[CONTRIBUTION FROM THE GEORGE HERBERT JONES LABORATORY OF THE UNIVERSITY OF CHICAGO]

Mechanism of Alcoholic Fermentation.[1] The Fermentation of Glucose-1-C^{14}

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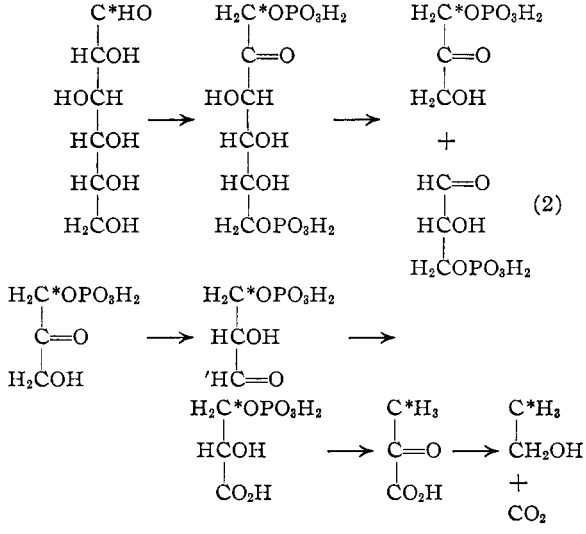
Glucose is fermented by yeast organisms to give alcohol and carbon dioxide in almost quantitative yield.

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 \qquad (1)$$

The currently accepted mechanism for the reaction, elucidated through the work of Meyerhof, Embden, Neuberg, Warburg, Cori and others,[3] describes the reaction in sufficient detail to allow the prediction of the pathway, during fermentation, of the individual carbon atoms of glucose. From the simplified outline of the mechanism shown below, it can be seen that all the carbon atoms originally present in the aldehyde group of glucose should eventually appear in the methyl group of the alcohol produced. The theory can, therefore, be tested on a quantitative as well as a qualitative basis by fermenting glucose labeled in the 1-position and determining the distribution of radioactivity in the fermentation products. Since the mechanism for the alcoholic fermentation probably differs only in minor respects from the anaerobic metabolism of carbohydrates in mammalian and plant tissues, and in bacteria, the results should be of significance beyond the particular reaction in question. (The sugar formulas are written in open-chain rather than in cyclic form to emphasize the reaction path of the carbon atom at position 1.)



(2)

In the work here described the glucose-1-C^{14} has been synthesized using a modified Fischer–Kiliani cyanohydrin synthesis, and the radioglucose fermented with Fleischmann household yeast under various conditions. The products were then analyzed for radioactivity; the alcohol was degraded in order to determine separately the activities present in the methyl and alcoholic ($-CH_2OH$) groups. In a previous communication[4] the results of some preliminary experiments were reported. In the present work, the experimental details and the results of experiments with glucose of higher specific activity are described.

Experimental

Synthesis of Glucose-1-C^{14}.—The glucose-1-C^{14} was synthesized from *d*(−)-arabinose and $HC^{14}N$, using the Fischer–Kiliani cyanohydrin synthesis[5] modified (a) by substituting the catalytic reduction of Glattfeld and Schimpf[6] for the sodium amalgam reduction used by Fischer and (b) by using carrier techniques to avoid the brucine salt procedure for the separation of gluconic acid from its epimer.

A solution of 0.25 millicurie of sodium cyanide[7] and 2 millimoles of inactive sodium cyanide in 1.0 ml. of water was placed in side-arm A of the reaction vessel shown in Fig. 1a. A solution of 2 millimoles of *d*(−)-arabinose and 0.035 millimole of ammonia dissolved in 0.5 ml. of water was placed in C, and 3 ml. of sirupy phosphoric acid in B. After the apparatus was evacuated, the cyanide and acid were slowly mixed; the $HC^{14}N$ was condensed in side-arm C, which was immersed in a Dry Ice–acetone-bath. When the hydrogen cyanide had been distilled over, the receiver was warmed to obtain a homogeneous solution (0–5°) and then placed in an ice-bath. After twenty-four hours at 0° and one week at room temperature, the gluconamide and mannonamide were converted into the corresponding aldonic acids by successive treatments with barium hydroxide and sulfuric acid, as described by Fischer. Inactive carrier gluconolactone (2.25 millimoles) was added, the solution evaporated to dryness, and the sirup dissolved in glacial acetic acid. The liquid was seeded with pure glucono-deltalactone, and the acetic acid slowly removed at room temperature under reduced pressure; the mixed lactones crystallized almost completely.

The dry crystals, free from acetic acid, were dissolved in 20 ml. of ice-water and transferred to the hydrogenation vessel which already contained 20 ml. of cold ethanol and 0.6 g. of platinum catalyst.[8] (The aqueous alcohol here employed led to better yields than did the solvent (water) used by Glattfeld and Schimpf.) The lactones were reduced by shaking the mixture vigorously (350 cycles per minute) for one hour under a pressure of three atmospheres of hydrogen. The catalyst was removed by centrifugation, 20 millimoles of inactive carrier glucose was added, and the solution evaporated to a sirup. The glucose was first crystallized from aqueous acetic acid, and then recrystallized alternately from acetic acid (from which α-glucose separates) and from pyridine (from which a com-

(1) Presented at the American Chemical Society Meeting, Sept. 20, 1949.

(2) Department of Chemistry, Harvard University, Cambridge, Mass,

(3) A good discussion, and references to the extensive literature, are given by J. R. Porter, "Bacterial Chemistry and Physiology," John Wiley and Sons, New York, N. Y., 1946.

(4) D. E. Koshland, Jr., and F. H. Westheimer, THIS JOURNAL, **71**, 1139 (1949).

(5) E. Fischer, *Ber.*, **23**, 2611 (1890); H. Kiliani, *ibid.*, **19**, 3029 (1886).

(6) J. Glattfeld and G. Schimpf, *ibid.*, **57**, 2204 (1935).

(7) Radioactive $NaC^{14}N$ was purchased from Tracerlabs, Inc., Boston, Mass.

(8) Adams, Vorhees and Shriner, in "Organic Syntheses," Coll. Vol. I, 2nd ed., John Wiley and Sons, Inc., New York, N. Y., 1941, p. 463.

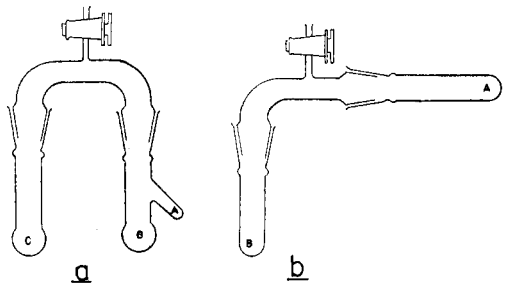


Fig. 1.

plex of pyridine and β-glucose separates[9]) until a specific activity constant to ±1% was obtained. From the specific activity of the purified glucose, the total activity present as glucose was calculated to be 0.025 millicurie, giving an over-all radiochemical yield of 10% based on the activity originally present as $NaC^{14}N$. The yield is lowered primarily by the preferential formation of mannonocyanohydrin in the first step of the synthesis, and secondarily in the catalytic reduction; the other steps involve only small losses. The synthesis compares favorably to those recently reported by Sowden[10] and by Mahler,[11] both of whom used the nitromethane condensation[12]; Sowden reported an over-all radiochemical yield of 5%, based on the C^{14} initially present as methanol.

Fermentation Conditions.—The radioglucose was fermented with Fleischmann's household yeast in phosphate buffers (0.07 molar KH_2PO_4) neutralized to the indicated *p*H. The apparatus (Fig. 1a) and all reagents except the yeast itself were sterilized in an autoclave at 115° for fifteen minutes, and the usual sterile techniques were observed throughout the work. The radioglucose solution (approximately 0.2 g. of glucose in each experiment) was placed in side-arm A, washed yeast (suspended in the buffer solution) in B, and carbonate-free sodium hydroxide in C. The air in the apparatus was replaced by purified nitrogen, the solution in A and B mixed, and the apparatus gently shaken in a thermostat at 25° for the stated intervals.

In the experiments with live yeast, the cells were washed with phosphate buffer just before using. The amount of yeast introduced was determined by drying and weighing an aliquot of yeast suspension similar to that used in fermentation. In the experiments with dried yeast, the cells were washed successively with buffer and distilled water, dried over phosphorus pentoxide, pulverized in the presence of a little toluene, and redried. In the experiment with toluene treated yeast, the suspension of cells was shaken with toluene (1.0 ml.) for one-half hour before the addition of glucose.

Separation of Fermentation Products.—On completion of the fermentation, side-arm C was warmed and B cooled, to return to B any alcohol which might have distilled. The apparatus was then opened, and the carbonate in the alkaline solution precipitated as barium carbonate. The precipitate was separated by centrifugation, washed twice with water and once with absolute alcohol, dried and counted. (The alcohol wash effectively prevents exchange between the barium carbonate and the carbon dioxide of the air.) A sample of the barium carbonate from one experiment which showed a counting rate (above background) of 2.1 ± 0.2 counts/minute (using the apparatus later described) was acidified with hydrochloric acid and the liberated carbon dioxide again precipitated as the carbonate. The second barium carbonate sample had a counting rate of 1.8 ± 0.2 counts/minute. This decrease is small compared to the variation from experiment to experiment.

The yeast cells were removed from the fermentation mixture by centrifugation, washed once with distilled water, dried, weighed and counted.

After removal of the yeast, the solution was acidified, and the alcohol distilled out of the solution. The aqueous alcoholic distillate was made basic with sodium hydroxide and redistilled. The alcohol was then oxidized to acetic acid and degraded as described below.

The residual liquors from both the acid and base distillations were made alkaline, evaporated to dryness and counted.

The solution of alcohol was made 4 molar in sulfuric acid, and the organic compound oxidized by heating it in a sealed tube for several hours at 100° with 0.5 g. of potassium dichromate. Control experiments showed that the oxidation, under these conditions, was essentially quantitative. The acetic acid was distilled from the solution, exactly neutralized with barium hydroxide, and the resulting solution evaporated to dryness. (The yield in the subsequent pyrolysis is adversely affected by an excess of base.) The barium acetate, prepared as indicated above, was placed in side-arm A of the apparatus shown in Fig. 1b, and the apparatus evacuated. Side-arm B was kept at −80° by immersion in a Dry Ice–acetone solution. In order to heat the salt as rapidly as possible, the side-arm containing the barium acetate was warmed gently with a bunsen burner and then thrust rapidly into an electric furnace heated to 530°; the sample was kept in the furnace for ten minutes. The acetone which collected in B was precipitated with an ice-cold saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. The crystallization of the hydrazone was complete in a few hours at 0°; the counting rate of a recrystallized sample of this compound was 0.99 that of the crude material. The residual barium carbonate from the pyrolysis was acidified, and the carbon dioxide liberated, reabsorbed as described above, and counted as barium carbonate.

$$\begin{array}{l} C^*H_3\text{—}CH_2OH \xrightarrow[H_2SO_4]{K_2Cr_2O_7} C^*H_3CO_2H \xrightarrow{Ba(OH)_2} \\ CH_3\text{—}CH_2OH \qquad\qquad CH_3CO_2H \end{array} \quad (3)$$

$$(C^*H_3CO_2)Ba(O_2CCH_3) \xrightarrow[\text{Vacuum}]{530^\circ} C^*H_3COCH_3 + BaCO_3$$

Some experiments were carried out with methyl-labeled acetic acid[13] to test the radiochemical efficiency of the pyrolysis. In four experiments with barium acetate from this acetic acid, and under the conditions outlined above, the specific activities of the barium carbonate relative to that of the initial barium acetate as 100, were 0.1, 0.3 and 0.4. In one experiment in which the barium acetate was brought up to 530° more slowly, the amount of activity in the barium carbonate was 1.7. Aronoff, Haas and Fries[14,15] showed that pyrolyses conducted at 450° resulted in extensive "mixing" of the carbon atoms in methyl-labeled barium acetate, and even at 530° they found from 0.8 to 3.0% of their activity in the barium carbonate fraction. The difference between their results and ours may be due (a) to a difference in the rate of heating or (b) to the fact that their pyrolyses were conducted in a stream of argon.

Counting Methods and Calculations.—All samples were counted as solids under standard conditions using an end-window Geiger tube connected to a Nuclear Instruments Development Laboratory Scaler Model 163. The samples containing the major portions of radioactivity were counted long enough to measure a total of more than 10,000 counts, so the standard statistical counting error was less than 1%; the low

(9) A. Mangam and S. Acree, THIS JOURNAL, **39**, 965 (1917).

(10) J. C. Sowden, *J. Biol. Chem.*, **180**, 55 (1949).

(11) H. Mahler, A. E. C. Document 2400 (December, 1948).

(12) J. C. Sowden and H. O. L. Fischer, THIS JOURNAL, **69**, 1963 (1947).

(13) The methyl-labeled acetic acid was kindly supplied by Dr. Konrad Bloch of the Department of Biochemistry.

(14) S. Aronoff, V. Haas and B. Fries, A. E. C. Document No. 1832, February, 1948.

(15) S. Aronoff, V. Haas and B. Fries, *Science*, **110**, 476 (1949).

activity fractions, *i. e.*, the barium carbonates obtained from the pyrolysis and from the fermentation carbon dioxide, were counted until the statistical counting error was less than 0.1% based on the glucose activity as 100. For example, the counting rate of the barium carbonate from the pyrolysis in experiment 1 (Table I) was determined to a statistical accuracy of ±0.2 count/minute; this is a statistical deviation of only 0.01 based on the glucose fermented. The counter was standardized by daily comparison with a Ul standard, and the results corrected accordingly.

To convert the results obtained from counting different materials to a common basis, samples of glucose and barium acetate were burned to carbon dioxide, using the wet oxidation method of Van Slyke and Folch.[16] Since the barium acetate sample was also pyrolyzed to acetone, empirical factors were obtained to relate the counting rate of the glucose and of the acetone 2,4-dinitrophenylhydrazone to that of the barium carbonate. Thus, in effect, all samples were counted as barium carbonate equivalents, while avoiding the necessiy of burning all the samples obtained in each experiment. Incidentally, the standardization of the acetone 2,4-dinitrophenylhydrazone by way of barium acetate was necessary, since there is considerable difficulty in the wet combustion of the former compound. Incomplete oxidation, accompanied by high counting rates for the resulting carbon dioxide, were the rule. Although thorough combustion, with a large excess of reagent, gave values approaching those here reported, the standardization by way of barium acetate is preferred.

The empirical factor relating the counting rate of the barium carbonate obtained from the fermentation carbon dioxide, was related to the counting rate of glucose as follows

$$\frac{\text{counting rate of 0.40 g. of glucose-1-C}^{14}}{\text{counting rate of 0.40 g. of BaCO}_3\text{ from the combustion of glucose-1-C}^{14}} \times \frac{2}{6} = 1.86 = f \quad (4)$$

The factor 2/6 takes account of the fact that two carbon atoms of glucose appear as carbon dioxide from fermentation; all six as carbon dioxide from combustion. For acetone 2,4-dinitrophenylhydrazone, the standard weight was 0.20 g. and the empirical factor 1.5. For counting purposes, the active hydrazone was routinely diluted with inactive acetone 2,4-dinitrophenylhydrazone.

The radioactivity present in the yeast and residual materials was calculated as the per cent. of *total* radioactivity. In order to make this calculation, it was necessary to assume that these fractions had the same counting characteristics as does glucose. Since the radioactivities present were small, any errors introduced by this assumption were insignificant for the purposes of this experiment. The radioactivity of both the yeast and the glucose was corrected for self-absorption by Libby's method.[17]

The counting was performed with samples spread over an area of 5 sq. cm. Thus for experiment 1 (Table I), Libby's equation 7′ shows that the activity present in 0.16 g. of the glucose, spread in a weightless layer, would increase the counting rate $(0.16 \times 5)/(5 \times 0.020)$ times; his equation 7 shows that the rate for yeast would be increased 1/0.36 times. An additional empirical factor of 1.1 takes into account small differences in geometry[18]; these differences could be neglected without appreciably affecting the final result. All the corrections relating to self-absorption and geometry are here incorporated in a single correction factor, g. For experiment 1 (Table I)

$$g = \frac{1}{0.36} \times \frac{5 \times 0.020}{5} \times 1.1 = 0.061 \quad (5)$$

The per cent. *total* activity in the yeast for experiment 1 is given by the expression

$$\frac{\text{counting rate of yeast cells} \times 100 \times g}{\text{counting rate of glucose} \times \text{weight of glucose used}} = \frac{230 \times 100 \times 0.061}{1950 \times 0.16} = 4.5 \quad (6)$$

A similar calculation can be made from the data of Table I for the per cent. of total activity in the residual materials.

For convenience in interpreting the data, the radioactivities of the fermentation products are *not* given as per cent. of *total* activity, but are related to the *specific* activity of glucose by using the stoichiometry of equation 1 and by assigning an arbitrary value of 100 to the specific activity of glucose. The relative specific activities of the products are independent of the yields obtained. They are equal to the percentages of total activity when the reaction goes quantitatively as written; they will be approximately equal to the percentages of total activity when, as in present case, the extent of the side reactions is small. However, the sum of the specific activities of all the products may exceed 100 in the event that a by-product, produced in small amount, nevertheless has high specific activity. A sample calculation for the results of experiment 1 (Table I) is given below.

$$\text{Hydrazone specific activity} = \frac{\text{counting rate of 0.20 g. of hydrazone}}{\text{counting rate of 0.40 g. of glucose}} \times 100 \times \text{dilution factor} \times f = \frac{569}{1950} \times 100 \times 2.05 \times 1.5 = 90 \quad (7)$$

Similar calculations, from the data of Table I, can be made for the activity of other products.

The counting was complicated by the fact that, to get maximum counting rates, it was necessary

(16) D. Van Slyke and J. Folch, *J. Biol. Chem.*, **136**, 509 (1940).

(17) W. F. Libby, *Ind. Eng. Chem., Anal. Ed.*, **19**, 2 (1947).

(18) D. Koshland, Jr., Dissertation, Chicago, 1949.

TABLE I

FERMENTATION RESULTS

Experiment number	1	2	3	4	5	6	7	8
Condition of yeast	Live	Live	Live	Live[a]	Tol.[b]	Dried[c]	Live	Live
*p*H	5.2	5.2	5.2	5.2	5.2	5.7	6.3	6.5
Temperature, °C.	25	25	25	25	25	30–35	25	30–35
Fermentation time, hr.	70	70	70	48	20	70	70	24
Glucose fermented, g.	0.160	0.192	0.200	0.239	0.170	0.155	0.286	0.170
Obsd. counting rate of 0.400 g. of glucose	1950	1950	1950	1950	1950	121	1990	121
Yeast								
Yeast added, dry wt., g.	0.055	0.066	0.068	0.066	0.066	0.105	.....	.....
Yeast recov., dry wt., g.	.052	.061	.063	.061	.052	.....	0.060	0.028
Obsd. counting rate, counts/min.	230	227	244	322	80.6	.....	259	10.3
Correction factor, *g*	0.061	0.071	0.073	0.071	0.061	.....	0.069	0.039
Wt. of yeast counted, g.	.052	.061	.063	.061	.052	.....	.060	.028
% *total* activity in yeast	4.5	4.3	4.6	4.9	1.5	.....	3.1	2.0
Residual materials								
Total vol. of residual materials, ml.	15	15	15	16	17	.....	.....	.....
Aliquot taken, ml.	3	3	3	3	3	.....	.....	.....
Wt. of evap. aliquot, g.	0.12	0.14	0.16	0.12	0.11	.....	.....	.....
Obsd. counting rate of evap. aliquot, counts/min.	0.9	12.5	2.6	14.2	9.6	.....	.....	.....
Correction factor, *g*	1.10	1.10	1.10	1.10	1.10	.....	.....	.....
% *total* activity in residues	0.2	2.6	0.6	2.1	2.0	.....	.....	.....
Acetone 2,4-dinitrophenylhydrazone								
Hydrazone wt. before dilution, g.	0.110	0.075	0.122	0.118	0.085	0.089	0.080	0.101
Inactive hydrazone added, g.	.115	.149	.113	.166	.137	.416	.146	.133
Dilution factor	2.05	2.98	1.93	2.41	2.62	5.67	2.83	2.32
Wt. of hydrazone counted, g.	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Obsd. counting rate, counts/min.	569	417	653	499	492	13.8	436	26.9
Empirical factor, *f*	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Rel. *specific* activity of hydrazone	90	96	97	92	99	97	93	77
$BaCO_3$ from CO_2								
Wt. of $BaCO_3$ recov., g.	0.350	0.386	0.374	0.440	0.345	0.308	0.602	0.340
Wt. of $BaCO_3$ counted, g.	.350	.386	.374	.400	.345	.308	.400	.340
Obsd. counting rate, counts/min.	28	26	19	20	35	2.8	19	3.4
Obsd. counting rate cor. to 0.40 g. $BaCO_3$	28	26	19	20	35	2.9	19	3.4
Empirical factor *f*	1.86	1.86	1.86	1.86	1.86	1.86	1.86	1.86
Rel. *specific* act.	2.7	2.5	1.8	1.9	3.3	4.5	1.8	5.3
$BaCO_3$ from pyrolysis								
Wt. of $BaCO_3$ counted, g.	0.093	0.097	0.112	0.107	0.094	0.088	0.150	0.090
Obsd. counting rate, counts/min.	1.9	.4	15.0	22.6	2.2	.4	4.6	2.1
Counting rate cor. to 0.40 g.	2.0	.4	15.8	23.8	2.3	.4	4.8	2.2
Empirical factor *f*	0.93	.93	0.93	0.93	0.93	.93	0.93	0.93
Rel. *specific* act.	0.1	.0	.75	1.1	.1	.3	.2	1.7

[a] Yeast preaerated for forty-eight hours before fermentation. [b] Toluene added to yeast one-half hour before fermentation. [c] Yeast dried and pulverized.

to place the top of the samples close to the counter window (the distance was approximately 3.5 mm. in this case). Small differences in the thickness of the layer to be counted, therefore, resulted in detectable variation in the counting rate. This error was less than 1% with glucose and barium carbonate precipitates. To determine the counting error with acetone 2,4-dinitrophenylhydrazone, eight samples of a single large homogeneous batch of this compound were counted, at times long enough to reduce the standard statistical counting error to less than 1%. Calling the average 100, the individual values were 100, 94, 101, 97, 99, 102, 100 and 106, giving 3.5% for the standard counting error.

Results and Discussion

The results of the fermentations of glucose-1-C^{14} are listed in Table I.

The $-CH_3$ Group.—It is seen that (in all experiments except no. 8) the specific activity of the methyl group of the alcohol produced in

fermentation is about 95% of that of the glucose used. From the observations (see below) that the glucose is fermented directly with a minimum of side reactions and little dilution by endogenous metabolism, it would be deduced that the relative specific activity of the acetone fraction should be approximately equal to that of the glucose. This is the fact: the mechanism of Meyerhof, *et al.*, is thus strongly confirmed.

The standard error for the relative specific activity of the acetone-2,4-dinitrophenylhydrazone, again excluding the result of experiment 8 is 3.8. This agrees remarkably well with the value of 3.5 obtained for the reproducibility in counting this compound, and indicates that the main factor in the variability of the results is the counting error.

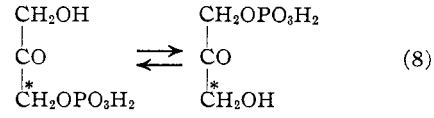
It is to be noted that no significant change in results was caused by varying the experimental conditions as shown in Table I. Live yeast gave results essentially similar to those obtained with dried or toluene-treated yeast. Changing *p*H or temperature was likewise without significant effect.

The $-CH_2OH$ Group.—From the experiments with methyl-labeled acetic acid, it may be expected that 0.4% of the activity present in the methyl groups of the alcohol may appear in the barium carbonate fraction because of "mixing" during the pyrolysis step. In five of the fermentations, the relative specific activity in this fraction was less than 0.4%, and it may reasonably be concluded that in these cases no activity, within experimental error, was present in the $-CH_2OH$ group of the ethyl alcohol. In the remaining cases here reported, the relative specific activity in the barium carbonate from pyrolysis was greater than 0.4%, and the activity in these cases is probably significant, although slight variations in the pyrolysis technique might conceivably account for these values, which average only 2%. The experiments here reported confirm the major premise of our earlier work,[4] which showed that the major part of the first carbon atom of glucose appears in the methyl group of the alcohol produced; however, the amount of activity previously reported for the $-CH_2OH$ group (around 5%) appears to be too high. Presumably the low level of activity in the glucose used in the early experiments is at least in part responsible for the discrepancy. In any event, it is clear that the deviation from the predicted mechanism, with respect to the $-CH_2OH$ group, is small.

The CO_2—The carbon dioxide obtained directly in fermentation contained an appreciable, although small, amount of the radioactivity originally present. The actual values varied somewhat in different fermentations, but average in conformity with the results previously[4] reported, around 3%. It can therefore be concluded that some separate pathway or slight modification of the postulated one exists which routinely produces small amounts of carbon dioxide from the carbon atoms originally present in the aldehyde group of glucose.

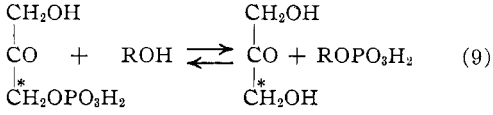
Pathways involving reactions associated with the oxidative metabolism of carbohydrates, such as the Krebs cycle or the oxidation *via* phosphogluconic acid should be minor under the anaerobic conditions used here. However, only small amounts of activity are observed in the fermentation of carbon dioxide and these reactions may account for some, if not all of it.

Other pathways which involve a simple extension of the currently accepted fermentation mechanism and which seem reasonable by analogy with reactions catalyzed by the phosphomutases and phosphorylases are: (a) an exchange reaction of phosphorylated dihydroxy acetone in which a phosphate group changes positions to give a structurally similar but radiochemically different molecule.



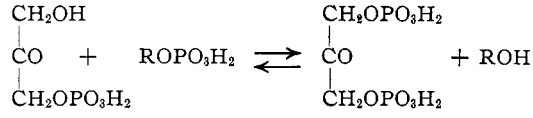
(8)

(b) a reversible phosphorylation to give free ketone



(9)

(c) a reversible phosphorylation to give a diphosphorylated compound



In these reactions ROH stands for any phosphate acceptor, *e. g.*, adenosine diphosphate. (Mechanism b is related to the hydrolytic mechanism postulated by Aronoff, *et al.*[15]) No independent evidence for the path or paths has been obtained in this work; the above suggestions are made to show that the activity in the carbon dioxide can be explained with simple extensions of the existing theory. It is clear, however, that if any of the above reversible equilibria occur, only a small fraction of the dihydroxyacetone undergoes the exchange.

Side Reactions.—Fermentation, under the conditions here employed, occurs with a minimum of side reactions. The weights of yeast before and after fermentation were essentially the same. The moderately low level of radioactivity in the recovered yeast indicates that some exchange did occur with the glycogen or other cellular constituents of the yeast, but the process occurred only to a small extent.

The residual materials which were obtained by evaporating the slightly alkaline residues from the distillations should contain non-volatile sub-

stances and volatile acids. Glycerol, for example, would not be present in this fraction, but succinic, lactic and acetic acids, as well as unreacted glucose, would be. The low level of radioactivity in this fraction indicated that the fermentation was essentially complete. Analyses for glucose[19] on several of the residual solutions likewise indicated complete fermentation.

Acknowledgments.—The authors wish to express their appreciation to Drs. W. Manning and G. Hennig of the Argonne National Laboratory for the use of laboratory space for some of this work, and to Drs. W. Libby and A. Turkevich of the Institute for Nuclear Studies for the use of their counting equipment.

Summary

1. Glucose-1-C^{14} has been prepared by a modified Fischer–Kiliani synthesis from $d(-)$-arabinose and $HC^{14}N$. An over-all radiochemical yield of 10% was obtained.

2. A method is described for the degradation of isotopically labeled ethyl alcohol to determine quantitatively the relative amounts of radioactivity in the CH_3 and $-CH_2OH$ groups.

3. Glucose-1-C^{14} has been fermented by yeast and the distribution of radioactivity in the products ascertained. About 95% of the total radioactivity fermented appeared in the methyl group of the alcohol obtained. This result gives quantitative support to the currently accepted mechanism for the alcoholic fermentation.

4. About 3% of the radioactivity of glucose-1-C^{14} appeared in the carbon dioxide produced in the fermentation. Several alternative explanations of this deviation from the fermentation mechanism are discussed.

(19) P. Shaffer and A. Hartmann, *J. Biol. Chem.*, **45**, 349, 365 (1921).

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[CONTRIBUTION FROM THE CHEMISTRY LABORATORY OF THE UNIVERSITY OF MICHIGAN]

Reactions of 2-Arylcyclohexanones. III. Cyanoethylations and Mannich Reactions[1]

BY W. E. BACHMANN AND L. B. WICK[2]

2-Phenylcyclohexanone reacted readily with acrylonitrile in the presence of Triton B (40% aqueous benzyltrimethylammonium hydroxide) at the 2-position to give a monocyanoethylated product 2-oxo-1-phenylcyclohexanepropionitrile (II).[3] The ketonitrile was hydrolyzed to the corresponding acid (III) in good yield by a mixture of hydrochloric acid and acetic acid. The successful monocyanoethylation of 2-phenylcyclohexanone, in contrast to the difficulty of obtaining other than polycyanoethylated products from cyclohexanone and 2-methylcyclohexanone[4] even with limited amounts of acrylonitrile, is attributed to the reactive hydrogen atom on the carbon atom attached to the carbonyl group and the phenyl group.

6-Benzal-2-phenylcyclohexanone (V) was cyanoethylated to the crystalline 3-benzal-2-oxo-1-phenylcyclohexanepropionitrile (VI) in 83% yield; the product was identical with that produced by reaction of benzaldehyde with II. Hence, in the preparation of VI from 2-phenylcyclohexanone, the order in which the two reactions, benzalation and cyanoethylation, are employed is not important. By carrying out the benzalation first, one deals with solids in both steps. These reactions definitely establish the structure of II.

In order to introduce the cyanoethyl group in the 6-position of 2-phenylcyclohexanone, the hydrogen atom in the 2-position was replaced by a bromine atom. The crystalline 2-bromo-2-phenylcyclohexanone (VII) was obtained in good yield by bromination of 2-phenylcyclohexanone in carbon tetrachloride. Treatment of the bromoketone with Triton B and acrylonitrile yielded a cyanoethylated product which was unsaturated and contained no halogen. This product (XI) must have resulted in virtue of the dehydrobrominating action of the Triton B either prior to or after cyanoethylation. Since it was observed that the solution of 2-bromo-2-phenylcyclohexanone remained neutral during the first additions of Triton B, cyanoethylation must have proceeded *via* the unsaturated ketone 2-phenyl-2-cyclohexenone (X) at least to some extent. The unsaturated ketone can be prepared in good yield by the action of hot 2,6-dimethylpyridine on the bromoketone. The unsaturated ketonitrile (XI) was hydrolyzed to the unsaturated ketoacid. Hydrogenation of the double bond and the carbonyl group followed by oxidation of the alcohol group yielded the crystalline 2-oxo-3-phenylcyclohexanepropionic acid (XII), which was shown to be different from the isomeric acid III. Only one racemic mixture of the two possible diastereoisomeric forms of XII was obtained in crystalline form.

6-Benzal-2-phenylcyclohexanone (V) reacted

(1) Paper II, Bachmann and Wick, THIS JOURNAL, **72**, 2000 (1950).

(2) From the Ph.D. dissertation of L. B. Wick. Present address: Department of Chemistry, Coe College, Cedar Rapids, Iowa.

(3) After our work had been completed, the preparation of this compound from 2-phenylcyclohexanone and β-chloropropionitrile was reported by Boekelheide, THIS JOURNAL, **69**, 790 (1947).

(4) Bruson, "Organic Reactions," Vol. V, p. 100 (1949).