite amounts of ammonia. The results given in table 4 were taken from experiments conducted with two bundles of sciatic nerves (70–100 mgm.) taken from frogs ranging in size of 19 to 24 cm. in length, under ordinary range of temperature variation (20–24°C.). The gas was collected exactly during 15 minutes of respiration starting it at about 10 minutes after the animals were decerebrated. The other nerves of the same frogs were stimulated and the NH_3 collected simultaneously with that of resting nerves.

In the following table, NH_3 production from stimulated and non-stimulated nerves are given to show the range of variation. If one compares two nerves of the same animal, we have no difficulty in showing increased NH_3 production during stimulation, but the actual amount of the gas given off by nerves of different animals varies considerably. Although these variations might be due to an error of the method which is necessarily great, yet we have evidence to show that there are other experimental and physiological conditions which have a great influence upon NH_3 production. An investigation is now under way in which these influences are more carefully studied. Until we shall know more about these factors, it is of prime importance to determine the amount of NH_3 given off by the unstimulated nerves of the same frog and compare it with the NH_3 produced by the other half under various conditions whose influence one wishes to investigate.

On the basis of 10 mgm. of the nerve for 10 minutes of respiration the unstimulated sciatic nerves of frogs (*Rana pipiens*) give 0.32×10^{-7} grams NH_3 on the average.

It is interesting to note that this amount of NH_3 corresponds to approximately $\frac{1}{17}$ of the weight of CO_2 given off under approximately the same conditions, and therefore for each mol. of CO_2, 1/6.5 of a mol. of NH_3 is given off, i.e., $\frac{1}{13}$ of equivalent weight of CO_2.

2. Stimulated nerve. In these experiments we used the ordinary method of electrical stimulation by weak induced current only, similar care being taken as described in our earlier work on CO_2 production. The remaining 2 of the nerves of the frogs, used in the experiment with the resting nerve were taken and stimulated side by side with unstimulated nerve, thus maintaining physiological and other experimental conditions as constant as possible.

The average amount of NH_3 by stimulated nerves is 0.68×10^{-7} grams as expressed on the basis of 10 mgm. tissue and 10 minutes'

respiration. Thus average amount of NH_3 given off during stimulation is approximately twice that of the resting nerve. This corresponds closely to the average increase of CO_2 in similar nerves during stimulation which was found to be 2.4 times that of the resting nerve.

TABLE 4

NUMBER OF EXPERIMENT	TEMPERATURE	WEIGHT OF NERVE	TIME ELAPSED FROM DECEREBRATION OF FROGS TO BEGINNING OF RESPIRATION	DURATION OF RESPIRATION	STIMULATION	TOTAL NH_3 GIVEN OFF	AMOUNT OF NH_3 GIVEN OFF, CALCULATED ON BASIS OF 10 MGM. OF NERVE AND 10 MINUTES OF RESPIRATION
	degrees C.	*mgm.*	*minutes*	*minutes*		*grams*	*grams*
2	23.0	105	9	15	−	4.0×10^{-7}	0.254×10^{-7}
		102	9	15	+	7.0×10^{-7}	0.457×10^{-7}
3	23.5	80	9	15	−	3.6×10^{-7}	0.30×10^{-7}
		80	8	15	+	10.8×10^{-7}	0.90×10^{-7}
4	20.0	90	11	15	−	4.8×10^{-7}	0.355×10^{-7}
		85	11	15	+	9.0×10^{-7}	0.705×10^{-7}
5	20.2	68	13	15	−	2.4×10^{-7}	0.235×10^{-7}
5a	20.0	95	13	19	−	5.6×10^{-7}	0.31×10^{-7}
		88	13	19	+	14.0×10^{-7}	0.83×10^{-7}
6	20.2	78	11	15	−	4.9×10^{-7}	0.419×10^{-7}
		72	11	15	+	7.0×10^{-7}	0.642×10^{-7}
8	24.0	80	11	15	−	4.2×10^{-7}	0.35×10^{-7}
		80	11	15	+	9.8×10^{-7}	0.81×10^{-7}
9b	23.0	80	13	15	+	4.8×10^{-7}	0.4×10^{-7}
Average for non-stimulated nerve							0.32×10^{-7}
Average for stimulated nerve							0.68×10^{-7}

3. NH_3 production following stimulation of the nerve. In these experiments, a series of test tubes containing exactly the same amount of MB-MR-acid solution was prepared in a group of 4. Two nerves were placed in the first tube and the other two in the next, the remaining two of the group acting as controls. The nerves in the first tube were stimulated for 15 minutes. At the end of 15 minutes, after usual procedure, both stimulated and unstimulated nerves were transferred into

corresponding tubes of the second group, no more stimulation being applied to the stimulated nerve during subsequent respiration.

The results of titration of each of these later tubes show that during the first 15 minutes following stimulation, the nerve gives off always more than the unstimulated nerve. Under ordinary condition, the amount of NH_3 produced by the unstimulated and the stimulated nerve during post-stimulation becomes equal at the end of 45 to 60 minutes. We have not yet succeeded in establishing the exact condition under which the two nerves give exactly the same amount of NH_3 after a definite time. One thing is certain, however, that an increase in NH_3 production due to stimulation keeps on for some time after the external stimulation is stopped.

Whether this increased NH_3 production during post-stimulation is due to an increase of NH_3 formation due to hyper-irritable condition of the nerve during post-stimulation, diffusion of preformed NH_3 produced during previous stimulation, or due to release of NH_3 by virtue of gradual oxidation of lactic acid, which if formed should tend to hold a part of NH_3 formed during stimulation, can not be decided without further experiments.

4. Effect of injury. We have shown before that the nerve, when mechanically injured, gives off more CO_2 than the uninjured, and attempted to explain the fact by considering the traumatic injury to be analogous to an extreme form of stimulation. Since a stimulated nerve gives off more NH_3 in a similar manner to CO_2, we expected to produce more NH_3 in the nerve. The results were, however, diametrically opposite. The crushed nerve not only does not give off more NH_3 than the resting, but gives far less, the amount varying from $\frac{1}{2}$ of that of the resting to none at all.

The mere fact that the nerve gives off less NH_3 under these conditions than uninjured and unstimulated nerve, does not necessarily, of course, rule out the possibility of an increase NH_3 production during trauma. On the contrary, in spite of these facts, there are reasons for believing that an actual formation of NH_3 is increased under all forms of stimulation.

Whether or not the nerves produce lactic acid under certain conditions has not been experimentally settled. If the nerve behaves similarly to muscle, then during trauma even if NH_3 might have been formed, we shall not be able to detect it by our present method unless such acid is removed by either being converted to a neutral substance or oxidized away. In case of muscle, however, Fletcher and Hopkins

(5) have shown definitely that the lactic acid formed during trauma can not be removed in the manner that happens to lactic acid produced during functional activity.

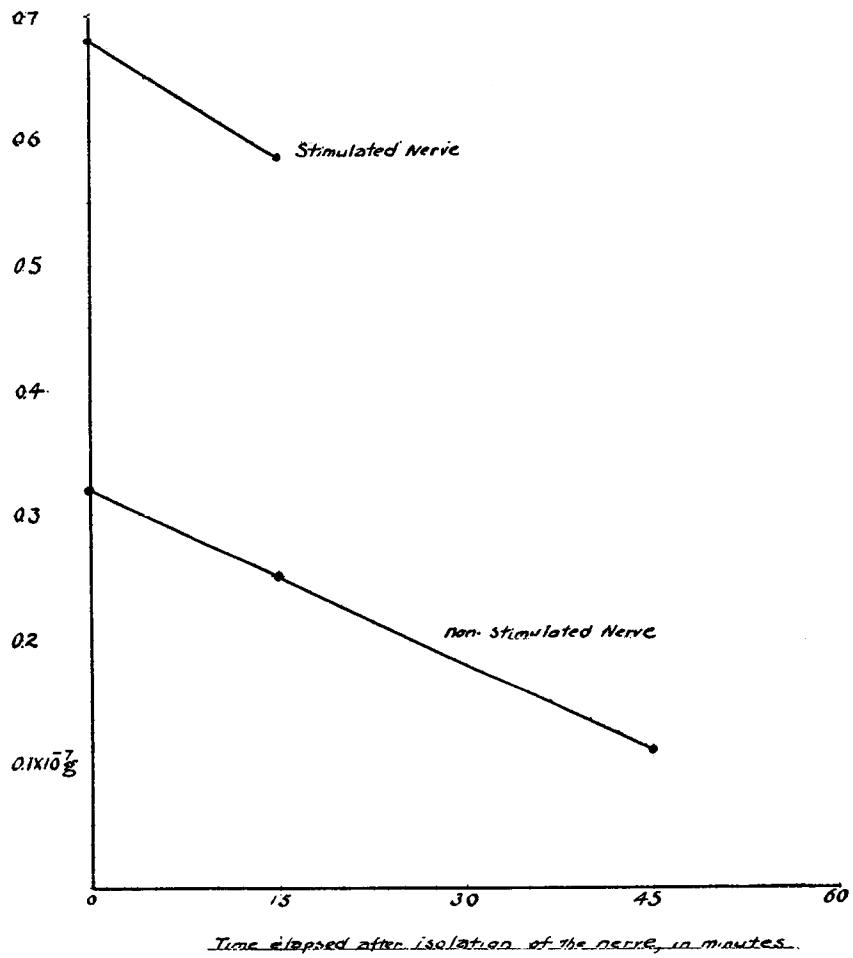
In the case of ammonia formation in muscle we can demonstrate these two different modes of behavior of lactic acid. As will be shown in another article, both injured muscle and muscle fatigued by successive stimulation give off practically no NH_3, but during recovery process, fatigued muscle will gradually give up NH_3, but not the injured muscle even if we let them "respire" more than 2 hours.

In the nerve, during a long period of respiration, the injured nerves seem to give up a part of NH_3. Whether this indicates formation of lactic acid in injured nerve, which holds NH_3, but which gives off a part of it later by partial oxidation of the acid unlike that of injured muscle or it means that traumatic injury was not complete and does not completely eliminate formation of NH_3 in the nerve, or that a certain amount of NH_3 keeps on coming independently of physiological process is a question to be determined more in detail.

5. Effect of standing on nerve NH_3. A knowledge of NH_3 production in the nerve during successive intervals after isolation from the animal is necessary in order to know the source of the gas. Gad-Anderson (6) has shown that muscle urea is spontaneously decomposed into NH_3 during post-mortem change, and certain bacterial or other decomposition is known to set in at a surprisingly early state of post-mortem period, such as histamine formation. Either one of these processes might give rise to NH_3 gas formation in a comparatively fresh tissue.

In general, isolation of a tissue from a body produces two opposing phenomena. From this point on, the survival physiological process gradually descends to zero, but post-mortem change gradually ascends to a certain climax. Whether the NH_3 we measured here is formed by survival physiological process or due to post-mortem phenomena unrelated to process in normal body, will be decided by the nature of the curve of NH_3 production during successive periods after the nerve is isolated from the body.

The gradual decrease of NH_3 production shown in the following chart, shows that NH_3 production in the nerve must be due to physiological process. We have not yet extended our observation on a large interval to see just when the physiological process reaches a minimum and to compare this point to the death point of the nerve determined by irritability and CO_2 method.



Curve 1

SUMMARY OF QUANTITATIVE RESULTS. The summary of quantitative estimation of NH_3 produced by the nerve under various conditions is given in table 5.

6. Quantitative results with nephelometer. In order to check the results obtained by the method based on base-forming property of gas, a nephelometric determination was made. N/800 H_2SO_4 is used to

TABLE 5

Summary of NH_3 production from the sciatic nerves of frog, Rana pipiens, under different conditions

CONDITIONS	RESPIRATION PERIOD	AVERAGE AMOUNT OF NH_3 GIVEN OFF, CALCULATED ON BASIS OF 10 MGM. OF THE NERVE AND 10 MINUTES RESPIRATION	TEMPERATURE
		grams	*degrees C.*
Resting nerve.....	15 minutes immediately after*	0.32×10^{-7}	20–24
	Next 15 minutes	0.25×10^{-7}	20–24
Stimulated nerve ..	15 minutes immediately after	0.68×10^{-7}	20–24
	Next 15 minutes	0.59×10^{-7}	20–24
Crushed nerve.....	15 minutes immediately after	0 to 0.15×10^{-7}	20–24

* "immediately" is used for those cases when the respiration was started within 7 to 10 minutes after decerebration of the animal.

TABLE 6

NUMBER OF EXPERIMENT	TEMPERATURE	WEIGHT OF NERVE	TIME ELAPSED FROM DECEREBATION OF FROGS TO BEGINNING OF RESPIRATION	DURATION OF RESPIRATION	STIMULATION	TOTAL NH_3 GIVEN OFF	AMOUNT OF NH_3 GIVEN OFF CALCULATED ON BASIS OF 10 MGM. OF NERVE AND 10 MINUTES OF RESPIRATION
	degrees C.	*mgm.*	*minutes*	*minutes*		*grams*	*grams*
AN 1	25.0	101	11	20	+	5.3×10^{-7}	0.26×10^{-7}
		103	11	20	−	3.8×10^{-7}	0.18×10^{-7}
AN 2	23.0	123	11	15	+	4.6×10^{-7}	0.25×10^{-7}
		141	11	15	−	3.8×10^{-7}	0.18×10^{-7}
AN 3	19.0	86	9	15	+	4.6×10^{-7}	0.35×10^{-7}
		87	9	15	−	2.8×10^{-7}	0.21×10^{-7}
AN 11	23.0	84	16	20	+	3.3×10^{-7}	0.20×10^{-7}
		87	16	20	−	2.6×10^{-7}	0.14×10^{-7}
AN 12	22.5	101	12	20	+	4.4×10^{-7}	0.22×10^{-7}
		90	12	20	−	2.6×10^{-7}	0.14×10^{-7}
AN 14*	24.0	103	12	20	+	6.4×10^{-7}	0.31×10^{-7}
		103	12	20	−	4.4×10^{-7}	0.21×10^{-7}
Average for non-stimulated nerve							0.17×10^{-7}
Average for stimulated nerve							0.27×10^{-7}

* Determined by Nessler.

absorb the gas, and treated with Graves' reagents as described on page 525. Both the Kober and our own modification of Dubosque colorimeter were used to determine resulting turbidity. On account of difficulty of the method, large numbers of readings were necessary for each experiment and our data are not extensive. The results obtained by this method are given in table 6.

In the sense of ordinary quantitative analysis, these two results shown in tables 4 and 6 cannot be said to be in a close agreement. Unfortunately like most so-called super-michrochemical methods, our method is subject to a much larger per cent of error than that which ordinary quantitative accuracy permits. It is highly probable that in our method there may be more than one error which is common to all our determinations. The absolute amount of NH_3 recorded here will no doubt be revised by some who will devise a more accurate method. In spite of this, however, we are quite certain that the relative amount of NH_3 gas produced by the nerve under various conditions will stand regardless of any method, within physiological variation.

Considering, therefore, the amount of the gas we are dealing with, these data obtained by methods based on entirely different chemical properties are close enough to show that the gas is ammonia.

CONCLUSION

However curious it may seem, the facts are that the nerve undergoes chemical reactions in which both acid-forming and base-forming substances are produced, and that the increase of CO_2 production during stimulation is accompanied with an increase of NH_3 production. Although the physiological and biochemical significance of these facts has not yet been investigated, they raise many interesting questions.

Where does NH_3 come from? We have endeavored to show that it is neither produced by bacterial decomposition nor from urea. Considering the minuteness of its amount, one might naturally suppose that inasmuch as the blood always contains a little NH_3, the nerve might receive it from the blood and retain it in an amount which will be in direct equilibrium with the blood, or lymph, and that this NH_3 will gradually diffuse out to a medium from which it is constantly removed. That this plausible explanation will not hold will be shown in later papers where we shall present evidence to show that NH_3 production from the different tissues is not the same, but varies within a large range, and that these variations are not proportional to anatomical variation, but due to some other physiological and biochemical factor.

Thus the process of elimination leads us to the speculation that this NH_3 must come from protein directly.

What becomes of it? According to the estimate made by Professor Donaldson,[9] an average adult human being weighing 150 pounds has 1,620 grams of total nervous tissues. If these tissues give off NH_3 in the same ratio as that of the sciatic nerve of the frog, we see that the daily output of NH_3 will amount to approximately 0.7 gram with corresponding variation during stimulation. Since the daily output of a normal man usually does not go beyond 1 gram of NH_3 and some other tissues also give off the gas in different degrees, and since by external factor alone one can almost completely abolish NH_3 output, even this entirely unqualified calculation tells us that the nerve NH_3 must be converted either entirely or partly into something else. It is not extraordinary speculation to consider that it is urea into which this ammonia is converted. Since no constituent of the urine is more variable than the urea, if NH_3 does go into urea, a variation of NH_3 production due to the changes in the nervous activity might easily be lost sight of.

How far this NH_3 influences acid-base balance in the body, and at what point it is converted to urea or something else can not even be speculated upon without further experimentation such as determination of the NH_3 production from the other tissues as well as an accurate determination of the NH_3 in the blood—a procedure which is one of the most difficult and unreliable of biochemical analyses.

What relationship has NH_3 with the irritability of the nerve? Does the fact that stimulation increases NH_3 production, suggest a decrease of NH_3 production during anesthesia? Muscle gives off far less am-

[9] Personal communication. For a man weighing 150 pounds, and who is 68 inches tall, his estimate of the total nervous system is as follows.

	grams
Brain	1400
Spinal cord	27
Sympathetic	30
Cranial nerves	12
Spinal nerves	151
Total weight	1620

Except weight of sympathetic which he considers more or less as a guess, these calculations are based on accurate anatomical data (8), (9), (10). For this information and many other suggestions, the author is greatly indebted to Prof. H. H. Donaldson.

monia than the nerve. Doctor Mathews suggested that this fact might be responsible for the extreme sensibility of muscle and relative immunity of the nerves to ammonia. Is there any relationship between NH_3 production and fatigue in view of the fact that the ratio between NH_3/CO_2 in activated nerve is far greater than that of the contracting muscle? What will be the NH_3 production from the nerve during prolonged refractory periods which are supposed to be more susceptible to a continued activity?

The accumulation of insoluble calcium salts at one point must be intimately concerned with a metabolism which forms bases. Drew (7) has shown that denitrifying bacteria can precipitate out $CaCO_3$ from sea water. In the physiological process of calcification and the pathological cases of softening of bone as well as pathological process of calcification, ammonia production in the tissues must be a dominant factor. This supports the idea that conditions like osteomalacia may be intimately related to disturbances in protein metabolism. Experiments to determine NH_3 production in the bone under different conditions will test these hypotheses.

In a series of papers on studies of alkaligenesis in the tissue, we hope to be able to answer some of these questions. Meanwhile we should emphasize the fact, as shown elsewhere (3), that any method which attempts to measure the small increase of CO_2 in the activated nerve should not ignore this exceedingly small but definite amount of NH_3 which is simultaneously formed in the nerve fiber.

SUMMARY

1. Evidence is given to show that resting nerves give off exceedingly minute quantities of a volatile base-forming substance which during stimulation is greatly increased, and that this substance is probably ammonia.

2. Methods are described by which we can measure NH_3 as small as 0.000,000,1 gram.

3. Quantitative data are given to show NH_3 production by the nerve under various conditions.

4. Various questions were raised concerning the rôle of NH_3 in the general physiological problem.

BIBLIOGRAPHY

(1) TASHIRO: This Journal, 1913, xxxii, 107.
(2) MOORE: Journ. Gen. Physiol., 1919, i, 613.
(3) TASHIRO AND HENDRICKS: Proc. Amer. Soc. Biochem., 1921, v, 15.
(4) GRAVES: Journ. Amer. Chem. Soc., 1915, xxxvii, 1171.
(5) FLETCHER AND HOPKINS: Journ. Physiol., 1906–7, xxxv, 261, 288.
(6) GAD-ANDERSON: Journ. Biol. Chem., 1919, xxxix, 267.
(7) DREW: Carnegie Inst. Washington Publ. no. 182, 1914, 21.
(8) DONALDSON AND BOLTON: Amer. Journ. Psychol., 1891, iv, 224.
(9) DONALDSON AND DAVIS: Journ. Comp. Neurol. and Psychol., 1903, xiii, 19.
(10) INGBERT: Journ. Comp. Neurol. and Psychol., 1904, xiv, 209.