now known, excepting melibiose and possibly stachyose, cannot be carried out by the method of a double enzymotic hydrolysis with as much accuracy as has been obtained for the sucrose and raffinose mixtures. In the experiments given in Table II this conclusion is tested.

TABLE II.—THE ESTIMATION OF RAFFINOSE IN THE PRESENCE OF SUCROSE AND OTHER SUGARS.

	Sugars in 100 cc. solution (grams).				Polarizations (corr.).		
		Raffinose.					
No. Expt.	Sucrose added.	Added.	Found.	Other sugars.	Original.	After first hydrolysis.	After second hydrolysis.
1	3.00	None	None	None	+11.53	— 3.69	— 3.69
2	None	3.00	2.95	None	+21.42	+11.04	+ 2.65
3	1.50	1.50	1.49	None	+16.47	+ 3.68	— 0.56
4	1.50	1.50	1.53	1.50 glucose	+21.21	+ 8.39	+ 4.05
5	1.50	1.50	1.50	1.50 fructose	+ 8.25	— 4.64	— 8.91
6	1.50	1.50	1.46	1.50 invert	+14.05	+ 1.96	— 2.18
7	1.50	1.50	1.45	1.50 lactose	+21.07	+ 8.28	+ 4.16
8	1.50	1.50	1.53	1.50 maltose	+27.59	+14.77	+10.42
9	1.50	1.50	1.46	0.75 invert 0.75 maltose	+21.30	+ 8.46	+ 4.32
10	1.50	1.50	1.46	0.75 invert 0.75 lactose	+18.00	+ 5.16	+ 1.00
11	1.50	1.50	1.50	1.50 trehalose	+31.43	+18.60	+14.35
12	1.50	1.50	1.52	1.50 cellose	+19.46	+ 6.61	+ 2.27

Table II records the data on the estimation of raffinose in admixture with sucrose and glucose, fructose, invert sugar, lactose, maltose, cellose or trehalose. A comparison of the third and fourth columns indicates that the accuracy of the method is quite sufficient.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY.]

STUDIES ON THE CULTURE MEDIA EMPLOYED FOR THE BACTERIOLOGICAL EXAMINATION OF WATER.

III. THE COMPOSITION OF THE GASES FORMED IN LACTOSE-PEPTONE FERMENTATION TUBES.

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In our second paper we have shown[1] that when lactose-peptone media are inoculated with the mixed flora of sewage or with bacteria from the feces of man or animals, the volumes of the gases formed by the fermentative action of these microörganisms are proportional to the amount of peptone, meat or liver present in the media inoculated.

Since in routine water examinations the "gas ratios" are generally ascertained for the purpose of record and diagnosis, it became essential to learn whether the composition of the gases was affected by concentration changes in the media, as well as the total gas volumes.

[1] THIS JOURNAL, 37, 1949 (1915).

The authors intend to discuss neither the value of the "gas ratio" nor to review the voluminous literature relating to the numerical expressions which have been reported for the *B. coli* group. For the purposes of this paper it is sufficient to state that the gas ratios ascribed to this group of bacteria vary between 1:3 and 1:1.

Our object is merely to point out several of the factors affecting this value and to emphasize the necessity of great care in the preparation of carbohydrate culture media and in the interpretation of results. The whole question of fermentation gases and gas ratios, although one of such complex biochemistry as to be admittedly beyond the scope of the investigations of the water analyst, yet is of necessity so closely associated with his work of diagnosis as to force upon him some consideration of the facts.

Apparatus and Methods of Analysis Employed.—For the collection of large volumes of gases huge fermentation tubes of the form shown in Fig. 1 were employed. The gas collecting tube A had a content of approximately 300 cc., while the reservoir R corresponding to the bulb of a standard fermentation tube had a volume of 600 cc. The bore of the stopcock *S* was 5 mm.

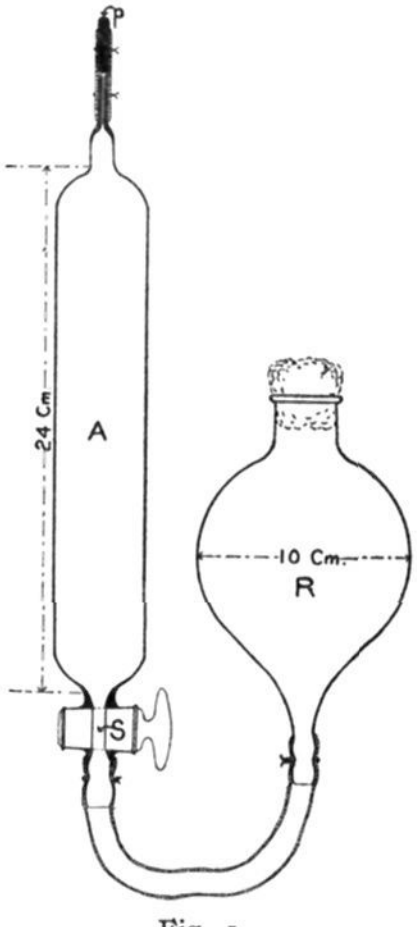

Fig. 1.

The tube was filled with media, a glass plug, *p*, inserted in the rubber tube and held tightly in place with fine copper wire. The opening of the reservoir was plugged with cotton and the entire apparatus on its stand sterilized at 110° for twenty minutes in an autoclave. After inoculation and incubation, a pinchcock was applied between the capillary tube and glass plug, the latter removed, and the gases of fermentation transferred to a Hempel buret for analysis.

In order to study the composition of the gases formed in ordinary fermentation tubes a short piece of capillary tubing was fused to the upper end of the closed arm. A short piece of rubber tube and a piece of glass rod as a plug was then attached, the fermentation tube filled with media and sterilized at 110° in an autoclave.

The gases were analyzed by means of Hempel burets and pipets, using mercury as the confining liquid in the burets.

Carbon dioxide was absorbed in KOH.

Heavy hydrocarbons were tested for in every case by exposing the gases to fuming sulfuric acid in the usual manner. No measurable con-

traction could in any case be observed with the 100 cc. samples of gas employed.

Carbon monoxide was also tested for, but none could be detected in 100 cc. samples by either ammoniacal cuprous chloride or hydrochloric acid cuprous chloride.

Oxygen was determined by absorption in the phosphorus pipet, but several checks were also made with alkaline pyrogallol.

For methane the Dennis combustion pipet was employed[1] which gave simultaneously a determination of the hydrogen present, but the hydrogen values were checked by the copper oxide method of Dennis.[2]

The residue of unabsorbable gas remaining upon completion of the analysis was called nitrogen. In the analyses tabulated below nitrogen has been taken by difference.

The Composition of the Gas.—As the concentration of peptone, meat or liver in lactose media increases, the volume of gas increases. This increase in gas volume is quite rapid up to 4% peptone, but after 5% peptone is reached, the increase is but slight in the volume percentage of gas collecting in the closed arm of the fermentation tubes. Preliminary experiments having shown that the composition of these gases remained substantially the same in the case of media having peptone concentrations above 5%, it was thought to be unnecessary to make complete gas analyses of the gases from all tubes of high peptone concentration, since CO_2 determinations alone would yield the information desired.

The averages of the results obtained are given in Table I.

TABLE I.

	Percentage of peptone in media.									
	1%.	2%.	3%.	4%.	5%.	6%.	7%.	8%.	9%.	10%.
Carbon dioxide	26.9	34.2	37.7	38.0	39.0	39.5	..	39.8	39	40
Hydrogen	70.2	63.2	62.3	58.3	59.0	60.5	..	60.2	61.0	60
Nitrogen	2.9	2.6	..	2.7	2.0	..	..	..	..	..
Methane	0	0	0	0	0	0	0	0	0	0
Heavy hydrocarbons	0	0	0	0	0	0	0	0	0	0
Carbon monoxide	0	0	0	0	0	0	0	0	0	0
Oxygen	0	0[3]	0	0[3]	0	..	..	..	..	..

In order to render easier the interpretation of these results the curves for the values of CO_2 and H have been plotted in Fig. 2.

The variations in the percentage composition of the gases obtained in different runs with media prepared at different times and inoculated with different sewage samples were less than we anticipated. In Table II are given the results obtained with gases from 4% peptone. These

[1] Dennis, "Gas Analysis," 1913 ed., 147.

[2] Dennis, *Ibid.*, 198.

[3] In a single sample of both 2% and 4%, oxygen was present in from 0.1 to 0.2%.

analyses are typical examples of the variations obtained with other concentrations.

TABLE II.

	Lactose media.					Dextrose media.	
	1.	2.	3.	4.	5.	6.	7.
Carbon dioxide	38.0	38.1	38.6	35.0	37.2	38.8	38.4
Hydrogen	59.9	60.0	59.4	61.6	55.6	59.2	59.5
Nitrogen	2.0	1.9	2.0	3.4	6.6	2.0	2.1
Oxygen	0.1	0	0	0	0.6	0	0
Methane	0	0	0	0	0	0	0
Heavy hydrocarbons	0	0	0	0	0	0	0

Gas samples 1, 2, 3, 4 were obtained from the large tubes shown in Fig. 1. Sample 5 consisted of the gases collected from a large number of ordinary fermentation tubes, while Samples 6 and 7 are gas samples from

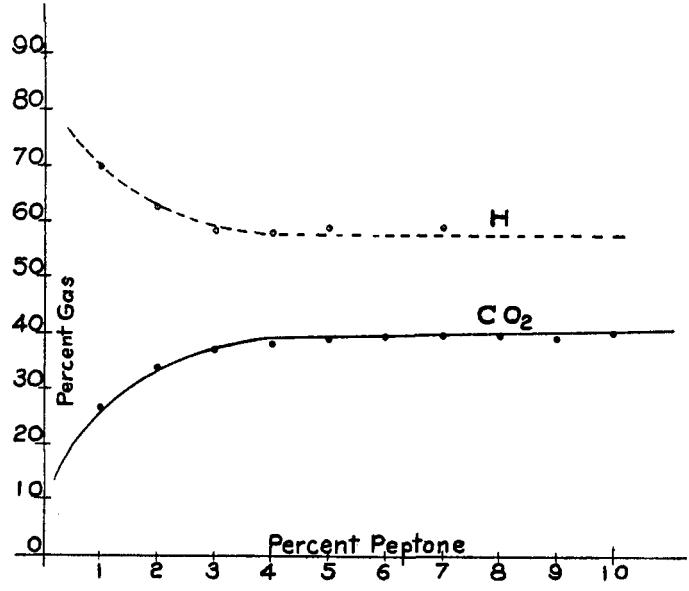

Fig. 2.

large tubes containing dextrose-peptone media, and have been incorporated in the table to afford a comparison between lactose and dextrose-containing media. It will be noted that the gases are substantially identical in character.

In only three cases was any evidence obtained of the presence of oxygen in the gases. Nor could we obtain any measurable volumes of CO_2 after combustion, showing, that if methane is present, it can be only in minute traces. It must be borne in mind, however, that these analyses were made as soon as the fermentation tubes reached the maximum fermentation,

i. e., as soon as the gases ceased to increase in volume, a condition usually reached at the end of from 20 to 26 hours.

This system of procedure was adopted so as to obtain as nearly as possible a correct gas composition. For it is obvious that upon standing for some hours there will be marked changes in the composition of the gases because of the relatively high solubility of CO_2 in aqueous solutions.

In the case of dextrose, Baginsky[1] was the first to point out that methane is sometimes formed, a fact later confirmed by Jesse.[2] It remained for Pennington and Küsel[3] to prove that methane is formed only when oxygen is present in solution in the media: this they did by excluding oxygen from the bulb of the fermentation tube by attaching thereto an absorption tube containing alkaline pyrogallol.

Since the passage from the closed arm to the bulb or reservoir of our fermentation tubes was relatively small, we may properly consider that we have an anaerobic growth in the closed arm, and that therefore the observations of Pennington upon the fermentative action of the *B. coli* group upon dextrose is confirmed for lactose.

It occurred to us, in this connection, that it might be of interest to try and obtain a production of methane by introducing oxygen into media and tubes. With this end in view a number of experiments were tried, using media containing in one series 3.5% and in a second series 4% peptone, and in all cases 1% lactose, 0.6% potassium chloride and an acidity of 1%. Into the large fermentation tubes (Fig. 1) various volumes of pure sterile oxygen were introduced and, after inoculation with sewage, incubated at 38° for twenty-four to twenty-six hours. The gases obtained were then carefully analyzed. The results given in Table III may be considered as typical of the phenomena observed.

TABLE III.

	Media shaken with pure oxygen until saturated.	15 cc. pure oxygen in closed arm.		42 cc. pure oxygen in closed arm.		50 cc. pure oxygen in closed arm.	
		Composition.	Corrected for excess oxygen.	Composition.	Corrected for excess oxygen.	Composition.	Corrected for excess oxygen.
Gas formed.....	60.4	58.3	..	58.3	..	60.4	..
Carbon dioxide.....	39.6	40.4	42.3	31.9	42.4	30.7	41.3
Hydrogen.....	59.2	53.6	55.9	40.8	54.2	40.2	54.1
Methane.....	0.2	0.2	..	0.4	..	0.2	..
Heavy hydrocarbons.	0.0	0.0	..	0.0	..	0.0	..
Carbon monoxide....	0.0	0.0	..	0.0	..	0.0	..
Oxygen.....	0.1	4.25	..	24.8	..	25.7	..
Nitrogen.....	1.6	1.6	..	2.1	..	3.2	..

[1] Baginsky, *Z. physiol. Chem.*, **13**, 352 (1889).

[2] Jesse, *Univ. Ill. Bull.*, **20**, IX, 47 (1912).

[3] Pennington and Küsel, THIS JOURNAL, **22**, 556 (1900).

These results appear to still further substantiate the results of Pennington and Küsel that it is only when oxygen is present that methane is formed, although we were able to obtain merely a very small yield of this gas with an atmosphere of pure oxygen and analyses made at the end of twenty-four hours.

It will be noted upon consulting Table II that the total volume of gas formed upon completion of fermentation, *including* the oxygen atmosphere in the tubes, was substantially constant, that is to say that when part of the closed arm of the fermentation tube is occupied by gas, less fermentation gases can collect therein. The gas volume is, therefore, as might be expected proportional to the volume of liquid in the closed arm, since it is only from this material that the gas can be formed and collect above the liquid. To prove this a large number of experiments were tried with fermentation tubes with enlargements of different capacities in the closed arms. In every instance the volumes of gas formed by sewage organisms were proportional to the volumes of culture media in the closed arms, and independent of the length of the closed arm.

A biochemic problem of great interest relative to mixed sewage floras is indicated by the results tabulated above. This is the relatively enormous quantity of oxygen utilized by these bacteria without very materially affecting the composition of the gases of fermentation, yet an excess of oxygen appears to be followed by an increased percentage of CO_2 and a decreased percentage of H. Although these biochemic questions had little bearing upon the main objects of the investigation it was thought that determinations of final acidity might indicate how the oxygen had been used by the microörganisms. Much to our surprise, no change in final acidity could be noted.

The "gas ratio" of an organism, as has been stated, is commonly expressed as the ratio of gas absorbed by potassium hydroxide to that unabsorbed by this reagent. The unabsorbed residue is generally called hydrogen, and the ratio expressed as $CO_2 : H = a : b$. The analyses given above indicate that the unabsorbed gas consists of hydrogen plus a small percentage of nitrogen and in rare cases some oxygen and perhaps methane. Calculated in the usual manner the average gas ratios which were obtained with lactose-peptone media determined as soon as fermentation ceased (20 to 26 hours), may be expressed as follows:

Peptone.....	1%	2%	3%	4%	5%	6%	8%	9%	10%
	1:2.71	1:1.92	1:1.65	1:1.63	1:1.56	1:1.53	1:1.51	1:1.56	1:1.50

We may, therefore, conclude that the "gas ratio" of a mixed sewage flora or of fecal bacteria will probably vary from $CO_2 : H = 1 : 3$ with low peptone concentrations to $CO_2 : H = 1 : 1.5$ (or perhaps even lower) with high peptone concentrations.

Standard lactose media made with 500 g. of meat per liter and 1%

peptone, comports itself toward this class of bacteria in a manner similar to media containing from 1.5 to 2% peptone. If, therefore, such media are inoculated with relatively large volumes of a water sample the concentration of nitrogenous material will be thereby reduced to a point such that fairly wide variations in the volumes of CO_2, H, and gas ratios may be expected.

When high peptone concentrations to which meat had also been added were employed the gases obtained were usually about 2% higher in CO_2 than when meat was absent. A correspondingly lower percentage of hydrogen was then observed.

The presence of "enriching agents," such as bile, bile-salt, or phenol, appeared to affect neither the total volume of gas formed nor the composition of these gases.

Pure cultures of *B. coli* isolated from sewage, from contaminated water and from the feces of horses, cows, calves, sheep and pigs gave gas volumes and CO_2 percentages increasing with increasing peptone concentrations, similar to the values obtained with the mixed flora of sewage.

Summary.

1. The gases formed by fecal bacteria in lactose-peptone media increase in volume with an increase in peptone concentrations.

2. The percentage of CO_2 present in these gases of fermentation increases with an increase in peptone, meat or liver until an equivalent of approximately 4% peptone is reached, after which the CO_2 per cent. remains substantially constant.

3. Hydrogen decreases with a rise in peptone until 4 to 5% of peptone is reached after which the per cent. of this gas remains substantially constant.

4. The "gas ratio" varies with the concentration of the nitrogenous material present in the medium.

5. No methane appears to be formed unless oxygen (air) has free access to the media and the inoculated medium stands for over twenty-four hours.

6. A small but nearly constant amount of nitrogen is found in the gases of fermentation.

7. An excess of oxygen retards gas formation and tends to increase the percentage of CO_2.

8. The total volume of gas formed is proportional to the volume of liquid contained in the closed arm of the fermentation tube.

9. The presence of enriching agents retards gas formation but does not materially alter the composition or total volume of gas formed.

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