CCCXXIX. APPARATUS FOR THE MICRO- DETERMINATION OF CERTAIN VOLATILE SUBSTANCES.

IV. THE BLOOD AMMONIA, WITH OBSERVATIONS ON NORMAL HUMAN BLOOD.

By EDWARD JOSEPH CONWAY.

*From the Department of Physiology and Biochemistry, University College,
Dublin.*

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I. INTRODUCTION.

THE determination of the free ammonia of circulating blood has long been regarded as one of outstanding difficulty. The early literature, dating from the researches of Nencki [Nencki *et al.*, 1895; Nencki and Zaleski, 1901] has been reviewed elsewhere [*e.g.* Parnas and Heller, 1924; Parnas and Klisiecki, 1926 *etc.*]. Folin and Denis [1912] were the first to find values under 0.10 mg. ammonia-N per 100 ml. If the special conditions likely to affect a determination be not taken into consideration it is possible to obtain results as high as 2.0 mg./100 ml. and many published results are of the order of 0.3 mg./100 ml. A value of about 30 γ (0.03 mg.) per 100 ml. has been considered to represent the free ammonia-N in circulating human blood. It will be shown however that the true value is in fact less than one-tenth of this, or less than one part in thirty millions. There are certain reasons why this fact has hitherto escaped observation, and why when this low value was suggested by Henriques and Gottlieb [1924] and by Fontes and Yovanovitch [1925] on quite insufficient grounds, it was then with good reason believed to be erroneous [Parnas and Klisiecki, 1926]. In the first place the methods used did not permit of the ready accumulation of data within the first few minutes after shedding. It was therefore not noticed that the blood ammonia formation was represented by a steep curve which terminated at about the fifth minute. Also the blood was not received directly into a carbon dioxide atmosphere, and maintained there for subsequent analysis; in this way only is the initial rapid formation abolished and the blood ammonia held at its true value for a certain time after shedding.

Methods hitherto in use for blood ammonia determination are either developments of the original Folin technique [*e.g.* Nash and Benedict, 1921, using special absorbers] or a steam-distillation method elaborated by Parnas and Wagner [1921]. The ammonia from 5 ml. blood—or 2 ml. with the Parnas method—is finally present in about 4 or 5 ml. fluid. It is then determined colorimetrically after adding Nessler's reagent. With regard to the possibilities of nesslerisation Van Slyke and Hiller [1933] state in a recent paper that amounts of ammonia-N corresponding to 0.02 mg. ammonia-N per 100 ml. (obtained from 5 ml. blood) are not "visible at all with Nessler's solution". This represents a dilution of 0.001 mg. ammonia-N in 5 ml. Parnas and Heller [1924], on the other hand, speaking of the same volume of fluid say "man kann bei der Analyse von 2 ccm Blut unterscheiden, ob der Ammoniakgehalt etwa 0.045 oder 0.050 mg. in 100 ccm Blut beträgt". They can in other words distinguish between a dilution of

0.001 mg. and 0.0009 mg. ammonia-N in 5 ml. Comparing these two statements it will be noted that marked differences in the reagent sensitivity were found. This may be attributed to some difference in the composition or manner of preparation of the Nessler's reagent. Van Slyke and Hiller make use now of a reagent attributed to Berthelot by Thomas [1912; 1913] who first utilised it for quantitative determination. The reagent is an alkaline solution of phenol and hypochlorite which on heating with ammonia gives a blue colour. It appears to be more sensitive than the Nessler's solution.

In the method to be described the ammonia is finally present in about 0.7 ml. of standard acid (0.0002 *N* HCl in which is incorporated 20 % alcohol and methylene blue and methyl red). In determining this ammonia a titration method is used. A definite change at the end-point is clearly visible to any ordinary observer on the addition of alkali corresponding to 0.00003 mg. ammonia-N. With the method used for the ammonia absorption this will correspond to 0.006 mg. (6 γ) ammonia-N per 100 ml. The alkali used is 0.0005 *N* barium hydroxide. The titration error sets the limit of delicacy for the determination since no appreciable error is introduced by the ammonia absorption itself. Practice with the method enables this error to be still further reduced. The titration is carried out with a special burette [Conway, 1934] which delivers 0.1 ml. with the same accuracy as the standard 2 ml. Bang pipette delivers 1.0 ml. or the standard 50 ml. burette delivers 15 ml., namely with a coefficient of variation of 0.12 %.

When titrating 0.0002 *N* acid under the above conditions the standard deviation is directly proportional to the volume at the end-point and may be stated as 1.42×10^{-6} millimols per ml. For 0.7 ml. acid with 0.9 ml. at the end-point this corresponds to 0.018 γ ammonia-N. For 5 ml. it would correspond to 0.13 γ .

This figure for the standard deviation is readily reproducible with the described conditions. Theoretically a reduction of the error to about one-third of this, but no further, remains as a possibility [Conway, 1934].

In the present method the liberation, absorption and determination of the ammonia are all conducted in the one small glass "unit", the ammonia being absorbed in about 0.7 ml. of standard acid.¹ There is no aeration or distillation. Each "unit" corresponds to the complete apparatus of other experimenters. This "unit" has been already described in a previous communication [Conway and Byrne, 1933]. It is similar to a small Petri dish with thick walls from the floor of which a circular inner wall arises to about half the height of the outer. When covered with a glass lid smeared with a suitable fixative (here a mixture of pure solid and liquid paraffin) the "unit" forms a closed compartment with inner and outer chambers. In the centre is placed for the present determination about 0.7 ml. of standard acid, accurately measured, the outer chamber containing the blood and potassium carbonate. The ammonia passes by simple diffusion into the central chamber. In the ordinary use of the "unit" absorption at room temperature takes 2 hours under such conditions, which is too long a period for blood ammonia determinations as there is a steady formation of ammonia in the presence of the alkali. When we consider however that each "unit" is turned out from the same mould with the same dimensions and surface relations, and further, that since after the first few seconds the fluid surfaces are perfectly still a uniform passage of ammonia may be expected and in fact occurs. An error of less than 5 % is introduced by cutting short the full absorption, and for the present determination this is of no consequence. Whatever

¹ "Units" of standard size are now available from Messrs A. Gallenkamp, Finsbury Square, London.

accuracy may be lost here is more than compensated for in comparison with other methods by the entire freedom from contamination during the absorption period. In about 10 min. 50 % of the ammonia is absorbed at room temperature and in 15 min. about 66 %. Either of these times may be selected or any intermediate time. The ammonia formation in the presence of the alkali over this period is very small. The exact correction for this ammonia as well as the factor for calculating the complete absorption may be read from a table or curve (see Table I).

The determination of blood ammonia by the present method offers in the opinion of the writer little more difficulty than that of an ordinary ammonia determination when the "units" have been adequately cleaned and after the blood has been collected. The manner of collecting the blood is however of the greatest importance. Collected straight from the vessel through a paraffined tube into an atmosphere of alveolar air or of pure carbon dioxide, human venous blood has practically no free ammonia.

The importance of collecting in a carbon dioxide atmosphere was first appreciated from a study of a large number of analyses of human blood collected from a vein directly through a hypodermic needle (without syringe) into a small paraffined Erlenmeyer flask. This would correspond to the ordinary manner of collecting—the blood falling through air.

The concentration of blood ammonia plotted against the time of shedding showed a steep rise in the first few minutes reaching then the level of concentration hitherto accepted as representing the normal concentration in the circulating blood. After 3 min. the change of concentration entered upon the slow and somewhat uniform rise described by other workers [*e.g.* Parnas and Heller, 1924]. If the first curve were extrapolated to zero time it passed directly through the origin. If the second were extrapolated it cut the ordinate at 38 γ ammonia-N/100 ml., a figure of about the same order as that given by Parnas as the normal content of ammonia in circulating human blood. From a consideration of the results it was thought that the initial stages of ammonia formation after shedding were most probably linked with the corresponding sudden fall in carbon dioxide tension and content of the blood, a hypothesis which proved to be correct on examination. It is very likely therefore that the first stage of ammonia formation is linked with the breakdown of a carbamino-compound, since the slight change of p_H can scarcely be held to account for so marked a difference.

II. PROCEDURE OF THE DETERMINATION.

Preparation of the "units".

The whole success of the determination depends on the careful cleaning of the "units". This procedure however may be quite simply and effectively carried out. The special cleaning of the "units" for blood ammonia determination should begin on "units" cleaned and dried in the ordinary way [Conway and Byrne, 1933] or cleaned and stored in weak acid. A little ether is poured into the centre of a "unit" and then discarded, or if the "unit" is taken from weak acid it is treated with alcohol and ether. The whole "unit" is now filled with approximately 0.005 N H_2SO_4 and kept 1 hour. The acid is then discarded and the "unit" well rinsed with tap water and subsequently a few times with distilled water, the last drops being shaken out and the "unit" allowed to dry in air, or if not required that day filled with distilled water. With this simple routine excellent results are obtainable from the beginning. Where the "units" have been stored in weak acid further soaking may seem redundant. In practice however the routine outlined

will probably be found the safest, owing to the occasional inadequate soaking of crowded "units". We have found the ordinary laboratory distilled water quite suitable for use. It should however give an approximately neutral tint with methyl red-methylene blue indicator. Pyrex lids are cleaned at the same time and washed with distilled water, being dried in air or with clean filter-paper.

Into the central chamber of each dry "unit" is now run 0.7 ml. of 0.0002 *N* HCl (in which is incorporated methyl red-methylene blue indicator and 20 % alcohol—see below). The acid is delivered from a straight-tube pyrex pipette without blowing, the tube being marked at 0.7 ml. approximately and drawn out at the end. It is standardised for exact delivery without blowing out. Into the outer chamber is delivered in a similar manner 1 ml. of saturated K_2CO_3 from a similar pipette with 1 ml. mark. A pyrex lid smeared with the fixative (a mixture of pure liquid and solid paraffins as described below) is placed in position and the "units" so treated are left for 30 min. Any final traces of ammonia in the carbonate or "unit" will have passed within that time into the central chamber. The ammonia blank value should not exceed 0.1 γ ammonia-N.

The collection of the blood.

The manner of collection will depend on the nature of the investigation. If the real concentration of free ammonium ions in the circulating blood is required the blood must be received into a carbon dioxide atmosphere. For this purpose alveolar air will suffice. In the present determinations a hypodermic needle was inserted into an arm vein. A tube coated with solid paraffin internally was attached to the needle by the minimum quantity of rubber tubing at the joint, the rubber being also coated. The first few drops of blood were discarded and then the collecting tube dipped down to the end of a tonometer of about 300 ml. capacity lined internally with solid paraffin and filled previously with alveolar air, and containing a little ammonia-free potassium or lithium oxalate. After collecting the required amount the tube was at once removed, alveolar air again introduced, the tonometer finally stoppered and the blood mixed. The alveolar air was first passed through two small wash-bottles, the first containing weak acid and the other serving as a trap. It is possible that this procedure was unnecessary, but it ensured freedom of the air in the tonometer from ammonia. Where pure carbon dioxide was employed it was delivered into the tonometer from a cylinder after passing through two similar wash-bottles.

The ammonia determinations.

When introducing the blood into the "unit" 1 ml. is removed from the flask in a long pyrex pipette similar to those previously used and delivered into a "unit" without blowing. The "unit" for this purpose is slightly tilted by resting on a spare lid, the exact delivery time being noted. The pipette should deliver the blood rapidly. After the introduction of the blood the slightly detached lid is closed at once and the fluids mixed by rotating about 20 to 25 times. After 10–15 min. the lid is removed and the acid titrated with 0.0005 *N* $Ba(OH)_2$ solution from the burette already described [Conway, 1934]. The strengths of acid and alkali given are suitable for blood concentrations up to 0.2 mg. ammonia-N/100 ml.

Calculation of the result. The standardisation of the burette divisions in terms of ammonia titrated may be made from the alkaline or acid side. The 0.0005 *N* $Ba(OH)_2$ remains quite stable over a long period in the little reservoir bottle of the burette. No deterioration has been observed after a fortnight, provided that

the contents of the delivery tubes are firstly run out before a series of titrations, so that the alkali used in titrating has not long left the reservoir bottle. The barium hydroxide is made up at the beginning from distilled water which gives the same reaction as the desired end-point. Traces of carbonate in the water do not interfere with the titrating alkalinity. The delivery volume of the burette is then given by a constant factor, the ammonia absorbed being calculated with the aid of Table I.

Table I.

Time allowed for absorption min.	Allowance for the extra ammonia produced in the presence of the alkali and absorbed in the time allowed γ ammonia-N	Factors for obtaining the full absorption of the blood ammonia
10	0.025	1.95
11	0.029	1.84
12	0.035	1.74
13	0.040	1.66
14	0.046	1.59
15	0.052	1.52
16	0.059	1.46
17	0.065	1.42
18	0.071	1.38
19	0.078	1.34
20	0.084	1.31

The above data are valid for the "unit" of dimensions described in a previous communication [Conway and Byrne, 1933]. They apply to the addition of 1 ml. blood to 1 ml. saturated potassium carbonate at 18°. For other temperatures the absorption factor in the third column is decreased 2% for each increase of 1°. Where the room temperature differs by more than a few degrees from the standard temperature it is well to keep to the shorter absorption times since the allowance for the ammonia produced in excess in the presence of added alkali is then very small, and changes therein will have only a slight influence.

Example: 1 ml. of blood analysed at room temperature of 18°.

Blank value	21.7 cm. on scale
Each cm. on burette scale	0.070 γ ammonia-N
Filled unit titrated after 11 min.	19.6 cm.
Allowance for ammonia produced and absorbed in presence of the alkali over 11 min. (see Table I) ...	0.029 γ ammonia-N
Factor for full ammonia absorption corresponding to 11 min. (see Table I)	1.84

Ammonia-N in 1 ml. blood is therefore

$$\{(21.7 - 19.6) \times 0.070 - 0.029\} \times 1.84 = 0.217 \gamma.$$

Ammonia-N/100 ml. is therefore 21.7 γ (0.0217 mg.).

The absorption factors in column 3 of Table I are in experimental and theoretical agreement with the equation

$$\frac{x}{a} = (1 - e^{-0.072t}),$$

where x is the fraction of the total a absorbed after the time t . (The equation has the same form as that for a unimolecular reaction.) For the computation of the figures in column 2 the formation of ammonia in the presence of the alkali has been experimentally determined for human blood as 0.0088 γ ammonia-N per min. The amount absorbed from this cause could be simply determined without very appreciable error by regarding half the amount formed during the period

to be present as an initial concentration. It may be more accurately determined from the equation

$$x = K_2 t - \frac{K_2}{K_1} (1 - e^{-K_1 t}),$$

where x is the amount absorbed in time t , K_2 is the constant expressing the rate of formation, namely 0.0088γ per min. and K_1 the absorption constant or 0.072 . The values calculated will depend as before on the temperature, but for the shorter times this effect can be neglected.

Notes on solutions required.

0.0002 N HCl containing reagent. This is made from stock $0.01 N$ acid, and stock reagent formed from 50 ml. of 0.1% methylene blue in alcohol and 200 ml. 0.1% methyl red in alcohol. The stock reagent is stored in a brown bottle and keeps indefinitely. 2 ml. of the reagent are run into a 200 ml. volumetric flask and 40 ml. of pure alcohol. Distilled water is added to within about 5 ml. of the mark and the mixture brought to the end-point colour (complete absence of reddish tint) by dropping in approx. $0.01 N$ $\text{Ba}(\text{OH})_2$ from a 1 or 2 ml. pipette. 2 ml. of the stock $0.01 N$ acid are now added and the mixture brought to the mark with distilled water. The distilled water used should have the reaction of the required end-point or deviate but little from it.

0.0005 N $\text{Ba}(\text{OH})_2$. This is formed by pipetting 2.5 ml. of $0.2 N$ $\text{Ba}(\text{OH})_2$ (or 5 ml. of $0.1 N$) into distilled water in a litre flask and making up to the mark. The burette reservoir is filled at once with this solution and will remain for a long period quite unchanged in strength. When the burette reservoir is filled and attached *etc.* the solution may be allowed to settle overnight.

Saturated K_2CO_3 . A large stock of saturated carbonate is made up in the ordinary way. About 200 ml. are decanted into a pyrex Erlenmeyer flask with glass stopper and some beads added. This alkali is boiled vigorously for about 20 min. If much water is lost it may be made good by addition. On cooling some solid crystals of carbonate should be in evidence.

Oxalate (ammonia-free). About 50 ml. of a strong solution of potassium oxalate are evaporated in an open pyrex dish after bringing to p_H 10 or 11 by adding a little alkali, the reaction being tested by a universal indicator. The water volume is maintained by occasional addition. The mixture is kept boiling for about 10 min. and then evaporated until the solid separates; after cooling the crystals are filtered off on a small Büchner funnel and preserved in a desiccator.

The fixative. The same fixative is used as described for chloride determinations [Conway, 1935]. 40–50 g. paraffin, m.p. 49° , are melted with 80 ml. of pure liquid paraffin and cooled. Vaseline should not be used as it contains traces of ammonia.

Stock ammonia standard. 0.471 g. of pure $(\text{NH}_4)_2\text{SO}_4$ is dissolved and made up to a litre with distilled water. 0.1 or 0.2 ml. *etc.* of this diluted to 100 ml. correspond to 10, 20 γ *etc.* of ammonia-N/100 ml.

Ammonia-free water. This may be made up in the usual manner by distilling dilute sulphuric acid and discarding the middle portion of the distillate. The distilled water of the laboratory was in fact found to be almost perfectly free from ammonia.

III. EXPERIMENTAL.

Blank values.

A series of 24 blank determinations was carried out with the above technique, and standard solutions. The mean of the series (Table II) gave 21.4 cm. reading on the burette scale, corresponding to 0.214 ml., the tube being calibrated to 0.001 ml. divisions. Direct titration of the acid gave a mean value of 22.3 cm.

Table II.

Burette reading cm.	Deviation from the mean value cm. on scale	Deviation from the mean value as γ ammonia-N
21.8	+0.4	+0.028
21.4	0.0	0.000
21.6	+0.2	+0.014
21.5	+0.1	+0.007
21.3	-0.1	-0.007
20.6	-0.8	-0.056
21.8	+0.4	+0.028

The table gives the first seven of a series of 24 blanks. Omitting one aberrant value the standard deviation is 0.30 cm. or 0.021 γ ammonia-N, a figure practically the same as that resulting from immediate titration of a succession of acid deliveries when expressed in the same manner.

The standard deviation of the individual blank titration, omitting two aberrant results, was 0.30 cm., corresponding to 0.021 γ ammonia-N. The standard deviation for the titration procedure carried out directly with a succession of deliveries in the one unit is 0.018 γ N. The blank values therefore show no significant difference from the titration itself with regard to variability, and are in this sense therefore practically perfect. Table II gives the first seven blank titrations of the series. Omitting the two aberrant results 20 of the remaining 22 blank values did not exceed 0.4 cm. deviation from the mean, corresponding to 0.028 γ N, and the greatest deviation was 0.8 cm. or 0.056 γ ammonia-N.

Determinations of standard solutions.

From the stock $(\text{NH}_4)_2\text{SO}_4$ described above a number of standards were made up as required containing 10, 20 and 100 γ ammonia-N/100 ml. respectively (0.1, 0.2 ml. of the stock made up to 100 ml.). Table III gives a summary of the

Table III. *Standard solutions.*

Strength of standard used γ ammonia-N/100 ml.	No. of determinations	Standard deviation of the individual determination γ ammonia-N/100 ml.	Maximum deviation of a single determina- tion from theoretical γ ammonia-N/100 ml.
10.0	11	3.5	7.5
20.0	8	4.7	8.1
100.0	5	5.9	8.8

Table IV. *Determinations of the standard 10 γ ammonia-N/100 ml.,
absorption being terminated after 10-15 min.*

Burette reading subtracted from blank value cm.	Time of absorption min.	Ammonia absorbed γ ammonia-N	Theoretical value for the absorption time γ ammonia-N	Difference from theoretical γ ammonia-N	Ammonia-N found in standard γ ammonia- N/100 ml.
1.1	10	0.077	0.051	-0.026	15
0.7	11.5	0.049	0.056	+0.007	9
1.0	12.5	0.070	0.059	-0.011	12
0.8	13	0.056	0.060	+0.004	9
0.7	14	0.049	0.063	+0.014	8

The blank value for the above table was determined as the average of five results. The table gives the first five of a series of eleven determinations of the standard 10 γ ammonia-N/100 ml. using the method of suspended absorption. The standard deviation of a single ammonia absorption in the unit from the theoretical is 0.017 γ ammonia-N. This may be compared with 0.018 γ for the titration procedure itself and 0.021 for the blank values. The standard deviation of the individual determination of the ammonia content in the standard is 3.5 γ ammonia-N/100 ml.

results obtained, from which it will appear that the accuracy of the determinations is only limited by the titration error, the ammonia absorption itself giving rise to no appreciable error. Thus, for the 10 determinations of 1 ml. of the 10 γ /100 ml. standard given in Table IV, 9 of the 10 did not deviate from the theoretical quantity of ammonia expected by more than 0.026 γ which is a result nearly identical in value for the blank determinations or in turn for that of the pure titration error. The standard deviation of the ammonia divergence from the theoretical for these lowest determinations is in fact 0.017 γ ammonia-N, which compares with 0.018 for the pure titration and 0.02 for the blanks. The difference between these values is merely an expression of sampling error.

The absorption rate of ammonia from blood-carbonate mixture.

It was expected before investigating this rate that some divergence would be found from that of standard solution-carbonate mixture. Such divergence however would be of a constant nature and expressible by a factor or incorporated in the factors of Table I. Two experiments carried out to determine the point gave results however showing no appreciable divergence (Table V). In these experiments 0.2 ml. of standard (NH₄)₂SO₄ containing 2.24 γ or 1.12 γ ammonia-N was introduced into the empty outer chamber of a "unit", the central chamber containing the acid as before, 1 ml. of freshly shed blood (10 min. after shedding into an open vessel) was introduced, the lid placed in position and 1 ml. of saturated carbonate then run in *etc.* The absorption was terminated after 10 min. and the acid titrated. A similar experiment was set up, water being substituted for blood; also corresponding blank experiments using 0.2 ml. water instead of the standard (NH₄)₂SO₄. From Table V it will appear

Table V. *Absorption rate of ammonia from carbonate-blood mixtures.*

Contents of outer chamber before adding 1 ml. saturated K ₂ CO ₃	Ammonia added γ N	cm. on burette subtracted from blank	Rate of ammonia absorption per γ NH ₃ -N added cm. per 10 min.
1 ml. blood <i>plus</i> 0.2 ml. standard ammonia solution (0.0008 N)	2.24	13.3	5.9
1 ml. blood <i>plus</i> 0.2 ml. standard ammonia solution (0.0004 N)	1.12	6.4	5.7
1 ml. water <i>plus</i> 0.2 ml. standard ammonia solution (0.0004 N)	1.12	6.6	5.9
Corresponding blank values.			
1 ml. blood <i>plus</i> 0.2 ml. water	0.00	18.2	—
1.2 ml. water	0.00	21.8	—

The absorption for the above experiments was terminated after 10 min. Duplicate determinations were carried out in each case. In the experiments above the carbonate was added at once after the mixing of blood and standard ammonia solution.

that the percentage of the added ammonia absorbed after 10 min. is the same with blood as with water, within the error of the determination. In these experiments the added ammonia was only in momentary contact with the blood before addition of the alkali. It is to be noted that the total outside fluid before the carbonate addition was 1.2 ml. so that the absorption rate is slower than where only 1 ml. is present [see Conway and Byrne, 1933].

The effect of saturated potassium carbonate on the ammonia formation in shed blood.

In determining this effect 25 ml. of recently shed blood from each of two subjects were mixed with 25 ml. of saturated K_2CO_3 in a 50 ml. Erlenmeyer flask provided with a paraffined rubber stopper. The flasks held in fact somewhat more than 50 ml. so that mixing could be adequately performed on inserting the stopper. When a sample of blood was required 2 ml. of the mixture were taken and run into the outer chamber of a prepared "unit" containing 0.7 ml. of the standard acid in the central chamber. The sample was quickly taken from the flask after inserting the pipette down to the bottom of the flask and the cork replaced at once. The ammonia vapour diffusing into the small air space left and in equilibrium with the mixture was considered negligible, and the removal of the sample from the bottom of the flask would in any case ensure against loss of ammonia. The samples in the "unit" were allowed 14 min. for absorption. In each case samples were taken after 4 to 8 min. and then after about 1 hour as

Table VI. *The effect of saturated potassium carbonate on blood ammonia formation.*

	Time after mixing equal vols. blood and carbonate min.	Time of absorption in "unit" min.	cm. on burette, corresponding to the absorption	Ammonia-N absorbed γ N
Blood I	4.5	14	3.2	0.22
	6.4	"	3.9	0.27
	7.7	"	3.6	0.25
Blood II	6.7	"	3.8	0.27
	7.8	"	3.7	0.26
Blood I	57.5	"	8.4	0.59
	58.0	"	7.8	0.54
Blood II	52.5	"	7.1	0.50
	53.0	"	6.6	0.46

Ammonia absorbed in "unit" after short period in Erlenmeyer flask, 0.25 γ (6.4 min. mean time in Erlenmeyer flask).

Ammonia absorbed in "unit" after long period in Erlenmeyer flask, 0.52 (55.2 min. mean time in Erlenmeyer flask).

Therefore $(0.52 - 0.25) \times 1.59 = 0.43$ ammonia-N represents the amount formed in 48.8 min. action of carbonate on blood. (The factor 1.59 arises from the suspension of absorption after 14 min. See Table I.) The ammonia formation per min. in the presence of carbonate is therefore 0.0088 γ ammonia-N at room temperature.

shown in Table VI. From the mean of the short and long time periods the ammonia formation in the presence of the carbonate appears as 0.0055 γ ammonia-N per min. with the 14 min. period of absorption or 0.0088 γ ammonia-N altogether, the ammonia formation during the absorption period being the same in both the short and long periods disappears in the subtraction.

The percentage error involved in the suspension of the ammonia absorption.

From the foregoing paragraphs of this section it is clear that as the ammonia for determination approaches minimum quantities, the only error involved in the determination is that of the titration itself. In a simple and easy manner therefore all extraneous contamination with acid or alkali or ammonia from other sources is removed. At the same time the question arises of the degree of error involved in the 10-15 min. restriction of absorption for a given quantity of ammonia. This had been previously examined in a number of early experiments

with the "unit". Sixteen determinations of 1.0 ml. $(\text{NH}_4)_2\text{SO}_4$ solution containing 46.4 γ ammonia-N were carried out, the ammonia being absorbed in 1 ml. of 0.00333 N HCl in the central chamber and subsequently titrated with 0.001333 N Ba(OH) $_2$ from a Bang burette. The absorption was terminated in each case after 10 min. The mean absorption at 17° was 49% of the added ammonia. The coefficient of variation of the individual determination from the mean value was 2.8%. All the sixteen determinations were under 5% deviation from the mean, the two highest deviations being 4.9%.

IV. RESULTS.

The ammonia content of normal human blood.

(a) *The blood ammonia value determined after collecting in an open vessel.* Here the blood was collected from an arm vein through a hypodermic needle. The blood was allowed to fall in a stream into a small Erlenmeyer flask lined with solid paraffin, containing a little ammonia-free oxalate, the exact time of shedding being noted with a stop-watch. About 25 ml. were collected as a rule. After shedding and quickly mixing with the oxalate, 1 ml. volumes were pipetted successively into a number of prepared units, the exact times of mixing with the carbonate being noted. In this way a large number of determinations could be grouped around the first few min. after shedding. The determinations were made on 7 subjects, 12 separate samples of about 25 ml. blood being taken. Up to 2.75 min. after shedding there are 29 separate determinations of the blood ammonia, and after this period 43 determinations. The results are summarised in Table VII, and in Figs. 1 and 3. In Fig. 1 the two stages of the blood ammonia

Table VII. *Blood collected in a paraffined Erlenmeyer flask.*

Time range after shedding min.	Ammonia concentrations as γ ammonia-N/100 ml.												Mean time after shedding min.	Mean concentration γ ammonia-N/100 ml.
	1 E.C.	2 F.K.	3 F.K.	4 E.C.	5 E.C.	6 E.C.	7 R.S.	8 G.F.	9 E.E.	10 D. O'S.	11 T.D.	12 E.C.		
0.75- 1.25	—	—	14(2)	—	15(2)	29	0	41	22	6	—	5	0.95	16.5
1.25- 1.75	—	22	20	18	—	24	31	28	—	—	34	24	1.45	25.2
1.75- 2.75	53	30	21(2)	46	—	31	—	45	31	12	41	24	2.22	33.4
2.75- 4.75	40	35(4)	34(3)	—	—	37(3)	45(2)	27(2)	33(2)	32	44	—	3.41	36.1
4.75- 10.0	—	—	40(2)	—	42(2)	38(4)	—	—	—	—	—	—	7.27	40.1
10.0 - 50.0	—	—	—	—	—	—	51(2)	—	54(2)	—	—	—	42.3	53.0
50.0 - 300.0	—	—	68	—	63(2)	80(2)	88(2)	68	—	—	—	—	97.4	73.5
1100.0 - 1500.0	—	—	—	—	—	—	204(2)	200(2)	—	—	—	—	1170.0	202.0

The bracketed figures indicate the number of determinations made, the mean value being stated. Figures without brackets are from a single determination.

Mean room temperature = 16°.

formation are clearly shown. The first rise extrapolated over 0.75 min. passes through the origin. The same result may be observed from the table where the rates of the ammonia formation are given. If the rate during the first 0.75 min. is as fast as that in the next min. the original ammonia must be either zero or practically indistinguishable therefrom. Fig. 2 shows the course of the formation for a single subject (F. K.), a rapid succession of analyses being carried out immediately on shedding. Eight determinations were made for times up to 4 min. after shedding. The means of each two analyses in order of time are given in the graph. The same characteristics are observed as in the general graph.

In general after the first rapid rise the rate of formation of blood ammonia within the succeeding few hours is 0.37γ ammonia-N per min. per 100 ml. around a temperature of 16° . Fig. 3 shows the effect of incubating the blood after shedding (upper curve) at 22.5° . This upper curve was constructed from determinations on two subjects. The rate at 22.5° after the first few min. is 0.82γ ammonia-N per min. per 100 ml.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 1. The curve is constructed from the means of a number of determinations on 12 blood samples (about 25 ml. each) from seven different subjects. The blood was collected in each case direct from an arm vein through a hypodermic needle into an open Erlenmeyer flask lined with solid paraffin. Mean temperature of room, 16° .

Fig. 2. The figure shows the blood ammonia formation after shedding into an open vessel for a single subject (F. K.). Each circle is the mean of a duplicate.

Fig. 3. Curve B is the same as in Fig. 1 on a ten times smaller time scale. Curve A represents the ammonia formation in blood from two subjects maintained at 22° .

Fig. 4. Curve A represents the blood ammonia formation for two subjects, E. C. and F. K., the blood being collected in an open Erlenmeyer flask. Curve B gives the formation of ammonia in blood from the same two subjects collected in alveolar air. Curve C represents the blood ammonia formation after collection in pure carbon dioxide at atmospheric pressure (for the single subject E. C.). This last curve represents the mean line through 11 determinations made up to 128 min.

(b) *The blood ammonia determined after collecting in alveolar air.* The peculiar nature of the true curve of formation after shedding in an open vessel suggested a connection with some factor altering with like rapidity within the first few min. Such a factor would be the carbon dioxide tension and content of the blood. For this reason it was thought desirable to collect the blood directly

from an arm vein into a tonometer filled with alveolar air. To the hypodermic needle was attached a pyrex tube lined with paraffin, the connection being made with the minimum quantity of paraffined rubber tubing. The tonometer, about 250 ml. in capacity, was also lined internally with solid paraffin. It contained some ammonia-free oxalate and was filled with alveolar air which had passed through a small wash-bottle containing dilute acid and another acting as a trap. The taps and stopper of the tonometer were lubricated with liquid paraffin. After insertion of the needle the first few drops of blood were discarded and the collecting tube plunged at once to the bottom of the tonometer. After collecting about 15 ml. blood, the tube was withdrawn, the blood mixed and more alveolar air passed through, the tonometer being then stoppered. Samples of blood for analysis were taken in quick succession, by means of a long-tube pyrex pipette with a mark at 1 ml., the stopper being opened and closed immediately after each sample, alveolar air being occasionally sent through. The blood donors were two subjects whose blood has been previously well investigated by the open method (E.C. and F.K.—Table VII). The blood was taken and analysed under exactly similar conditions and at the same time of day (about 4 p.m.). The surprising result was obtained that for the first 12 min. after shedding the mean value of 11 determinations (6 from one and 5 from another subject) was almost indistinguishable from zero. The initial rise had entirely disappeared. For the first 12 min. practically no distinction could be made between the results returned. The average of 11 such results gave $3.1 \pm 2.2\gamma$ ammonia-N/100 ml. (± 2.2 gives the standard deviation of the mean) with a standard deviation of the individual result of 7γ N/100 ml., the highest deviation obtained being 11γ /100 ml.

When the ammonia formation is followed beyond 12 min. however it appears that a slow increase is occurring from the period of shedding corresponding to the secondary rise in blood collected in an open vessel. This is shown in Table VIII

Table VIII.

Time after shedding min.	Collected in alveolar air			Collected in open vessel		
	No. of determinations	Mean time min.	Mean conc. γ ammonia-N/100 ml.	No. of determinations	Mean time min. after shedding	Mean conc. γ ammonia-N/100 ml.
0- 3	4	1.8	2	9, 7	1.6, 1.7	22, 28
3- 6	3	4.0	3	6, 7	4.0, 4.1	36, 40
6- 12	4	10.5	8	5, 2	7.4, 9.2	37, 35
12- 50	4	40.4	29	—	—	—
50-100	—	—	—	4, 2	59, 73	74, 63
> 100	4	148	62	—	—	—

Comparison of the 4th and 7th columns shows the marked difference resulting from collecting in alveolar air as compared with collecting in an open vessel. The figures after the commas—for collection in an open vessel—refer to data assembled 2 years before the preceding values. These latter were taken a day or two before the determinations with alveolar air. Roughly equal numbers of determinations were made for each time period on the blood of each subject. The figures found being very similar for each donor are incorporated together for economy of presentation.

and in Fig. 4. The table summarises the data obtained from two subjects (E. C. and F. K.) the blood from whom had been previously well investigated for open vessel collection. Fig. 4 (upper two curves for the same two subjects) shows a linear ascent of concentration for collection in alveolar air (middle curve, the upper curve being for open collection) up to 40 min., the line of formation pointing directly into the origin.

The blood ammonia determined after collecting in carbon dioxide at full atmospheric pressure.

The effect of collecting the blood in an atmosphere of pure carbon dioxide at atmospheric pressure was also investigated, the blood being collected in the same manner as for alveolar air. In this case the tonometer was filled with pure carbon dioxide derived from a cylinder and passed through two wash-bottles as before. The blood donor here was one of the subjects (E. C.) investigated for open vessel collection and for the alveolar effect described in the last section. Table IX gives

Table IX.

Time after shedding min.	Open vessel collection	Alveolar air collection	Collection in carbon dioxide
	γ ammonia-N/100 ml.	γ ammonia-N/100 ml.	atmospheric pressure γ ammonia-N/100 ml.
0- 3	29 (8)	8 (2)	7 (2)
3- 6	41 (7)	3 (1)	1 (3)
6- 12	36 (5)	4 (2)	6 (2)
12- 50	—	25 (2)	3 (2)
50-100	72 (4)	—	—
100-200	—	53 (2)	7 (1)

The numbers in brackets refer to the number of determinations made.

a summary of the results obtained, the different results of the three methods of collection being compared for one subject. Even up to 128 min. after collection in carbon dioxide the blood ammonia concentration is but little different from zero. The mean value of the 10 determinations made on blood up to this period is $4.4 \pm 1.3\gamma$ ammonia-N/100 ml. The mean of the last three observations made at an average time of 68 min. was 4.3γ ammonia-N/100 ml. The mean of the first three at 1.8 min. was 6.0γ ammonia-N/100 ml., no increase occurring therefore over 66 min. After 128 min. one observation gave 7.0γ ammonia-N/100 ml. For a long period after shedding therefore pure carbon dioxide at atmospheric pressure completely prevents all formation of ammonia in human blood and the mean value obtained is only one part in 23 millions. The data for collection in pure carbon dioxide are summarised in Table IX and Fig. 4 (lowest curve).

Effect of pure carbon dioxide at atmospheric pressure on blood ammonia already formed.

In experiments carried out to determine this effect, blood collected in a paraffined Erlenmeyer flask was allowed to develop ammonia over 2-3 hours. The ammonia was then determined and at the same time some of the blood was introduced into a tonometer filled with carbon dioxide at atmospheric pressure. The blood was rotated and mixed in the tonometer, the carbon dioxide having been renewed a few times. After 10-15 min. of action the blood was again analysed for free ammonia. The results are shown in Table X. The duplicate

Table X.

Blood	Time after shedding into open vessel	Ammonia-N/100 ml.	Ammonia-N/100 ml. after carbon dioxide
	min.	γ	(760 mm.) action γ
I	176	99	101
II	121	107	100

determinations on blood from two different subjects gave 99γ and 107γ ammonia-N/100 ml. before, and 101γ and 100γ ammonia-N/100 ml. after the

carbon dioxide action. It appears therefore that carbon dioxide of this tension although preventing any formation of ammonia over a long period is without effect in reversing the formation once the free ammonium ion has appeared in solution.

V. DISCUSSION.

Accuracy of method.

With the "unit" described and utilised in previous communications it is shown to be possible to determine ammonia in 1 ml. of standard solutions to an ultimate standard deviation of 2γ ammonia-N/100 ml. For the blood ammonia determination as described above, the absorption was limited to 50–70 % of the total (10–15 min.). This limitation with standard solutions comes to mean a determination of ammonia-N to an ultimate standard deviation of 4γ . These are exact statements of accuracy and are readily reproducible. How they apply in the actual practice of blood determination will appear from an analysis of the 21 determinations made on blood shed into a carbon dioxide atmosphere within the first 12 min. after shedding. The standard deviation of the individual value in these 21 determinations was 5γ ammonia-N/100 ml. (mean value 3.7). These values include however the 11 values in alveolar air which, from the curve over a long subsequent period, we may suppose to have risen slightly. For the 10 values in pure carbon dioxide the standard deviation found was 4γ ammonia-N. For 29 determinations in all carried out on 7 subjects for open collection within the 3rd to the 10th min. afterwards the standard deviation of the individual determination was 6.6 (mean value 38γ). The latter result is in itself remarkable not only for establishing the accuracy of the determinations but also indicating a constancy of ammonia formation under such conditions, since the standard deviation carried out on the same standard solution would in fact be 4γ ammonia-N, and a slight rise is also occurring over the 3rd to the 10th min. in blood collected with the open method. For determinations of the blood ammonia described by other workers there are no such precise statements of accuracy. We may select for comparison however the four results published by Van Slyke and Hiller [1933] on the same blood sample. They used 5 ml. blood with each determination in accordance with the Nash and Benedict technique. The mean value was 48γ ammonia-N/100 ml. and one of the four determinations deviated by 7γ /100 ml. This may be compared with the results of 11 determinations on blood samples from two different subjects, analysed between 3 and 12 min. after shedding. The maximum deviation found was 5γ ammonia-N/100 ml. from a mean value of 38γ ammonia-N/100 ml. A more exact comparison of the accuracy found by Van Slyke and Hiller, so far as may be judged from their published data, can be made in the following way. In their published figures there are 6 duplicates given and 4 determinations on the same blood, which latter may be regarded as 2 duplicates, and in all therefore, 8. For such minimum quantities the absolute rather than the percentage error is significant and from the following formula we may determine the standard deviation of the single determination

$$2\sigma^2 = \frac{1}{N} \sum (X_1 - X_2)^2,$$

where X_1 and X_2 are the members of a duplicate and σ is the standard deviation required. From the data in question and considering that 5 ml. were used the result is 0.19γ ammonia-N. From the results of the present method we have 0.04γ ammonia-N (or 4γ N)/100 ml. which shows therefore five times the accuracy in absolute quantities and permits the same relative accuracy with one-fifth the quantity of blood.

As mentioned above, Van Slyke and Hiller had the advantage over other workers with this method of a more delicate reagent than Nessler's.

The present method permits of the rapid and easy accumulation of large numbers of determinations. For example, with one blood sample from the beginning of the period of shedding up to the 4th min. 8 determinations were set up and completed within 26 min. In all 159 determinations were assembled, 96 in a later group, in 6 working days.

The true content of ammonia in human venous blood.

The examination of the general time concentration curve where the blood was collected in an open vessel (paraffined Erlenmeyer) shows the erroneous conclusions hitherto arrived at from an extrapolation of the secondary rise over the first few min. The true curve of formation rises steeply and apparently from the origin to a concentration level of 38γ ammonia-N/100 ml. which is reached about the 3rd min. It enters then on the slow ascent described by other workers.

An entirely different picture is presented if the blood is collected in an atmosphere of alveolar air. The initial steep rise disappears and for the first 12 min. after shedding the mean value of 11 determinations is $3.1 \pm 2.2\gamma$ ammonia-N/100 ml.

Is the value in fact zero? It may be noted that even 3.1γ ammonia-N/100 ml. represents one part in 32 millions, and is one-tenth of the very minute value previously thought to represent the true ammonia content of circulating blood. The latter may in fact be zero particularly in the light of the following considerations. Examining the ammonia content after collecting in alveolar air, what seemed when the first 12 min. only were taken into consideration to be a complete cessation of ammonia formation showed itself to be similar to the general slow and secondary rise with the open vessel collection when much longer time periods were investigated (see Fig. 4). The curve of this formation which appears as a straight line up to about the first hour points unmistakably into the origin. The rate of formation as indicated by this line is 0.66γ ammonia-N/100 ml. per min. If we consider that the mean time for the 11 determinations over the 12 min. period is 5.6 min., then in this period we may expect 3.7γ ammonia-N/100 ml. formed. The mean of the 11 determinations is however 3.1γ ammonia-N/100 ml. We can therefore conclude that the ammonia content of circulating human blood is zero from these findings or so small that it is quite beyond any present method of determination. Both the initial steep rise to 38γ ammonia-N/100 ml. and the secondary slow rise are stopped by pure carbon dioxide. For a long period (at least 2 hours) after collection in pure carbon dioxide only minimum quantities of ammonia are present. The average up to 128 min. of 10 observations is 4.4γ ammonia-N/100 ml.

There now arises for consideration the following question: Is the absence of free ammonia from circulating blood merely an analytical result due to the combination of the free ammonia liberated after adding alkali with free carbon dioxide? Such a point was already raised by Rehberg [1926]. It is conceivable that the ammonia could in this way combine with free carbon dioxide (which presumably would have insufficient time to disappear as carbonate ion) and form ammonium carbamate. This in turn would be slow to disintegrate at the high p_H . The following facts however completely decide the point. Small quantities of ammonia immediately added to blood should not be recoverable at all or only in very reduced amounts. In fact they are recovered practically quantitatively. Again if the free carbon dioxide operates in such a fashion an increase of twenty times its value should have an extremely marked effect. It has been shown

however that once the ammonia is formed in the blood after shedding, subsequent treatment with pure carbon dioxide at atmospheric pressure has no appreciable effect on its concentration. This completely rules out the above conception of an instantaneous carbamate formation with subsequent slow disintegration.

The mother substances of the ammonia formed after shedding.

From the period of the discovery of Embden *et al.* [1928] of the free ammonia in muscle arising from adenylic acid a similar origin for the ammonia formation in shed blood was a recognised possibility. Adenylic acid in blood was quantitatively determined by Buell and Perkins [1928] and a parallel formation of free purine bases and ammonia in blood was demonstrated by Mozolowski [1929] who showed the process of formation to pass through inosinic acid which subsequently broke down to form free purines. The purine-nitrogen formed however only accounted for a fraction of the total ammonia-N. That at least two substances were involved was further demonstrated by Heller and Klisiecki [1935]. Adenylic acid corresponds to their substance B. With intact corpuscles adenylic acid in fact does not break down at room temperature for 24 hours or more. Even at 37° the beginning of this process is only demonstrable after 24 hours. The ammonia formation considered above would therefore, from these findings, have nothing to do with adenylic acid breakdown. The formation would correspond to the substance A of Heller and Klisiecki, which gives rise to upwards of 500 γ ammonia-N/100 ml. From the experiments described in this communication it seems very likely that at least the first stage of the formation is due to the breakdown of a carbamate or carbamino-compound. The existence of carbamino-compounds in blood is very probable from the work of Henriques [1928] and in particular that of Meldrum and Roughton [1933]. (This work has been reviewed by Roughton [1935].) Whether however the secondary slow formation of ammonia in shed human blood (using an open vessel) is also associated with a similar breakdown is more doubtful, since although atmospheric tension of carbon dioxide completely excludes it over a long period the p_H change produced by this tension enters also as a possible factor. The action of carbon dioxide on the ammonia formation 5 min. after shedding was in fact also examined by Parnas and Heller [1924] who used it however with the object of producing hydrogen ion changes and explained the effects produced by reference to this factor. Such results may now be explained very differently, namely by a specific action of carbon dioxide. From the results of Parnas and Heller [1924] at tensions of carbon dioxide ranging from 155 mm. to 0.2 mm. the rate of formation within the first 5 hours or so varied according to the tension from 2 to 5 γ ammonia-N/100 ml. per min. (at 20°). At the same time the reaction altered from p_H 6.7 to 8.4. When the reaction becomes markedly alkaline the rate of breakdown of a carbamino-compound becomes considerably reduced, so that in fact with changes of carbon dioxide tension we should have an interplay of two effects on the rate of breakdown. For the second slow rise it will probably be an easy matter to dissociate the p_H effect and the specific effect of carbon dioxide, though the introduction of new reagents such as phosphate buffers is not altogether free from objection. It is interesting in this connection that Krebs and Henseleit [1932] have also noticed and commented upon a specific carbon dioxide effect in the formation of urea in liver. The rate of formation is markedly reduced when the carbon dioxide content falls, even though the reaction be maintained by the use of phosphate and a sufficiency of carbon dioxide be present for urea production.

Considering that the breakdown of a carbamino-compound is required to explain the first rapid change with open shedding, the question arises is carbamic

acid itself responsible? The instability of carbamic acid and its general association with the ammonium ion in solution would on a first consideration render it very unlikely to account for the change. It is necessary to inquire however if any fraction may be assigned to its breakdown. From the following two equations

we may derive

$$\frac{[\text{NH}_4^+]}{[\text{NH}_2\text{COO}^-]} = \frac{K_G \times [\text{H}^+]}{K_{\text{NH}_4} \times [\text{HCO}_3^-]}.$$

Here K_G has been determined by Faurholt [1922, 1, 2] as 0.33 (being very little affected by temperature) and inserting approximate values for the other components on the right side of the equation we have

$$\frac{[\text{NH}_4^+]}{[\text{NH}_2\text{COO}^-]} = 10^{3.1}.$$

The ammonium ion concentration must therefore be a thousand times greater than that of the anion of carbamic acid. But this latter is about half or a very appreciable factor of the whole carbamic acid present if we accept the dissociation constant given by Faurholt. This constant was very roughly determined as 10^{-7} – 10^{-8} . It was in fact placed considerably lower by Wegscheider [1922], namely at 5.5×10^{-10} , a figure which would necessitate the salts of carbamic acid being considerably dissociated. Such dissociation was not found by Faurholt. Considering that no measurable ammonia is present in the circulating blood it is clear that no appreciable amount of carbamic acid can exist therein. The substance in question accepting it as a carbamino-compound must therefore be a derivative of carbamic acid. Such a compound even at the carbon dioxide tension of alveolar air would be practically undissociated into the free amine. Also when the amine is formed a further process of deamination is necessary to explain the appearance of the free ammonia. Further experiments are clearly necessary to warrant any further speculation on such lines.

In conclusion it may be pointed out that apart from the nature of the precursor present, the results described above for the normal human subject remove any real significance from previous determinations of the blood ammonia which purport to represent the actual conditions in circulating blood.

VI. SUMMARY.

1. A method is described for the determination of very minute quantities of ammonia (less than 1γ) in which the ammonia absorbed is determined to an ultimate standard deviation of 0.02γ (0.00002 mg.) ammonia-N. It consists of some additional refinements introduced into the method described in a previous communication [Conway and Byrne, 1933]. Applying the method to the determination of ammonia in 1 ml. blood, the absorption period being limited to 10–15 min., the standard deviation for the determination of minimum quantities of ammonia is 0.04γ ammonia-N. In the 10–15 min. period, 50 to 66% of the whole ammonia present is absorbed. The relative error involved in this limitation of the absorption period with the special "units" may be expressed as a coefficient of variation of 2.8%.

2. It is shown that the free ammonia present in shed human blood depends very largely on the manner in which the blood has been collected. When received into a small Erlenmeyer flask directly from a vein a steep rise occurs which

terminates about the 3rd minute, and passes into the slow ascent previously described by other workers. When the first curve is extrapolated over the first 0.75 min. after shedding it passes through the origin. When the second curve is extrapolated it cuts the ordinate at 38 γ ammonia-N/100 ml. corresponding to the concentration previously accepted as representing the concentration of free ammonia in circulating blood.

3. When the blood is received into an atmosphere of alveolar air for about the first 12 min. after shedding the quantity found is scarcely distinguishable from zero, being 3.1 γ /100 ml. It is quite indistinguishable at zero time when allowance is made for the slight ammonia formation in the period as calculated from the subsequent rise over 2 hours. The first steep rise is completely absent, the secondary slow ascent being still present. This curve of formation is a straight line up to about 1 hour and points directly into the origin.

4. Blood received into pure carbon dioxide at atmospheric pressure forms no ammonia over a long period after shedding. The average of 11 observations up to 128 min. after shedding is $4.4 \pm 1.3\gamma$ ammonia-N/100 ml.

5. When ammonia is once formed after shedding subsequent action of carbon dioxide even at full atmospheric pressure has no noticeable effect in diminishing the concentration present.

6. It is concluded from the controlling influence of the carbon dioxide tension on ammonia formation that the first stage of ammonia formation in shed blood is associated with the breakdown of a carbamino-compound.

7. The action of saturated potassium carbonate on blood when mixed in equal quantities has also been investigated. It is shown that 0.0088 γ ammonia-N per min. per ml. blood is formed in the presence of the alkali.

The results summarised here are based on a large number of determinations (159 in all) on human blood.

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