XXXI. FURTHER DEVELOPMENTS OF RAPID VOLUMETRIC METHODS FOR THE DETERMINATION OF AMINO-ACIDS, ORGANIC ACIDS AND BASES.

II. RAPID QUANTITATIVE REMOVAL AND DETERMINATION OF THE CARBONIC ACID RADICLE, ESPECIALLY IN BACTERIAL CULTURES, WITHOUT EXPOSING OTHER CONSTITUENTS TO RISK OF LOSS OR CHANGE.

By FREDERICK WILLIAM FOREMAN.

*From the Animal Nutrition Research Institute,
School of Agriculture, Cambridge.*

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IN a new method described in the preceding paper [Foreman, 1928] "freed" volatile bases in the "alcohol extracts" of biological fluids are very rapidly expelled by means of a current of steam. It seemed possible that "freed" volatile acids might respond similarly, but on investigation they were found to behave in the directly opposite manner, showing less tendency to escape the higher the alcohol concentration. During the collection of the earliest small fractions of their steam distillates, solutions as highly alcoholic as "alcohol extracts" (87-88 % by vol.) were very highly retentive of "free" volatile acids. As the alcohol percentage fell and consequently as the temperature approached nearer to 100°, the volatile acids, in spite of the progressive reduction in their concentration, passed over at a gradually increasing rate which reached a maximum by the time the liquids were practically alcohol-free.

The phenolphthalein titration values of the first one-minute distillates from 105 cc. quantities of 92 % alcohol containing (a) acetic acid to the value 9.0 cc. *N*/10, (b) butyric acid to the value 9.43 cc. and from 55 cc. quantities of 88 % alcohol containing (c) acetic acid to the value 9.0 cc., (d) butyric acid to the value 9.43 cc., were 0.16, 0.17, 0.32, 0.31 cc. *N*/10 respectively. The one-minute periods were timed from when the distillates commenced to run into the neck of the condenser. Series of successive one-minute distillates obtained in the case of (b), (c) and (d) required *N*/10 alkali as follows:

(b) 0.17, 0.17, 0.2, 0.3, 0.42, 0.7, 0.7, 0.72, 0.71, 0.6, 0.5 cc.

(c) 0.32, 0.6, 0.75, 0.72, 0.72, 0.72, 0.6.

(d) 0.31, 0.47, 0.82, 1.2, 1.14, 0.94, 0.69, 0.55.

The condenser was well washed down between each fraction. 105 cc. and 55 cc. of 87 % alcohol lost practically all their alcohol in 5 to 6 and about 4 minutes respectively. In (b) the liquid in the flask gained an average of about 8 cc. water per minute as condensed steam.

It will be noted that only very small percentages of the volatile acids present were expelled during the first minute.

50 cc. water containing butyric acid to the value 11.14 cc. *N*/10, warmed nearly to 100° before commencing the steaming, gave the following successive one-minute values:

2.8, 1.35, 0.98, 0.86, 0.7 cc. *N*/10.

A brisk current of steam kept as uniform as possible throughout these experiments was used.

The high retentiveness of 87 % alcohol for volatile acids when steamed, even though the temperature was probably as high as 85°, coupled with another observation that CO₂ escapes extremely readily from its partially saturated solution in alcohol on exposure to the air at room temperature, gave rise to the suggestion that at temperatures well below 85°, a current of air passed through a solution of carbonic acid and "free" volatile acids in 87 % alcohol, causing practically no change in the alcohol concentration, might very rapidly remove the CO₂ completely and leave the whole of the volatile acids behind. These expectations were fulfilled and a simple rapid room temperature process for completely¹ removing and estimating the carbonic acid radicle in "alcohol extracts" without exposing other constituents (including volatile bases) to risk of loss or change, was evolved.

DESCRIPTION OF THE METHOD.

Rapid elimination of the carbonic acid radicle from "alcohol extracts." The "Stage 2" value of the "alcohol extract" to be examined is first accurately determined exactly as if only amino-acids were present [see Foreman, 1928, p. 211]. Another aliquot portion, 25 cc. if plenty, and 50 cc. if little, of the carbonic acid radicle is present, is placed in the 250 cc. Erlenmeyer flask (*A*), and a quantity of accurate *N*/10 HCl equal to the "Stage 2" value added² from a burette. The tightly fitting rubber stopper containing tubes (*a*) and (*b*) is then fixed securely in position so that the lower end of tube (*a*) is as near to the bottom of the flask as possible without actually touching. Tube (*a*) is 4½ to 5 mm. internal diameter. Its lower end should be a simple straight cut-off at right angles, and the opening should not be reduced in size. It is attached to the 250 cc. wash-bottles each containing about 50 cc. of 1 in 2

¹ It was found that, in addition to introducing errors in subsequent estimations, precipitation processes, *e.g.* with barium hydroxide, do not eliminate the carbonic acid radicle satisfactorily.

² This addition of HCl was originally introduced for liberating carbonic acid from its salts and to fix volatile bases during the CO₂ removal. Probably much less would suffice. Later findings indicate that in the great majority of cases the addition of HCl may be dispensed with altogether (see p. 226).

potassium hydroxide. A very brisk¹ current of air, but not so fast that the splashes approach too near tube (b), freed from CO₂ by the potassium hydroxide, is drawn by means of the pump through the alcoholic liquid for 3 minutes if 25 cc., or 5 minutes if 50 cc., of the "alcohol extract" is used. Tube (a) is then closed by clip (f) and air admitted *immediately* through tap (d). The rubber attachments on tubes (a) and (b) are then removed, the stopper containing these tubes is raised, and tube (a) washed down with 5 cc. rectified spirit from a pipette. Finally the inlet tube of B₂ is sealed by clip (e) in order to preserve the potassium hydroxide for further estimations.

Fig. 1.

The periods mentioned afford ample margins of time for securing the complete removal of the CO₂ at room temperature. If flask (A) is placed in a water-bath at 40 to 45° while the suction is applied, 3 minutes is more than sufficient even for 100 cc. (see p. 228), but although no loss of volatile acids occurs at this temperature yet, for the sake of saving a very little time, it seems inadvisable to sacrifice the assurance associated with a room temperature process that the other constituents have not undergone change. At the close of an operation if warming has been practised the lower end of tube (b) and the base of the stopper may show traces of condensed alcohol. Apparently the lower end of tube (a) being so near the bottom causes the splashes to be directed on to the walls of the vessel for about half the way up, the aeration being the more effective the less the depth of the liquid. Perhaps therefore a somewhat larger Erlenmeyer would be more suitable for 50–100 cc. of "alcohol extract."

Estimation of the remaining alcohol-titratable acid radicles. After the addition of a quantity of rectified spirit equal to ten times the volume of the N/10 HCl originally added, the "Stage 2" value of the alcoholic liquid resulting from the aeration is accurately determined by titrating to phenolphthalein with accurate N/10 carbonate-free alcoholic potassium hydroxide (standardised with phenolphthalein as indicator). The "Stage 2" value for

¹ In many successful estimations at room temperature and at 45° aerating about 30 cc. alcoholic liquid the pressure was reduced 50–80 mm. by the suction.

the whole of the alcohol-titratable acid radicles of the "alcohol extract" other than the carbonic acid radicle is given on subtracting the value of the $N/10$ HCl originally added from the reading of alcoholic potassium hydroxide.

This net "Stage 2" value may be differentiated into class values on applying the methods described [Foreman, 1928] to this solution, or the solution may be used for any estimation, *e.g.* volatile acids, with the advantage that, in the absence of the carbonic acid radicle, the results are more precise. Owing to the fact that a little of the CO_2 is caught in a volatile acids distillate, it is important, especially when the volatile acids content is small, to eliminate the carbonic acid radicle prior to their distillation.

Estimation of the carbonic acid radicle. The difference between the original "Stage 2" value and the net "Stage 2" value after the aeration gives the $-\text{HCO}_3$ value of the "alcohol extract." The "blank" value is usually very minute.

Loss of CO_2 in preparing and titrating "alcohol extracts." An advanced culture of bacteria in a highly concentrated unneutralised meat extract contained the $-\text{HCO}_3$ radicle at a concentration of about $N/15$. The culture was very nearly neutral to phenolphthalein, showing the CO_2 to be practically all in the form of bicarbonate. Examined by the alcohol methods, including the CO_2 process, the balance sheets [Foreman, 1928] showed that 15 % of the $-\text{HCO}_3$ radicle originally present in the culture had been lost, leaving an equivalent amount of "free" weak nitrogenous bases in the alcoholic liquid. Supplementary experiments showed that this loss was due to the escape of CO_2 during the short exposures involved in preparing and pipetting the "alcohol extract" and while agitating it during the "Stage 2" titration, apparently mainly in the titration.

Since the percentage of the total CO_2 lost in these exposures would fall to a largely increased extent the higher the degree of dilution, loss may be inappreciable when the carbonic acid content is very small or even when dilute media contain moderate amounts. However, bearing in mind that the advantages of carbonic acid elimination (see above) and perhaps other advantages [see Foreman, 1928, p. 212] are gained by direct applications of the method to ordinary "alcohol extracts," that part of the loss which occurs during the direct "Stage 2" titration, or the total loss if a figure for the whole of the carbonic acid of the culture is desired, may be avoided as follows.

Avoiding loss of CO_2 during a "Stage 2" titration. The first aliquot portion of the "alcohol extract" [Foreman, 1928, p. 211], withdrawn by gentle suction in the pipetting, is delivered with the tip of the pipette under the surface of a mixture of $N/10$ alkali sufficient to provide excess with any extra 97 % alcohol which may be needed to give 87 % alcohol by volume. The solution is then titrated back with $N/10$ HCl. The CO_2 is thus all fixed as NaHCO_3 at the commencement of the titration. This "Stage 2" value instead of that obtained by direct titration is then used in determining the $-\text{HCO}_3$ value as described above.

Avoiding all loss of CO_2 in estimating the $-\text{HCO}_3$ value of the culture. A full account of this process as suggested for highly concentrated cultures has been already given [Foreman and Graham-Smith, 1928, Appendix]. Briefly, a portion of the culture is delivered with the pipette lip under the surface into sufficient CO_2 -free sodium hydroxide to render just alkaline to phenolphthalein the solution obtained on subsequently adding two volumes of 97 % alcohol. Much undesirable matter with most of the pigment is precipitated in this 65 % alcohol but the whole of the NaHCO_3 and volatile acids are left in solution. An aliquot part of this solution is then delivered under the surface into a mixture of 1 cc. $N/10$ sodium hydroxide and sufficient 97 % alcohol to yield 87 % alcohol when the aliquot part is present, and $N/10$ HCl is run in drop by drop until the "Stage 2" end-point is reached. The measured quantity of $N/10$ HCl is then added, the CO_2 removed, and the liquor titrated with $N/10$ alcoholic potassium hydroxide, as described for ordinary "alcohol extracts" (p. 223). The difference between the value of the measured quantity of $N/10$ HCl and the reading of $N/10$ alcoholic potassium hydroxide gives, after subtracting the very small $-\text{HCO}_3$ value of the sodium hydroxide used, the $-\text{HCO}_3$ value of the culture. The same liquid is then used for estimating volatile acids which will be obtained in CO_2 -free condition.

Carbonic acid in the form of bicarbonates of weak nitrogenous bases in the aqueous solution behaves like "free" carbonic acid on aerating the "alcohol extract" at room temperature. The observation in regard to the loss of CO_2 from the "alcohol extract" of the neutral culture mentioned on page 225 suggested that "weak" nitrogenous bases had no power to augment the low retentiveness of strong alcohol for carbonic acid, and that it might be possible to eliminate the whole of the carbonic acid radicle by simple aeration without employing mineral acid. Using the apparatus (Fig. 1), a very gentle air current, passed for 10 minutes through 25 cc. portions of the "alcohol extract," at room temperature, completely removed the CO_2 and left the whole of the other constituents intact. No loss¹ of volatile bases occurred, estimations made before and after the aeration giving identical results. Unfortunately the effects of brisk air currents (p. 224) were not investigated.

By means of this modification it was found possible to show that ordinary salts of non-volatile amines, and not substances of the nature of amino-acids in possessing "internal salts," were responsible for the considerable extent by which the "total alcohol value" of the "alcohol extract" of this culture exceeded the comparable Sørensen-formaldehyde value [see Foreman and Graham-Smith, 1928, p. 61].

Whether or no the mineral acid may be omitted in the case of "alcohol extracts" of cultures containing "free" nitrogenous bases or of cultures in media neutralised with sodium hydroxide before inoculation, requires

¹ Since weak nitrogenous bases show no basicity to phenolphthalein in the "Stage 2" titration, loss of volatile bases during the aeration would not affect the $-\text{HCO}_3$ estimate. Loss of volatile bases, however, would vitiate certain subsequent estimations (p. 225).

investigation, but it seems possible that provided a sufficiency of alcohol-titratable acid radicles (other than the carbonic acid radicle) is present, the $[H^+]$ of the "alcohol extracts" may be high enough for a complete removal of CO_2 at room temperature without loss of volatile bases. The HCl addition, however, would be necessary in the case of "alcohol extracts" made from aqueous solutions of the carbonates or bicarbonates of "strong" bases alone or in the presence of less than an equivalent quantity of alcohol-titratable acid radicles.

Cultures fairly acid to phenolphthalein which have been incubated at 37° usually contain only traces or very small quantities of carbonic acid. In these cases, since CO_2 estimations are unimportant, it is now customary to introduce aliquot portions of the "alcohol extracts" to be used for other estimations straight into the apparatus and, without additions of any kind, to aerate gently for a few minutes at room temperature.

EXPERIMENTS.

Using the apparatus (Fig. 1), brisk air currents were drawn for differing periods through mixtures of 97 % alcohol, a half-saturated solution of CO_2 in 97 % alcohol and known quantities of standard solutions of common volatile acids, at different temperatures. The results are shown in the following table:

Table I.

Acid	97 % alcohol used cc.	$\frac{1}{2}$ sat. alcoholic CO_2 added		Aqueous acid added cc.	% alcohol in mixture	Suction period minutes	Temp.	Pp. value of aqueous acid added cc. N/10 NaOH	Result after allowing for spirit acidity cc. N/10 alc. KOH
		quantity cc.	$-HCO_2$ value cc. N/10						
Acetic	25	5	2.15	0	—	2	room	0	0.05
	25	5	"	5	83	2	"	9.0	9.0
	25	5	2.85	0	—	2	50°	0	0.02
	25	5	"	5	83	2	46°	9.0	8.99
	25	5	3.45	5	83	2	room	9.05	(a) 9.15
Formic	25	5	"	5	83	2	$45-50^\circ$	9.05	(b) 9.05
	25	5	about 3.0	5	83	3	room	9.43	9.43
Butyric	25	5	"	5	83	3	$40-45^\circ$	9.43	9.40
	50	5	"	5	89	3	room	9.43	9.40
	25	0	"	5	81	5	"	9.43	9.43
	25	0	—	5	81	5	$40-45^\circ$	9.43	9.38
	30	0	—	0	—	5	"	0	0.02
Hydrochloric	25	5	3.45	6	81	2	room	6.0	6.03

A comparison of the figures in the last two columns shows that the CO_2 is completely removed in a very short time and that even at about 45° the volatile acids, in such concentrations, are completely retained. Perhaps traces of butyric acid were removed in 5 minutes at $40-45^\circ$ from 30 cc. of 81 % alcohol (see table), but having regard to the very small percentage lost in the first minute of steaming (p. 222) this inference seems hardly justifiable. It may be mentioned that a mixture exactly as in the fourth acetic acid experiment which had been gently moved for 2 minutes in the open vessel placed in water at 90° (no suction) titrated 8.98 cc. (cf. 9.0 cc. for the acetic acid used).

Various tests of a similar nature were applied to an old "alcohol extract" of an advanced putrefactive culture. Since it contained nitrogenous bases it was important to take care that the requisite minimum of 86 % alcohol was present at the close of the titrations, and it proved convenient to use $N/10$ alcoholic potassium hydroxide. Mixtures of 10 cc. portions of the "alcohol extract," 50 or 100 cc. 97 % alcohol, 10 cc. half-saturated alcoholic CO_2 and a quantity of $N/10$ HCl equal to the previously determined "Stage 2" value were aerated as described. For the 75 cc. mixtures, 5 minutes at room temperature or 3 minutes at $40-45^\circ$ gave the correct titration value, and 2 minutes at 45° practically sufficed. Three minutes at $40-45^\circ$ was also practically sufficient for the 130 cc. quantities but 5 minutes at room temperature was not quite long enough. 30 cc. quantities of alcoholic liquid made from the "alcohol extract" of the culture referred to on page 225, exactly alike in content of "extract" and HCl were aerated 3, 6, and 10 minutes at room temperature. The final titration results were identical.

It was evident that the rapidity with which the CO_2 was expelled depended largely upon the depth of the alcoholic liquid. The deeper the liquid the slower the expulsion. Possibly some part of this effect was due to the fact that when the smaller volumes were aerated there was more room for the splashes and more vigorous air currents could be used without fear of contaminating the outlet tube. Consequently, using the 250 cc. Erlenmeyer flask (p. 223) it seems advisable to limit the volume of alcoholic liquid actually aerated to about 50 cc. for room temperature estimations, and to aim at volumes nearer 30–35 cc. with a "Stage 2" value of 2 to 5 cc. $N/10$ solution.

Possible explanations. Traces of CO_2 dissolved in distilled water are removed with difficulty even on boiling. Apparently the rate at which the CO_2 is expelled from water depends mainly upon the effects of temperature and dilution upon the molecular and electrolytic dissociations of the carbonic acid. The greater the mass of the water relative to the quantity of carbonic acid present, the greater the depression of the dissociation: $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$. Consequently the CO_2 is expelled at a slower rate as the dilution increases through CO_2 expulsion. Neglecting the possibility that the dissociation constant increases with rising temperature, the electrolytic dissociation would increase with increasing dilution, and it seems probable that the corresponding decrease in the concentration of un-ionised H_2CO_3 might produce a further adverse effect upon the molecular dissociation especially after boiling for some time, when the dilution would be extremely high. Perhaps the addition of other acids facilitates the complete expulsion of CO_2 owing to the increase in concentration of un-ionised H_2CO_3 caused by their H ions depressing the electrolytic dissociation.

The addition of alcohol to aqueous carbonic acid solutions virtually brings about the removal of water, thus tending to eliminate the adverse effects mentioned.

I have not made a systematic study of the rates at which CO_2 may be

expelled from aqueous solutions under varying conditions of temperature and dilution. Actual boiling is always regarded as essential, however, in those analytical processes which depend upon the complete expulsion of CO_2 after adding excess of mineral acid. In the case of one such process, namely that of Amos [1906] for estimating carbonates in the soil when the content is small, it is now generally recognised that the hot aqueous mineral acid exercises a decomposing effect upon the soil's organic matter to yield appreciable amounts of CO_2 . Therefore when organic matter is present application of heat is very undesirable.

It will be shown in another communication that the dissociation constants of "weak" nitrogenous bases seem to be lowered to about one-thousandth of the values exhibited in water on adding alcohol up to 87 % by volume to the aqueous solutions, their dissociation curves then occupying correspondingly higher positions in the $[\text{H}^+]$ scale. Apparently in 87 % alcohol their titration curves commence, whereas in water they finish, at about p_{H} 8.5. Hence alcohol added up to 87 % to aqueous solutions of salts of such bases would liberate the acids to an extent depending upon the resulting $[\text{H}^+]$ which would be determined by the "strength" of the acids. It appears therefore that the CO_2 was expelled from the "alcohol extract" of the culture which contained its CO_2 as bicarbonates of such bases (p. 225) practically as easily when no HCl had been added owing to the fact that it was virtually a solution of "free" carbonic acid which was aerated. Probably no loss of volatile bases occurred during this aeration because the organic acid radicles possessed the power of keeping the $[\text{H}^+]$ at a high enough level under the room temperature conditions.

SUMMARY.

Strong alcohol in which carbonic acid and small quantities of "free" volatile acids are dissolved very readily parts with the whole of the CO_2 but completely retains the volatile acids on aeration. In this solvent, bicarbonates of "weak" nitrogenous bases and "free" carbonic acid lose their CO_2 on aeration with equal readiness. A simple rapid room temperature process and a useful modification, applicable to fluids such as bacterial cultures, for estimating and eliminating the carbonic acid radicle, are based upon these observations. There is no risk of loss or change in any of the other constituents and subsequent determinations are more precise.

REFERENCES.

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Foreman (1928). *Biochem. J.* 22, 208.
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