Yeast.	Pabulum.	Detection of methyl alcohol in media. Nitromethane method.	 Yanagisawa's method after oxidation.	 Rimini's method after oxidation.
"Saké" yeast A. No. 34	"Koji"-extract	+	+	—
Wine yeast (Oppenheimer).	"	+ trace	+?	—
Beer yeast (Bottom)	"	+	++	+?
Distillery yeast	"	+	+	—
Wine yeast (Oppenheimer).	Raisin extract	+	+	—?
"Saké" yeast A. No. 34	Hayduck's solution	+	+(+)	—
do.	(1% glycocoll instead of asparagin in Hayduck's solution).	+	++	+?
Beer yeast	do.	+	+	+
Beer yeast	Hayduck's solution	+	++	+
Distillery yeast	" "	+	++	—

Thus, the formation of methyl alcohol by all kinds of yeasts examined is proved. Especially in the case of "saké" yeast, the quantity of methyl alcohol formed was increased when glycocoll was used instead of asparagin in Hayduck's solution. The other interesting fact to be noticed is the behavior of the wine yeast to be pabulum; the quantity of methyl alcohol formed was decidedly larger in the raisin extract than in "koji" extract, which, after the fermentation, was proved to be almost free of it.

Summary.

1. In the distillates of alcoholic beverages, which we have examined, we could not find formaldehyde directly when we distilled below 80°, but after the oxidation of the distillates there were found differences in regard to the quantities of methyl alcohol according to the kinds of alcoholic beverages. The smallest quantity was found in the case of "sake," except a special sample, in which there was a distinct evidence of methyl lactate.

2. In the case of a doubtful test a large sample was taken for analysis, followed by the redistillation of the distillate.

3. All the kinds of yeast tested, "saké-", beer-, wine-, and distillery-yeasts formed methyl alcohol in the saccharine fluids and the quantity of it is increased by the addition of glycocoll as a nourishment. This fact exactly coincides with Ehrlich's[1] theory of the fermentation of protein matter.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF WASHINGTON.]

ACCURATE METHODS FOR THE DETERMINATION OF TOTAL ACIDITY OF URINE.

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The purposes of this paper are (1) to show the influences of urea and

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[1] Ehrlich, *Brewer's J.* (New York), 38, 144 (1914).

ammonia carbonate on the present methods of determining acidity of urine, (2) to discuss methods by which urea and ammonium carbonate can be expelled without loss of acids and their disturbing influence thereby eliminated, and (3) to demonstrate that the original total acidity can be determined even when urines have undergone final bacterial decomposition.

If solutions of acids are titrated to a faint pink or just to a colorless solution by an alkali and phenolphthalein, such solutions are not affected by the addition of ammonium carbonate solutions.[1] However, if the ammonium carbonate is first treated with an acid and is then titrated with an alkali, far different results are obtained, owing to the escape of carbon dioxide, in accordance with the reaction

$$(NH_4)_2CO_3 + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + H_2O + CO_2. \qquad (1)$$

Even the weak acids of urine, as hippuric, benzoic, uric, etc., exercise this influence, as shown in the following table:

ACIDS FIRST ADDED.

Acid.	Cc. $(NH_4)_2CO_3$. 1 cc. = 0.0041 g.	Volume of of acid first added.	0.1 *N* KOH next added.	Final volume of acid required for 1 cc. 0.1 *N* KOH.
Benzoic	....	....	1.00	21.00
Benzoic	10.00	40.00	1.00	44.00
Benzoic	10.00	44.00	1.00	48.00
Benzoic	10.00	48.00	1.00	50.00
Benzoic	10.00	50.00	1.00	51.90
Benzoic	10.00	100.00 heated	1.50	66.66
Hippuric	....	....	1.00	72.00
Hippuric	10.00	100.00	1.00	132.00
Acetic	....	....	1.00	1.58
Acetic	10.00	1.00	1.00	3.20
Acetic	10.00	3.00	1.00	4.20
Acetic	10.00	4.00	1.00	5.10
Acetic	10.00	5.00	1.00	5.50
Uric	....	....	1.00	500.00
Uric	10.00	500.00	1.00	1500.00

Here it is observed that, as more and more of the acid is first added to the ammonium carbonate, a greater total quantity of acid is required to establish the neutral point. As a matter of fact, it was found that only by *boiling for some time with an excess of acid* to drive off all of the carbon dioxide[2] can a definitely established end point in such titrations be reached. When boiling with an acid is not resorted to, only a part of the carbon

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[1] Solid ammonium bicarbonate and ammonium carbonate added to water are slightly alkaline toward litmus.

[2] If an ammonium carbonate solution containing phenolphthalein is made slightly acidic and colorless and is then boiled, on cooling the red color appears, indicating that heat decomposes the ammonium carbonate and that carbon dioxide is more quickly expelled than the ammonia.

dioxide escapes but its equivalent of acid must be supplied, to unite with the ammonia and other bases so as to reach the neutral point.

It might be imagined that, if an excess of alkali is first added to the ammonium carbonate, a definite end point would be given with the acid used for titrating. That this is not the case may be observed in the following table:

ALKALI FIRST ADDED.

Titrations.	Ammonium carbonate.	N KOH.	Benzoic acid.
1	....	1.00	21.00
2	1.00	1.00	23.70
3	2.00	1.00	24.40
4	10.00	1.00	32.50
5	20.00	1.00	45.00

Here *local acidity*[1] causes a disengagement of carbon dioxide, in accordance with Equation 1, although the solution may be alkaline when made homogeneous by mixing.

It is further evident from the above experiments that when titration with acids and alkalies are made *back and forth* in the presence of ammonium carbonate, indecisive end points are reached and discordant data are obtained. Also it must become evident that the acidity of urines developing ammonium carbonate by bacterial transformation of urea, cannot be titrated with accuracy by the methods now in use. Of course, this fact is well known. However, that fresh urines are more or less subject to this same influence, apparently has not been recognized hitherto.

An Acidic Urine.—The following data were obtained from a fresh acidic urine. The indicator used was phenolphthalein; the numbers are cubic centimeters of 0.1 N acids or alkalies.

		Titrations made.			Titrations by the new method.		
	Urine.	Direct.	Back and forth.	After heating with acid.	Total acidity.	Volatile acidity.	Ammonia evolved.
(*a*)	10.00	6.40	5.85	4.78	9.53	0.00	5.00
(*b*)	10.00	6.50	5.60	6.41	9.43	0.10	5.30

Here it is observed that the total acidity, as determined by the following method, is considerably higher than the total acidity by variations of the direct method.

An Alkaline Urine.—The following data were obtained from a fresh alkaline urine:

ALKALINITY.

		Direct titrations.		New method.			
	Urine.	Litmus.	Ph-ph.	Litmus.	Ph-ph.	Volatile acidity.	Ammonia evolved.
(*a*)	10.00	4.15	1.50	4.20	2.50	0.60	19.92
(*b*)	10.00	3.30	0.70	4.11	2.40	0.20	16.67
(*c*)	10.00	3.50	2.01	...	...	...	....

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[1] That local acidity is produced in titrations is evident in the colorless portions of the mixture before shaking.

Here it is observed: (1) the urine was truly *alkaline*, (2) litmus is more reliable for these titrations than is phenolphthalein, (3) the new method gives more consistent results than the direct method.

An Aged Urine (7 mos.).—This urine, which had been permitted to stand in a glass stoppered bottle for seven months, was analyzed by the following method: The clear portion was obtained by filtering; the mixture was obtained by dissolving, in a measured volume of 0.1 *N* acid, the thoroughly mixed liquid and precipitate. The data are calculated on the basis of 10 cc. of urine.

	Alkalinity by direct titration.		Total acidity by new method.		Volatile acidity.	Ammonia evolved.
	Litmus.	Ph-ph.	Litmus.	Ph-ph.	Litmus.	Litmus.
(A) Clear (*a*).......	60.20	3.00	6.64	10.80	1.70	72.20
portion (*b*)......	64.39	5.00	6.58	10.70	1.80	72.00
(B) Mixture (*a*).....	68.20	40.60	4.30	9.80	1.50	77.74
(*b*).....	65.64	36.76	4.52	9.00	1.80	77.30

Here it will be observed:

(1) Though strongly alkaline on account of free ammonia, the urine reveals by the method employed the original acidity. This acidity of the clear portion is equivalent to 6.60 cc. of 0.1 *N* acid; of the mixture, 4.40 cc. By difference the alkalinity of the sediment is apparently equivalent to 2.20 cc. of 0.1 *N* alkali.

(2) The alkalinity cannot be estimated directly when phenolphthalein[1] is used as the indicator. Litmus paper gives approximate values by direct titration, as shown in the respective alkalinities and the ammonia evolved.

(3) As shown by the lesser acidity of the mixture than of the clear portion of the urine, the urinary sediment is alkaline. This was also proven by dissolving the filtered sediment in 0.1 *N* acid and titrating its solution. Inasmuch as the sediment contains ammonia in urates and triple phosphates, a moiety of true acidity is contained therein, though the final reaction is alkaline. The increase of evolved ammonia given in the table bears this out.

(4) Since acidities determined by the use of phenolphthalein are invariably high, it is questionable whether this indicator is of any value for urines containing ammonia.

Total Acidity Method.

Into a 500 cc. Kjeldahl flask accurately measure 10 cc. of urine[2] and

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[1] See Berthelot, *Compt. rend.*, **131**, 552 (1900).

[1] Greater volumes of urine than 10 cc. are disadvantageous for the reason that too large volumes of 0.1 *N* acid and alkali thereby are required. Moreover, 10 cc. gives the easiest basis for calculation of acidity in the total volume of urine. Since this method is applicable to neutral and alkaline urines that have precipitated ammonium urate and earthy phosphates containing ammonia, the necessity is obvious of their thorough mixing by shaking and of rapid measurement with a pipet.

10–100 cc. of 0.1 N H_2SO_4. The volume of the latter is dependent upon the ammonia concentration of the urine and should be sufficient to maintain during the subsequent distillation a colorless solution with a little solid[1] phenolphthalein[2] used as the indicator. Connect the flask with a Hopkin bulb, a condenser, and an Erlenmeyer flask used as a receiver. Distil until[3] all but 5–10 cc.[4] have been collected as distillate. Turn off the flame, so as to cool and to drain for a minute or more.[5] To the Kjeldahl flask add an accurately measured volume of 0.1 N NaOH or KOH, sufficient[6] to maintain during the subsequent distillation a deep red color with the indicator present. Connect the flask with the distilling apparatus, change receivers and distil until all of the ammonia has been expelled—assured[7] when only about 5 cc. of liquid remains in the distilling flask.[8] Cool the Kjeldahl flask, transfer[9] its contents to the Erlenmeyer flask containing the first distillate and titrate[10] the mixture with 0.1 N H_2SO_4,

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[1] An alcohol solution of phenolphthalein is not recommended for the reason that the formation of ethyl sulfate should be avoided. The quantity of phenolphthalein should be very minute because the indicator is an acid.

[2] Of course, other indicators may be used; litmus paper is especially recommended for the final titrations. The former indicator invariably gives higher acidity values than litmus does. The reason for this will be discussed in an early paper on indicators.

[3] When only about 5 cc. of liquid remains in the flask, the flame may advantageously be turned so low that the liquid boils but does not distil from the flask. If heating is now continued, all of the urea will ultimately be transformed to ammonium sulfate. The exact conditions for this transformation, the use of urease for this transformation, and the possibility of simultaneous estimation of urea and total acidity will be studied.

[4] If the urine becomes too concentrated, caramelization, charring, and intensification of color of the urochrome may mask the color of the phenolphthalein. In these cases litmus paper may be used as the indicator.

[5] At this stage the distillate may be titrated for *volatile acidity.* This acidity can be increased somewhat by distillation to practical dryness; concentrating the H_2SO_4 hydrolyzes certain of the components of urine to volatile acids. For example, hippuric acid yields an increased quantity of benzoic acid. See THIS JOURNAL, **36,** 2130 (1914).

[6] The volume of 0.1 N alkali used here should be sufficient (1) to neutralize all of the 0.1 N H_2SO_4 added, (2) to neutralize the original acidity of the urine, and (3) to liberate all of the ammonia originally present and formed from urea. The ammonia from 10 cc. of urine containing 2% of urea is equivalent to 66.6 cc. of 0.1 N alkali. Therefore, 70 cc. of 0.1 N alkali, plus a volume equal to the 0.1 N H_2SO_4 used in the first distillation will ordinarily be sufficient.

[7] A drop of the distillate tested with a drop of Nessler's reagent will usually give a color, but indicates only a trace of ammonia.

[8] At this stage, for very accurate determinations, an excess of 0.1N H_2SO_4 may be added and the mixture heated to expel carbon dioxide present in the 0.1 N alkali used and formed from the urea and ammonium cyanate. (Vide infra.)

[9] When the volatile acidity has been titrated separately, the titration of the residual urine may conveniently be made in the Kjeldahl flask.

[10] Since all of the ammonia has been removed in previous operations, the use of

and litmus paper[1] used as the indicator. The sum of the 0.1 N alkali used in the various operations, minus the 0.1 N H_2SO_4 used, is equal to the acidity of 10 cc. of urine in terms of normal acid.

Discussion of Method.

It is obvious that ammonium carbonate, as formed from urea by bacterial ureases, is decomposed by the acids present in acidic urines. The liberated carbon dioxide may partially or completely escape, or remain in solution, while the ammonia forms salts with the free acids. When all of the acidic components of urine have united with ammonia, the urine becomes neutral, and any increase of ammonium carbonate tends to remain as such. However, owing to hydrolysis of ammonium carbonate and the greater volatility of carbon dioxide than of ammonia, such urines become alkaline. Therefore, urines progressively become less and less acidic, then neutral, and finally more and more alkaline. These changes take place in the urinary tract, continue with great speed after being voided, in many cases yield the alkaline stage in a very short time, and in all cases yield the alkaline stage in a day or two. Hence it must be evident that *ammonia more or less neutralizes and masks original total acidity in all urines.* Certainly in all 24-hour samples analyzed for total acidity, this becomes an important or serious factor.

The above method involves the complete elimination of carbon dioxide and ammonia without a loss of the acids of urine other than carbon dioxide. The first distillation from the solution made strongly acidic by the addition of 0.1 N H_2SO_4, assures the complete dissipation of carbon dioxide, since this has little or no tendency to condense in the distillate. Of course, during this distillation, other volatile acids of urine,[2] as benzoic, acetic, etc., are more or less completely carried over; these volatile acids are completely distilled only by evaporation to dryness. Since this distillate is subsequently added to the urine to be titrated, no component of urine except ammonium carbonate (and some urea) is lost in the two distillations.

Now since ammonium carbonate and other ammonium salts are volatile with steam, the first distillation with an *excess* of sulfuric acid is necessary not only to expel the carbon dioxide but also to prevent ammonia being carried over as easily hydrolyzed and volatilized salts. In fact, a di-

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potassium oxalate to prevent precipitation of earthy phosphates, as required by Folin's method, may be dispensed with. *Loc. cit.* Also Van Slyke and Bosworth, *J. Biol. Chem.*, 19, 75 (1914); 20, 144 (1915).

[1] Since phenolphthalein is already present, titrations to the end points of it and of litmus paper may be made. With the latter, pouring back and forth, (from the Kjeldahl and Erlenmeyer flasks), as the 0.1 N H_2SO_4 is added, will enable one to judge of the color of the litmus paper.

[2] THIS JOURNAL, 36, 2118 (1914).

rectly distilled acidic urine may yield an acidic, a neutral, or an alkaline distillate, depending upon the ammonia concentration in the urine.

In the above method, therefore, sufficient sulfuric acid must be used in the first distillation, not only to eliminate the carbon dioxide but also to retain all of the ammonia. In the following experiment, with a strongly alkaline, six months' old urine very different results were obtained:

	Urine.	Distilled first with 0.1 *N* H_2SO_4.	Distilled next with 0.1 *N* KOH.	Total 0.1 *N* H_2SO_4.	Total 0.1 *N* KOH.	Excess of
(*a*)....	25.00	55.50	75.00	91.46	79.30	12.16 acid
(*b*)....	25.00	111.00	150..00	136.75	159.50	22.75 alkali

According to (*a*) the urine apparently contained 12.16 cc. of normal fixed alkali; according to (*b*) it was found to contain 22.75 cc. of normal acid, present as ammonium salts. The distillate from the 55.50 cc. of 0.1 *N* sulfuric acid in case of (*a*) was alkaline and yielded by redistillation with 1–2 cc. of concentrated sulfuric acid, a new distillate that was acidic, thus demonstrating that ammonium *salts volatilize* when insufficient 0.1 *N* sulfuric acid is used. Of course, the first distillation may be considered efficient (1) when the urine containing the phenolphthalein remains colorless, (2) when the distillate is acidic, or (3) when the distillate does not color with a drop of Nessler's reagent.

Clarens[1] treated urines with 0.1 *N* HCl to expel the carbon dioxide and then titrated the residual mixture with 0.1 *N* KOH and phenolphthalein. He thus obtained "either acidic or alkaline urines." Of course, as demonstrated above, he fell into error because the ammonia was not eliminated.

The use and the varied conditions of the second distillation, with 0.1 *N* alkali, will now be considered. If all of the ammonia, originally present or derived from urea by bacterial and hydrolytic decomposition, is not expelled, it will neutralize an equivalent quantity of original acid or 0.1 *N* sulfuric acid added in the first distillation. Therefore, invariably too little acidity will be found. In fact, for this reason, it is obvious that all previous methods for determining acidity, especially with aged urines, have yielded too low data. This is true not only for direct titration methods[2] but also for hydrogen-ion concentration methods[3] based on indicators.

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[1] *Compt. rend.*, 160, 814 (1915). See also Godoy's method, *Mem. Inst. Ostwaldo Cruz.*, 5, 256 (1914).

[2] Berthelot, *Compt. rend.*, 131, 552 (1900); Naegeli, *Z. physiol. Chem.*, 30, 313 (1900); Höber, Hofmeister's *Beiträge*, 3, 525 (1903); Folin, *Am. J. Physiol.*, 9, 265 (1903); Dreser, *Beitr. Chem. Physiol. Path.*, 6, 178 (1905); Vorarik, *Pflügers Archiv.*, 111, 473, 497, 526 (1906); Skramlik, *Z. physiol. Chem.*, 71, 290 (1911); Lematte, *Compt. rend.*, 154, 1445 (1912); Orlowski, *Deut. med. Wochschr.*, 38, 1041 (1912); Fränkel, *Ibid.*, 38, 1974 (1912); Morel, *Bull. soc. pharm. Bordeaux*, 20, 193 (1914); Kolthoff, *Pharm. Weekblad*, 53, 396 (1915); Williams, *Med. Record*, 90, 223 (1916).

[3] Sörensen, *Biochem. Z.*, 7, 45 (1907); 21, 131 (1909); 22, 352 (1909); Henderson.

It may be contended that the *original urine contains ammonia*[1] (about 0.04% and equivalent to 2 cc. of 0.1 *N* alkali) and that the acidity as determined by the present method will be high. This contention is valid but it must be remembered that ammonia is increased by ingestion of acids and in acidosis and, therefore, compensates[2] *in vita* a certain quantity of *true* acidity.

But may not acidity be developed from other components of urine, through boiling first with a dilute acid and then with a dilute alkali? Urea, hippuric and uric acids, the purine bases, sugars and proteins possibly may be so affected. Whether these effects are measurable, under the conditions of the method, will be determined by future studies.

Since Walker and Hambly[3] have made the observation "that urea, when heated at 100° with water, is partially transformed into ammonium cyanate," perhaps urea thus will add cyanic acid to the original acidity. This source of error is precluded in the first distillation, since ammonium cyanate is transformed by free acid into ammonia and carbonic acid. According to the data of these authors, in the second distillation, 2–4% of the urea then present could thus be transformed—on the basis of the original urea usually present in urine, this is equivalent to 1–2 cc. of 0.1 *N* acid. However, it must be remembered that much of the urea is converted into ammonium carbonate by distilling to low volumes with 0.1 *N* acid and alkali, therefore, this error tends to approach a negligible limit. With old urines this possible error approaches zero in value.

The effects on this method of cyanate formation, of hydrolysis of the larger components of urine, of application of urease for the elimination of urea, of bacterial increase or decrease of acids, of the partitions of components in urinary sediments, will be studied. Application of the present method to urine and to milk, but not to other fluids, is reserved.

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Ibid., **24**, 40 (1910); *J. Biochem.*, **9**, 403 (1911); **13**, 393 (1913); **14**, 81 (1913); **17**, 305 (1914); Ringer, *Z. physiol. Chem.*, 60, 341 (1909); Howe and Hawk, *Proc. Soc. Biol. Chem.*, **1913**, 48; Zemen, Kohn and Howe, *J. Biochem.*, 20, 26 (1915); Host, *Z. klin. Med.*, **81**, 266 (1915); Kelly, *J. Lab. Clin. Med.*, 1, 194 (1915).

[1] For the effect of ammonia on titrations of urinary acidity, see Spindler, *Schweiz. Wochschr.*, **47**, 767, 782 (1910). For its effect on acidosis see Hasselbalch, *Biochem. Z.*, **74**, 18 (1916).

[2] Since sodium carbonate in aqueous solution is alkaline, it cannot exist as such in acidic urines, hence it cannot affect the above method when applied to such acidic urines. True alkaline urines containing sodium carbonate, being decomposed by the 0.1 *N* H_2SO_4, give correct data of alkalinity. For urine acidity and ingestion of alkali carbonates see: Hosslin, *Münch. med. Wochschr.*, **56**, 1673 (1909); *Deut. Arch. klin. Med.*, **105**, 147 (1912); Sochanski, *Z. exp. Path. Ther.*, **13**, 246 (1913); Palmer, *Boston Med. Surg. J.*, **169**, 464 (1913); Edie and Whitley, *Biochem. J.*, **1**, 11 (1906).

[3] *J. Chem. Soc.*, **67**, 746 (1895).