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July 24, 1951

Berkeley, California

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ABSTRACT

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Resting cells of eleven microorganisms were exposed to radioactive carbon dioxide for 40 minutes. The radioactive compounds formed during this time were separated and identified by paper chromatography.

Resting cells of Lactobacillus casei fixed no carbon dioxide and growing cells fixed carbon dioxide primarily in malic and aspartic acids.

All of the radioactive compounds formed could have become radioactive by reversal of known decarboxylation reactions.

(1) The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

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The place occupied by the photosynthetic mechanism of energy conversion in the stream of biochemical evolution has been the subject of considerable speculation. (Van Niel, 1949; Blum, 1937) It is clear, however, that in order to establish such a place, a more or less definite notion of the nature of the entire evolutionary stream is necessary. In recent years the idea that the stream is flowing in the direction of the loss of synthetic biochemical abilities has gained a special prominence, particularly as a result of laboratory experimentation as well as the observation of naturally occurring systems. (Beadle, 1945; Tatum, 1946; Knight, 1950) Such a view quite clearly places the photosynthetic mechanism very near the beginning. However, a variety of very powerful arguments have been adduced (Oparin, 1938; Haldane, 1934) against the sudden appearance of the very complex, completely autotrophic organism containing extremely involved biosynthetic sequences in an essentially inorganic world.

A mechanism has been proposed (Horowitz, 1945) for the evolution of these very involved biosynthetic sequences leading to complex compounds by the gradual acquisition of synthetic abilities. Such a view of the evolutionary stream would, of course, place the photosynthetic energy converting system

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near the top or the end rather than at the bottom of the scale or near the beginning of the stream. It seemed to us not unreasonable to suppose that the entire evolutionary stream consists of both types of change, the one involving increasing biosynthetic abilities being the earlier leg, leading ultimately to completely autotrophic organisms, and the other involving the loss of biosynthetic abilities being a second leg and becoming the major direction comparatively later on the evolutionary time scale.

The fossil record that is left to us is, of course, of very recent origin on such a scale. Presumably we have no direct geological record of the events on the rising leg of such a stream. It is, however, conceivable that some heterotrophic organisms alive today might represent stages on the rising leg, and the problem becomes the one of seeking to find criteria to determine whether or not such an organism represents a precursor to an autotroph or a product of the loss of synthetic abilities from an autotroph.

The availability of methods, paper chromatography and radioautography, of observing the ability of a wide variety of organisms to fix carbon dioxide into organic compounds and to determine the nature of the organic compounds into which this carbon had been fixed suggested its use as a criterion for the classification of these organisms. The study herein reported was undertaken to determine the compounds in which carbon dioxide was incorporated in the dark by a variety of organisms. They were chosen to try and be representative of both the plant and the animal world and both heterotrophic and autotrophic. The general procedure was to expose a sample of the organisms to radioactive carbon dioxide and then analyze the fixed carbon by means of paper chromatography.

Experimental

Each organism was grown in an appropriate medium and harvested while substrate was still present and the cells were actively dividing.

Cell suspensions of Hansenula anomala and Tetrahymena geleii were prepared in the following manner. The cultures were centrifuged and washed once with distilled water and 1 ml. of packed cells suspended in 100 ml. of distilled water. 7.2 μM $NaHCO_3$ containing 44.8 μC of C^{14} was added to the suspension and shaken occasionally. After 40 minutes the suspension was poured into 400 ml. of boiling alcohol. The total amount of activity fixed in non-volatile products was determined immediately on the entire suspension and the amount of activity in soluble non-volatile compounds measured after filtration through celite.

Cultures of Allomyces arbuscula and Blastocladia pringsheimeii were harvested by filtration and the mycelial mats washed with water. Care was taken to prevent the mat from becoming completely dry. 1.7 g. of mycelium was then suspended in 100 ml. of water and treated as described above for Tetrahymena and Hansenula. Only the alcohol extractable activity was determined because of the difficulty of counting the mycelium.

One ml. of Azotobacter agilis cells was suspended in 14 ml. of water, 11.5 μM $NaHCO_3$ containing 71.7 μC C^{14} were added and the suspension shaken constantly. After 40 minutes the mixture was dumped into 56 cc. of boiling ethanol. The activity of the suspension was determined before and after filtration through celite. Cell suspensions that were not shaken fixed little activity.

Two obligate anaerobes, Butyribacterium rettgeri and Clostridium kluyveri, were treated in the following manner: 1 cc. of unwashed cells was resuspended in 5 ml. water and placed in a Thunberg tube. A solution containing 14.4 μM $NaHCO_3$ and 89.6 μC of C^{14} was placed in the side arm, frozen in liquid nitrogen and the system evacuated. The $NaHCO_3$ solution was then thawed, mixed with the cells and the solution shaken for 40 minutes. The cells were then killed and extracted with 20 ml. of boiling ethanol. The solution was filtered through celite and concentrated to 2 ml.

One ml. of washed cells of Physarum polycephalum was suspended in 3.5 ml. of water and 3.8 μM $NaHCO_3$ containing 40 μC of C^{14} added. After 40 minutes the cells were killed with 15 ml. of boiling ethanol and filtered.

Three photosynthetic algae, Scenedesmus D_3, Chlorella pyrenoidosa and Synechococcus cedorum, were exposed to carbon dioxide in the dark. One ml. packed cells was suspended in 100 ml. of distilled water, the flask covered with aluminum foil and placed in a completely dark room. A solution containing 7.78 μM $NaHCO_3$ and 48.5 μC C^{14} was added to the algae in the dark, shaken and after 40 minutes in darkness the algae were killed by dumping in 400 ml. of boiling alcohol. The total amount of activity fixed into non-volatile compounds was determined before and after filtration.

All of the filtered solutions were concentrated in vacuo to 2 ml. and aliquots chromatographed. A solution containing the 80% ethanol extractable material from 100 mg. wet weight of cells was found to give satisfactory chromatograms with all organisms. Two-dimensional descending paper chromatograms using water-saturated phenol as the first developing solvent and butanol-propionic acid-water as the second solvent were prepared.

The radioactive compounds present were determined by exposing the chromatograms to "No Screen" X-ray film. After location of the radioactive areas with the radiogram, the amount of activity in each spot was determined by placing a thin window Geiger-Muller counting tube over the spot. Radioactivity measurements from duplicate chromatograms agreed within 5%

The results are shown in Table I. The amount of carbon dioxide activity fixed during 40 minutes is reported as counts per minute per ml. of packed cells. Since each organism will have different packing characteristics and varying concentrations of C^{14} were used, no direct comparison should be made of the amount of carbon dioxide fixed by different organisms. The activity of each compound is expressed as the per cent of the total soluble non-volatile activity.

Results

The compounds containing fixed carbon dioxide may have been produced by exchange reactions or may represent a net synthesis. No attempt was made to determine by what reaction each compound was formed.

The data obtained from radiograms of the extract are only for non-volatile, soluble compounds. Carbon dioxide incorporated into cellular material such as proteins and complex polysaccharides would not be detected. The difference between total activity fixed and the 80% alcohol extractable material would give a measure of the amount of carbon dioxide incorporated into cellular material. This varies from 4% in *Tetrahymena geleii* to 59% in *Azotobacter agilis*. The alcohol-extract of *Azotobacter agilis* also contained a considerable amount of polysaccharides. The two water molds, *Allomyces arbuscula* and *Blastocladia pringsheimii* also fixed carbon dioxide

into polysaccharides. Fats are extracted by the 80% ethanol and would appear on the chromatograms. No radioactivity was found in fats of any of the organisms.

Volatile compounds would not be detected as they would be removed during the plating operations, the vacuum concentration and the chromatographic procedure. A striking example of this was with extracts of Butyribacterium rettgeri where a considerable amount of radioactive acetic and butyric acid was formed. With the chromatographic solvents used these acids were lost during development of the paper chromatograms.

With Hansenula anomala, an oxidative yeast, 75% of the fixed activity was found in aspartic, glutamic and succinic acids. Alanine, malic and citric acids were other compounds having measurable activity.

The protozon, Tetrahymena geleii, fixed 63% of the carbon dioxide activity in succinic acid. This is in agreement with the results of Van Niel, et.al. (1942) who found that resting cells of this orgainsm fixed carbon dioxide in succinic acid during the fermentation of glucose.

The two water molds, Allomyces arbuscula and Blastocladia pringsheimii formed mainly radioactive aspartic and glutamic acids. Relative smaller amounts of radioactive succinic acid was formed by these organisms (See Cantino 1949).

More than half, 59% of the activity fixed by Azotobacter agilis was found in insoluble cellular material. Of the soluble activity, 54% was found in compounds which are probably complex polysaccharides. Hydrolysis of these compounds with 1 N hydrochloric acid for 30 minutes at 100° gave glucose and another unidentified compound. This compound is not an acid degradation product of either glucose or fructose.

The anaerobic bacterium, Butyribacterium rettgeri, during fermentation of lactic acid fixes carbon dioxide in acetic and butyric acids. (Barker and Haas, 1944) The resting cells of this experiment also fixed carbon dioxide into acetic and butyric acids. Of the non-volatile compounds formed, the majority of the activity was in malic acid and tyrosine and phenyl alanine. No activity could be detected in alanine.

Another anaerobe, Clostridium kluyveri, fixed 78% of the activity in alanine.

A slime mold, Physarum polycephalum, fixed carbon dioxide mainly in aspartic, glutamic, malic and citric acids.

The pattern of carbon dioxide fixation in the dark for the three algae examined was similar to that for the non-photosynthetic organisms. The principal compounds labeled were aspartic, glutamic, succinic and malic acids. None of the phosphorylated compounds formed by carbon dioxide fixation in the light were observed.

Lactobacillus casei differed from the other organism in this study by its very slow rate of carbon dioxide fixation. Resting cell suspensions of Lactobacillus casei from 24 hour cultures did not fix any measurable amount of carbon dioxide during a 40 minute exposure. (See Gibbs, 1950) Therefore the organisms were grown in the presence of radioactive carbon dioxide, 11.5 μM carbon dioxide containing 71.7 μC carbon 14, for 16 hours. The total amount of activity fixed in the cells and supernatant was 88,000 cts./min. Of this, 4000 counts were incorporated in the cells. Over 90% of the activity in the supernatant was found in one compound, malic acid. The activity of the cells was in water insoluble materials. 6 N acid hydrolysis at 100° for 8 hours gave one radioactive compound, aspartic acid.

Discussion

It is clear from the foregoing results that the original purpose of the search has not been fulfilled; that is, a criterion for the distinction between an organism lying on the rising leg and one lying on the falling leg of the biochemical evolutionary stream is not apparent. Quite the contrary. The pattern of dark carbon dioxide fixation seems to be very much the same in all the organisms so far tested, and leads to a conclusion of the single origin of all of these biochemical systems in much the same way as the generalized Vitamin B nutritional requirements do. This is particularly true for *Lactobacillus casei* where its inability to fix carbon dioxide corresponds to its fastidious nutritional requirements.

Furthermore, the pattern of compounds into which carbon dioxide is incorporated is most readily and simply accounted for by the universal existence of a single, well-established reversible carboxylation reaction of pyruvic acid leading to oxaloacetic acid and malic acid. From these, via the well-known reactions of the tricarboxylic acid cycle, together with a few transamination reactions, the carbon could be transported into practically all of the compounds which we have observed.

The two most noticeable differences from the average carbon distribution are the small amount of glutamic acid found among the bacteria, together with the apparently larger quantities of tyrosine and phenylalanine found in the anaerobic bacterium, *Butyribacterium rettgeri*. It is quite possible that further more extensive and precise work on the distribution pattern might lead to systematic differences capable of classification utility. In this connection it should be mentioned that *Euglena gracilis* has been run and found to produce a pattern distinctly different from those herein reported. It will be discussed in a forthcoming publication.

Summary

Resting cells of eleven microorganisms were exposed to radioactive carbon dioxide for 40 minutes. The radioactive compounds formed during this time were separated and identified by paper chromatography.

Resting cells of *Lactobacillus casei* fixed no carbon dioxide and growing cells fixed carbon dioxide primarily in malic and aspartic acids.

All of the radioactive compounds formed could have become radioactive by reversal of known decarboxylation reactions.

Acknowledgments

We gratefully thank Dr. H. A. Barker for supplying cultures of *Azotobacter agilis* and *Clostridium kluyveri*, Mr. L. Pine for *Butyribacterium rettgeri*, Dr. R. E. Emerson for *Alloymces arbuscula* and *Blastocladia pringsheimii*, Dr. J. O. Thomas for *Tetrahymena geleii*, Dr. J. Tabachnick for *Hansenula anomala*, Dr. K. Hok for *Physarum polycephalum* and Dr. M. B. Allen for *Synechococcus cedorum*. Their generous assistance made this comparative study possible.

TABLE I

Organism	C^{14} Activity cts/min* Total	Soluble
1. Hansenula anomala	152,000	132,000
2. Tetrahymena geleii	494,000	476,000
3. Allomyces arbuscula	—	100,000
4. Blastocladia pringsheimii	—	52,000
5. Azotobacter agilis	260,000	106,000
6. Butyribacterium rettgeri	—	92,000
7. Clostridium kluyveri	—	75,000
8. Physarum polycephalum	—	18,000
9. Scenedesmus D_3	600,000	371,000
10. Chlorella pyrenoidosa	103,000	71,000
11. Synechococcus cedorum	36,000	25,000

(*) Counts per minute of activity fixed by 1 ml. of packed cells exposed to $C^{14}O_2$ for 40 min.

Table I (cont'd)

Organism	Radioactive Compounds formed % of total non-volatile soluble activity							
	Aspartic	Glutamic	Alanine	Succinic	Malic	Citric	Lactic	Fumaric
1. Hansenula anomala	25	25	12	26	6	4	—	—
2. Tetrahymena geleii	4	13	5	63	8	—	2	1
3. Allomyces arbuscula	22	13	2	4	6	2	—	2
4. Blastocladia pringsheimii	32	28	0.4	5	15	1	2	5
5. Azotobacter agilis	1	8	4	—	1	3	18	—
6. Butyribacterium rettgeri	5	6	—	—	23	2	—	—
7. Clostridium kluyveri	5	2	78	—	1	—	—	—
8. Physarum polycephalum	20	13	—	—	24	11	—	7
9. Scenedesmus D_3	34	44	—	0.7	7	14	—	0.4
10. Chlorella pyrenoidosa	10	43	4	7	21	4	—	5
11. Synechococcus cedorum	15	36	—	18	16	—	—	2

Table I (cont'd)

Organism	Radioactive Compounds formed % of total non-volatile soluble activity					
	Glycine	Glutamine	Asparagine	Poly-saccharides	Tyrosine	Phenyl alanine
1. Hansenula anomala	—	—	—	—	—	—
2. Tetrahymena geleii	2	—	—	—	—	—
3. Allomyces arbuscula	2	5	2	24	—	—
4. Blastocladia pringsheimii	—	2	1	6	—	—
5. Azotobacter agilis	2	—	—	54	—	—
6. Butyribacterium rettgeri	—	—	—	—	15	22
7. Clostridium kluyveri	—	—	—	—	—	—
8. Physarum polycephalum	—	—	2	—	—	—
9. Scenedesmus D_3	—	—	—	—	—	—
10. Chlorella pyrenoidosa	—	—	—	—	—	—
11. Synechoccus cedorum	—	—	—	—	—	—

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