THE MAGNITUDE OF THE ERROR DUE TO AMMONIA AND ITS SALTS IN THE VAN SLYKE AMINO NITROGEN PROCEDURE AS COMMONLY APPLIED IN STUDIES OF BACTERIAL METABOLISM

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Although Van Slyke definitely states that the presence of ammonia may cause a serious error in the determination of amino nitrogen by his method, many bacteriologists have apparently failed to appreciate the importance of this point. He warns that,

> For the accurate determination of amino nitrogen in digesting solutions, etc., it is advisable first to remove ammonia although good comparative results can be obtained in the presence of the relatively small proportion of ammonia usually present, if the reaction conditions of time, concentration, and temperature of solutions are kept constant so that the proportion of the ammonia decomposed is the same in each determination.

He even presents some data (table 1) showing the amounts of nitrogen liberated from an ammonium sulfate solution when allowed to react for various lengths of time.

A brief survey of recent studies of nitrogen metabolism of bacteria (Wagner Dozier and Meyer, Avery and Cullen) failed to show that these investigators had made any allowance for ammonia interference in their Van Slyke amino nitrogen determinations. Others (De Bord and Lamson) have criticized and discarded the method without considering the importance of the ammonia interference.

Kendall recognized the indefiniteness of the ammonia correction and therefore gave preference to the Sörensen method

for amino nitrogen. Reddish and Rettger apparently recognized the ammonia error since they removed the ammonia before analyzing for amino nitrogen by the Van Slyke method.

In view of the conflicting experiences of various investigators, it seemed worth while to undertake a brief physico-chemical investigation of the nature of the reaction of ammonium salts in the Van Slyke procedure, and to determine the magnitude of the error under various conditions.

TABLE 1

Used 10 cc. portions of ammonium sulfate solutions containing 28.02 mgm. of nitrogen. Temperature 24. Pressure 752 mm.

TIME OF REACTION	NITROGEN	WEIGHT OF NITROGEN	PER CENT OF TOTAL AMMONIA NITROGEN
minutes	*cc.*	*mgm.*	
3	12.1	6.86	24.5
5	18.4	10.16	36.3
10	31.5	17.38	62.1

EXPERIMENTAL

The experiments detailed in this paper were carried out with the micro-apparatus. Reagents of a satisfactory degree of purity were used. The sodium nitrite solution used was made up to the recommended concentration of 300 grams per liter, unless otherwise specified. The ammonium salt solutions used were made from the purest materials available and the concentrations were verified by subsequent analysis for ammonia.

The quantities of acetic acid and sodium nitrite solutions were carefully measured in the apparatus and the volume in the reaction chamber was carefully adjusted to the 4 cc. graduation before admitting the sample.

The rapidity of shaking was adjusted to 250 to 300 r.p.m., this being deemed sufficient to remove all liberated nitrogen. The temperature of the room was carefully controlled and the reagents were kept adjusted to this temperature. The latter precaution is of the utmost importance as will be shown subsequently. The writers are satisfied that the temperatures recorded represent those of the reaction mixtures to $\pm 0.3°C$.

The details of the Van Slyke procedure are too well known to require further comment. The various periods of the reaction were timed with the greatest possible precision and the liberated

TABLE 2

The amount of reaction is independent of the ammonium salt used

Temperature 25°C. Pressure 735 mm. Shaking three minutes. Sample = 1 cc.

AMMONIUM SALT USED	CONCENTRATION (NITROGEN PER CUBIC CENTIMETER	NITROGEN EVOLVED	PER CENT TOTAL NITROGEN EVOLVED
	mgm.	*mgm.*	
Acetate	5.0	1.630	32.6
Butyrate	5.0	1.680	33.6
Chloride	5.0	1.679	33.6
Acetate	2.5	0.825	33.0
Butyrate	2.5	0.816	32.6
*Gelatin + butyrate	2.5	0.865	34.6

* Five per cent Nutrient gelatin (Difco) + ammonium butyrate (2.5 mgm. nitrogen per cubic centimeter) gave the following:

Average corr. cc. nitrogen from 1 cc. above..... 2.24
Average corr. cc. nitrogen from 1 cc. gelatin control..... 0.63
From butyrate..... 1.61 cc. = 0.865 mgm.

TABLE 3

Data to determine the order of the ammonia reaction

Temperature 25.5°C. Pressure 745 mm. Sample = 1 cc. Concentration = 5 mgm. nitrogen per 1 cc.

TIME	NITROGEN EVOLVED		PER CENT OF TOTAL NITROGEN EVOLVED
minutes	*corr. cc.*	*mgm.*	
2	2.05	1.02	20.4
3	3.10	1.52	30.4
4	4.10	2.13	42.6
5	4.70	2.46	49.2
8	5.90	3.13	62.6
10	6.30	3.36	67.2
15	7.80	4.15	80.3

gases were removed with the utmost celerity after the desired reaction time had elapsed. These details are necessary to obtain concordant results with ammonium salts, although such

precautions are obviously unnecessary with amino acids where the reaction proceeds to completion before three minutes (the usual time of shaking) has elapsed.

The factors which characterize any chemical reaction proceeding with a measurable velocity are: (1) the order of the reaction, (2) the temperature coefficient, (3) the concentration of the reactants.

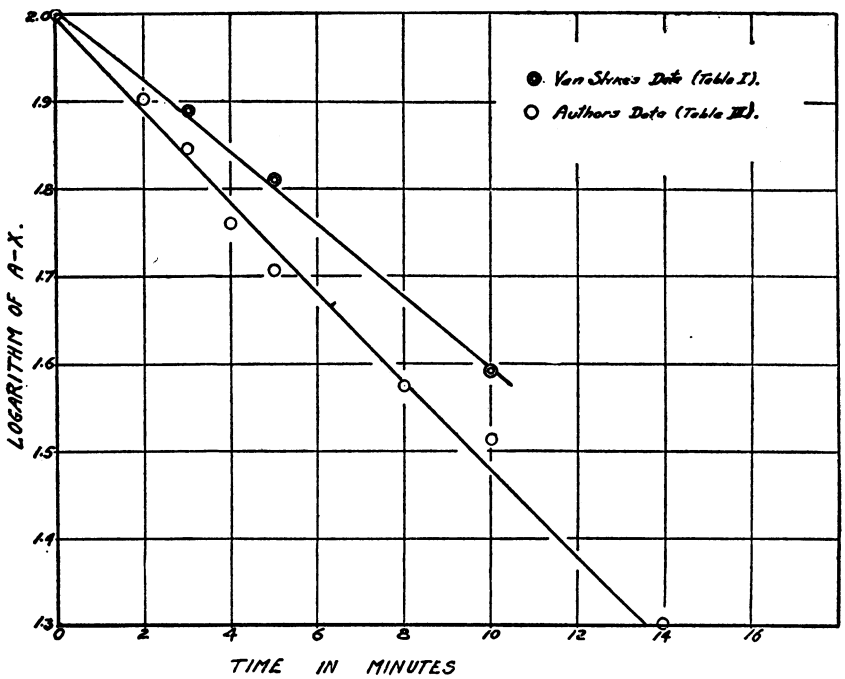

CHART 1. THE ORDER OF THE REACTION

It became necessary first to determine whether various ammonium salts reacted to the same degree. Table 2 shows the results obtained with a number of salt solutions.

It is evident from table 2 that the amount of nitrogen evolved is, within the limits of experimental error, independent of the salt used and is not influenced to any appreciable extent by the presence of even 5 per cent of gelatin.

The data given in table 3 were obtained with ammonium acetate to determine the order of the reaction.

If the reaction is monomolecular the logarithm of the concentration of the ammonium salt remaining in the reaction chamber at the end of time t plotted against time will be a straight line. The plots of the data in table 3 as well as the data of Van Slyke (table 1) are given in chart 1.

Both sets of results plotted in this way give straight lines. Their different positions on the chart are due to different reaction velocities resulting from different temperatures and different concentrations[1] of reagents in the reaction mixture.

TABLE 4

Calculation of velocity constant

t	x	$a-x$	$\frac{a}{a-x}$	$\log \frac{a}{a-x}$	$\frac{2.303}{t}$	k
			$a = 100$			
2	20.4	79.6	1.25	0.097	1.150	0.108
3	30.4	69.6	1.44	0.158	0.770	0.122
4	42.6	57.4	1.74	0.241	0.575	0.139
5	49.2	50.8	1.97	0.294	0.462	0.138
8	62.8	37.4	2.67	0.427	0.288	0.123
10	67.2	32.8	3.05	0.484	0.230	0.111
15	80.3	19.7	5.06	0.704	0.154	0.108
			Van Slyke's data (table 1) $a = 28.02$			
3	24.5	75.5	1.32	0.121	0.770	0.0875
5	36.3	63.7	1.57	0.196	0.462	0.0905
10	62.1	37.9	2.64	0.422	0.230	0.0970

Table 4 shows the velocity constants (K) calculated from the monomolecular reaction law[2] for the data in tables 1 and 3. For the sake of uniformity the results are calculated in percentages.

[1] Van Slyke used the macro-apparatus with 10 cc. of sample. This corresponds to the concentration obtained if a 2 cc. sample is used in the micro-apparatus. The results in table 3 were obtained from 1 cc. samples.

[2] The velocity of a monomolecular reaction is given by the equation

$$k = \frac{1}{t} \log_e \frac{a}{a-x}$$

where k is the velocity constant; t is the interval of time during which the reaction takes place, a is the initial concentration and x is the change in concentration, i.e., the amount of nitrogen evolved in the time t. For further details consult any text book of physical chemistry.

The values thus obtained for k are probably as constant as could be expected when one considers the experimental difficulties involved.

Since the reaction is undoubtedly monomolecular it follows that the rate of reaction is independent of the concentration of the ammonium salt, i.e., the per cent of the total nitrogen evolved in a definite interval will be the same regardless of the initial concentration of the ammonium salt.

TABLE 5

The amount of nitrogen evolved is independent of the concentration of ammonium salt

t = 25°C. Sample = 1 cc. Shaking = three minutes.

CONCENTRATION (NITROGEN PER CUBIC CENTIMETER)	NITROGEN EVOLVED	
mgm.	*mgm.*	*per cent*
1.0	0.32	32.0
2.5	0.82	32.8
5.0	1.68	33.6

These results show that within the limits of the probable experimental error the above statement is correct and may be taken as additional evidence that the reaction follows the monomolecular law.

TABLE 6

Per cent of nitrogen evolved between 20°C. and 35°C.

Shaking = three minutes. Concentration = 5 mgm. per cubic centimeter. Sample = 1 cc.

AMMONIUM SALT USED	NUMBER OF DETERMINATIONS	TEMPERATURE	AVERAGE AMOUNT OF NITROGEN EVOLVED	
			mgm.	*per cent*
Acetate	3	22.5	1.255	25.1
	2	23.5	1.405	28.1
	2	25.5	1.565	31.3
	1	27.0	1.940	38.8
	1	29.5	2.140	42.8
	2	32.5	2.725	54.5
Chloride	1	22.5	1.410	28.2
	1	25.5	1.720	34.4
	1	27.0	1.920	38.4
	1	32.0	2.580	51.6

The rate of a chemical reaction is usually doubled or tripled with each 10° rise in temperature. The temperature coefficient for this reaction between 20°C. and 30°C. may be stated thus

$$C = \frac{k_{30}}{k_{20}}$$

k being the velocity constant at the specified temperature. For the actual determination of C it was considered that the most

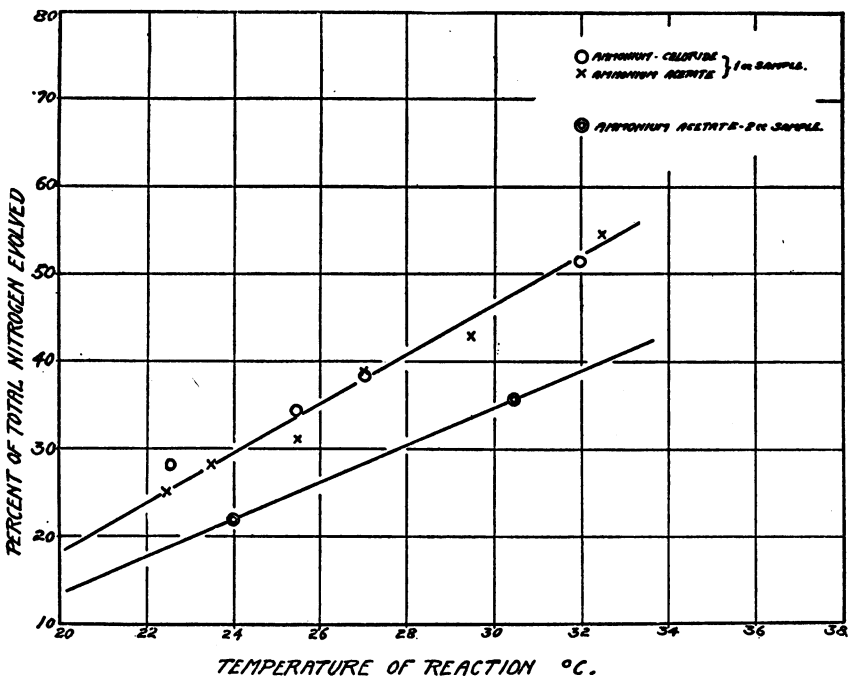

CHART 2. THE EFFECT OF TEMPERATURE AND SIZE OF SAMPLE ON THE AMOUNT OF AMMONIA NITROGEN EVOLVE

reliable result could be obtained by observing the amounts of nitrogen evolved in three minutes at various temperatures over this range.

Two sets of observations were made using two different ammonium salts. The results are recorded in table 6.

The results in table 6 are plotted in chart 2. The percentages of nitrogen evolved as taken from the curve at 20° and 30°C.

are 18 and 46 per cent respectively. This gives $k_{20} = 0.067$ and $k_{30} = 0.205$ and the temperature coefficient (20°C. – 30°C.).

$$C = 3.0$$

It is to be expected that the concentrations of the acetic acid and of the sodium nitrite will affect the reaction velocity. If this is the case, proportionate amounts of nitrogen will not be obtained from 1 cc. and 2 cc. samples. It is to be expected that the larger sample will give relatively less nitrogen. Table 7 shows the amounts of nitrogen obtained at two different temperatures from 2 cc. samples.

TABLE 7

Nitrogen evolved from 2 cc. samples of ammonium acetate

Sample = 2 cc. Shaking = three minutes. Concentration = 10 mgm. per 2 cc.

TEMPERATURE	NITROGEN EVOLVED		
	Milligrams	Average	Average per cent
°C.			
24.0	2.11	2.20	22.0
	2.27		
	2.22		
	2.22		
30.5	3.53	3.57	35.7
	3.57		
	3.50		
	3.65		
	3.60		

The values in table 7 are plotted in chart 2 and make possible a direct comparison with the results obtained with 1 cc. samples. The temperature coefficient (20°C. – 30°C.) calculated from this curve is 2.9 which is in good agreement with the value 3.0 obtained with 1 cc. samples. This speaks well for the consistency of the results.

DISCUSSION

It should be pointed out that the experimental results herein detailed are as close approximations as can be obtained with

the usual Van Slyke procedure. While greater precision could doubtless be obtained by introducing obvious refinements, it was rather the purpose of this work to observe the ammonium reaction under conditions usually employed.

The results show definitely that the reaction has a high temperature coefficient. This fact alone is sufficient to explain the failure of some investigators to obtain checks since in our experience the laboratory temperature may fluctuate 4° to 5°C. in a short period of time. Chart 2 shows that 18 per cent of the total ammonia is evolved in three minutes at 20°C., 32 per cent at 25°C. and 46 per cent at 30°C. Another factor which may lead to poor checks is failure to time the shaking accurately when ammonia is present. A smooth curve drawn from the data in Table 4 indicates that three and one-half minutes shaking yields 4 per cent more of the total ammonia than does a three minute shaking. This amounts to a very considerable error in many investigations, especially those studies of bacterial metabolism in which the ammonia nitrogen may be equal to or greater than the amino nitrogen.

Another factor which may give discordant results is the failure to measure accurately the reagents used, either initially, or in the deaminizing chamber at the time of admitting the sample. The effect of variation in the amount of sample is shown graphically in chart 2. In three minutes shaking at 25°C. the 2 cc. sample yields 24 per cent of its ammonia nitrogen as compared with 33 per cent for a 1 cc. sample. This difference is due to the dilution of the reagents by the larger sample.

The curves presented in chart 2 afford a possible basis for correcting results obtained from samples of which the ammonia content is known. The writers feel, however, that such a procedure is of questionable value since in most cases it is practicable to remove ammonia by some suitable method before making the Van Slyke determination.

The following results are offered to show what may be expected from a correction of Van Slyke analyses of material high in both amino and ammonia nitrogen. The sample used was a composite of several twenty-day-old nutrient gelatine cultures of *C. flabelliferum.*

	mgm.
Ammonia N (Folin)	345
Amino N (Van Slyke):	
1. NH_3 not removed, 1 cc. sample, 27°C	485
2. NH_3 removed by K_2CO_3 and aeration	334
3. NH_3 removed by $Ca(OH)_2$ and evaporation	371
Amino N (1) corrected[3] for NH_3	354

These results show that the corrected amino nitrogen is midway between the figures obtained after the removal of ammonia by two different methods commonly used. They further indicate that a study should be made to determine the most suitable procedure for the removal of ammonia from bacterial cultures.

TABLE 8

ANALYSIS NUMBER	OBSERVER	CORR. CC.	NITROGEN PER 100 CC.	DEVIATION FROM MEAN	
			mgm.	mgm.	per cent
1	WSS	5.36	142.0	−1.1	0.77
2	WSS	5.41	143.3	+0.2	0.14
3	WSS	5.36	142.0	−1.1	0.77
4	LBP	5.41	143.3	+0.2	0.14
5	LBP	5.41	143.3	+0.2	0.14
6	LBP	5.46	144.6	+1.5	1.05
Mean			143.1		
1	LBP	1.96	51.9	+0.54	1.05
2	LBP	1.94	51.4	+0.04	0.08
3	LBP	1.91	50.6	−0.76	1.48
4	LBP	1.98	52.4	+1.04	2.03
5	LBP	1.95	51.6	+0.24	0.47
6	WSS	1.95	51.6	+0.24	0.47
7	WSS	1.93	51.1	−0.26	0.51
8	WSS	1.90	50.3	−1.06	2.06
9	WSS	1.92	50.8	−0.44	0.87
10	WSS	1.96	51.9	+0.54	1.05
Mean			51.36		

Despite the difficulties experienced by Lamson and others which have caused some to discard the method, the writer's

[3] At 27°C. (chart 2) 38 per cent of the total ammonia nitrogen is evolved. This amounts to 131 mg. giving a corrected amino nitrogen of 485 − 131 = 354 mgm.

results, after the removal of ammonia, have been entirely satisfactory. The results of two series of consecutive analyses on two different samples of twenty-day-old *C. sporogenes* cultures in nutrient gelatin are presented in table 8.

As may be seen in table 8 the maximum deviation from the mean in two series of analyses by two different analysts was 2.06 per cent. This error is almost certainly within the limits of the accuracy of the measurements of the 2 cc. sample. Lamson's maximum deviations from the mean as computed from his Tables II and III, which are comparable in amino nitrogen content to the latter part of table 8, are 7.2 and 9.4 per cent respectively. The cause for these greater deviations is presumably due to his failure to remove ammonia.

SUMMARY

1. Data have been presented to show; the order of reaction, the temperature coefficient and the effect of dilution in the ammonia decomposition in the Van Slyke amino nitrogen procedure.

2. It has also been demonstrated that by removing ammonia satisfactory checks can *consistently* be obtained for amino nitrogen.

3. Results obtained without the removal of ammonia are too high by 18 to 50 per cent of the total ammonia nitrogen present depending upon the temperature and the size of the sample used. This may readily cause amino nitrogen values to be 50 per cent too high in cases where the ammonia nitrogen is equal to or greater than the amino nitrogen.

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