THE INFLUENCE OF AMINO ACIDS ON FRUCTOSE UTILIZATION IN THE LUMINOUS BACTERIUM, ACHROMOBACTER FISCHERI

STANLEY FRIEDMAN[1, 2]

Department of Biology, the Johns Hopkins University, Baltimore, Maryland

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Although a large number of investigations have been carried out on the luminescent system of the marine luminescent bacteria by both the Delft school and workers in this country, very little has been done in the way of characterizing their normal metabolic properties. Johnson (1936) investigated the oxidation of various carbon sources by resting cell suspensions of *Achromobacter fischeri*, and Doudoroff (1942*a*, *b*) made carbon balance studies of the fermentation of glucose by various members of this family of microorganisms. The most comprehensive work from a nutritional standpoint was that of Farghaly (1950) who made a general survey of the carbon and nitrogen requirements for growth of these bacteria. In this same study, he observed the effects of various environmental changes on their nutritional requirements.

The very scanty information available prompted a reinvestigation of some of the nutritional characteristics of this microorganism. An intensive study corroborated Farghaly's findings that glucose and glycerol were the only carbon sources utilizable for growth, but it was noted that in the presence of small amounts of amino acids, fructose also could be used. The present investigation indicates that fructose inhibits the growth of the bacteria even in the presence of high concentrations of glucose. Amino acids are effective in reversing this inhibition provided they are present in the medium during the early lag phase of growth. Both enzymatic and respiratory studies have been made in order to determine the nature as well as the site of inhibition by fructose. The results obtained suggest that fructose or compounds formed from fructose inhibit growth by reacting with amino nitrogen, thus reducing the free amino acid pool required for enzyme formation and growth.

[1] Work partially done during tenure as a Lalor Predoctoral Fellow at the Johns Hopkins University, Baltimore, Maryland, 1951–1952.

[2] Present address: Department of Entomology, University of Illinois, Urbana, Ill.

MATERIALS AND METHODS

For growth experiments, *A. fischeri* was cultured in optically standardized test tubes of 15 mm outside diameter by 125 mm length. A total of 5 ml of medium plus supplement was used per tube, and these were incubated at 23 C on a rotating shaker. The medium used for growth studies included NaCl, 30 g; Na_2HPO_4, 1.78 g; KH_2PO_4, 0.70 g; NH_4NO_3, 1 g; $MgSO_4$, 0.10 g; trace elements (McElroy and Farghaly, 1948), 0.05 ml; distilled H_2O, 1,000 ml; pH 7.3. The carbon source was added as required, the usual concentration for normal growth being 5 g per liter. Density measurements were made on a Klett-Summerson colorimeter using a 42 filter. For respiration studies and experiments involving cell-free extracts, the bacteria were cultured in minimal or complete (minimal plus 0.5 g yeast extract and one g hydrolyzed casein per liter) liquid medium, harvested on a Sharples high speed centrifuge, and washed and recentrifuged 2× with buffer.

Oxygen uptake was measured by the direct method of Warburg at 23 C. In most cases, the contents of the vessel were: 1 ml bacterial suspension in 0.015 M phosphate buffer pH 7.3, substrate and 3 per cent sodium chloride solution to bring the volume up to 2.2 ml. The substrates were made up in 3 per cent sodium chloride solution, and the buffer contained 3 per cent sodium chloride. Two-tenths ml of 20 per cent potassium hydroxide was placed in the inset cup. Vessels were equilibrated for 15 to 20 minutes, and at zero time the substrate was tipped in from the side arm.

Extracts were prepared by making acetone powders or grinding with "alumina (A-301)" cells which had been harvested and washed as described above. Optical measurements on cell-

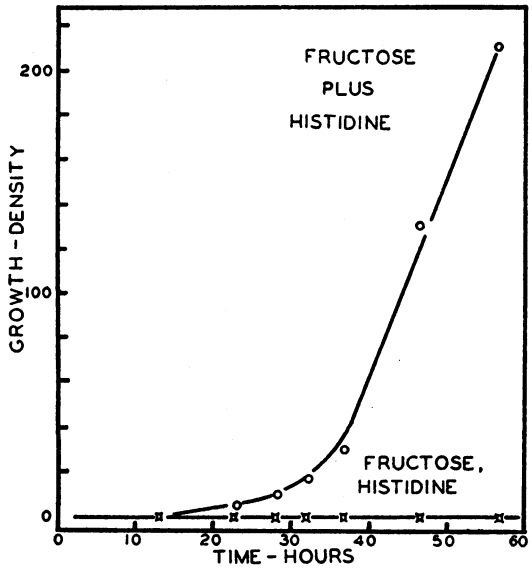

Figure 1. Growth of cells in fructose minimal medium in the presence of histidine. Fructose concentration: 0.5 per cent. Histidine concentration: 0.02 per cent.

free systems were made with a Beckman Model DU spectrophotometer. Triose phosphates were determined by the Sibley-Lehninger method (1948), pentoses by the method of Meijbaum (1939). Inorganic phosphate, before and after 7 minute hydrolysis in one N HCl at 100 C, was determined by the method of Sumner (1944).

Diphosphopyridine nucleotide and triphosphopyridine nucleotide were obtained from hog liver by the method of Horecker and Kornberg (*unpublished data*). Reduced diphosphopyridine nucleotide was prepared by the method of Pullman *et al.* (1952). Potassium adenosinetriphosphate was prepared from the Sigma dibarium salt. The sodium salts of 3-phosphoglyceric acid, glucose-6-phosphate, fructose-6-phosphate, and hexose diphosphate were prepared from the barium salts of these same compounds as purchased from Schwarz Laboratories. Aldolase and triose phosphate dehydrogenase were prepared by the method of Cori *et al.* (1948) from rabbit muscle. Lactic dehydrogenase was prepared from pig heart by the method of Straub (1940).

1. *Quantitative growth studies.* On the basis of the observation that *A. fischeri* could use fructose at a concentration of 0.5 per cent in the presence of low concentrations of amino acids (figure 1), the response of the organism to various fructose concentrations was investigated more intensively. It was found that the bacterium would grow to a limited extent without added amino acids on quantities of fructose not exceeding 0.3 per cent. In these cases, however, the rate of growth was found to vary inversely with fructose concentration, and it was not possible to approach the density obtained with glucose as a carbon source. At the highest concentration of fructose permitting growth of the organism, there was a very long lag period before growth commenced. The reversal of this inhibition by the amino acids seemed to be even more important when viewed in the light of these experiments so investigations were carried out in an effort to determine their place in the reaction scheme.

Eighteen amino acids were tested for the ability to permit utilization of fructose in high concentrations by the bacterium. Of all of these (arginine, proline, glutamic acid, tyrosine, glycine, aspartic acid, phenylalanine, lysine, histidine, tryptophan, threonine, alanine, leucine, methionine, cysteine, valine, serine, cystine) only two, serine and alanine, consistently showed an inability to stimulate growth. Other compounds were tested for this effect, including α-ketoglutarate, acetate, glucosamine, glutamine, *p*-aminobenzoic acid, adenylic acid, riboflavin, inositol, thiamin, and pyridoxine. Only glutamine would permit initiation of growth, and this after a long lag period. Increasing the concentration of NH_4NO_3 in the medium did not influence growth in the presence of fructose. It was shown that the amino acids themselves could not act as carbon sources. Inoculation of bacteria into a minimal medium with no carbon source except the amino acid (in concentrations as high as 0.5 per cent) resulted in no growth. The effectiveness of the amino acids in promoting growth on fructose varied considerably. The most efficient (utilized in lowest concentrations) were glutamic acid, histidine, methionine, and cysteine. The rate of growth of the organism on fructose also was found to be conditioned by the amount of amino acid present (figure 2). Increasing the concentration present in the medium from 0.01 to 1 mg per 20 ml increased the rate almost linearly.

Testing the effects of age of culture and inoculum density on fructose utilization showed that 0.5 per cent fructose in liquid minimal medium could not be utilized by organisms aged

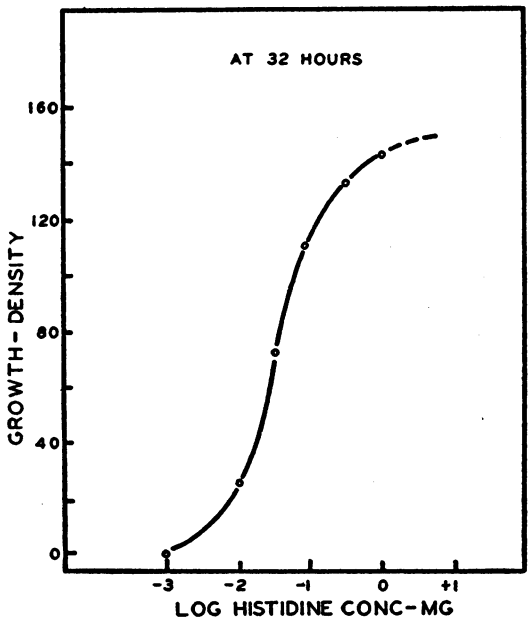

Figure 2. Rate of growth of cells on fructose minimal medium in the presence of various concentrations of histidine. Fructose concentration: 0.5 per cent.

12 to 33 hours when inoculated unless an amino acid were present. Increasing the density of the inoculum sixfold did not permit growth to occur.

The possibility that autoclaving the fructose in water would lead to formation of inhibitory products was rendered void by using Seitz filtered fructose and obtaining results similar to those obtained on autoclaving. A heavy metal inhibition also was ruled out by extraction of the fructose solution with diphenylthiocarbazone (0.004 per cent in CCl_4). The same inability to grow except in the presence of an amino acid was observed.

Several experiments were performed in which bacteria were preincubated with the growth promoting amino acid both in the presence and absence of fructose. These were as follows: (1) Preincubation with glutamic acid (1 mg/ml medium) for 60 minutes in a minimal medium minus a carbon source, then centrifugation of cells and inoculation into minimal medium plus 0.5 per cent fructose. (2) Incubation with fructose (0.5 per cent) plus histidine (1 mg/20 ml medium) for fifteen minutes in minimal medium minus other carbon and nitrogen sources, then treatment as in (1). In neither case did growth occur when the cells were returned to the fructose minimal medium.

It was apparent from these experiments that the amino acid had to be present in the medium in order that growth could occur. How long could the bacteria remain in contact with fructose alone and still grow when the amino acid was added? The results of these experiments are presented in table 1. If addition of the amino acid to the medium was delayed for four hours after inoculation, growth failed to take place.

The results of the above experiments made it of interest to learn whether or not the fructose was acting as a bactericidal agent. Experiments were set up in which the bacteria were incubated for four hours in 20 ml of liquid minimal medium in the presence of either fructose (0.5 per cent), fructose (0.5 per cent) plus an amino acid (1 mg/20 ml), or no carbon source. At the end of this time, 0.5 ml of each incubation mixture was removed and streaked onto complete plates. The cells which were incubated in fructose were much slower (12 to 24 hr) in developing on the plates than were the cells from the other two incubation mixtures. However, when growth finally did take place, there was no apparent decrease in cell number. Therefore, it was concluded that although fructose was not killing the cells, it had a definite inhibitory effect. An interference with growth on glucose was taken as confirmatory evidence of this effect. If fructose in low concentrations (0.02 per cent) was added immediately to a medium containing glucose (0.2 per cent), a long lag period occurred before growth com-

TABLE 1

Effect of glutamic acid addition on growth in fructose minimal medium

Fructose concentration = 0.5 per cent, Glutamic acid concentration = 0.01 per cent. Glutamic acid added at times noted after organisms inoculated into a fructose containing minimal medium.

SUBSTRATE	ADDITION	TIME OF ADDITION	DENSITY AT 30 HOURS
Fructose	None	—	0
Fructose	Glutamic acid	0	20
Fructose	Glutamic acid	1	30
Fructose	Glutamic acid	2	30
Fructose	Glutamic acid	3.5	5
Fructose	Glutamic acid	5	0

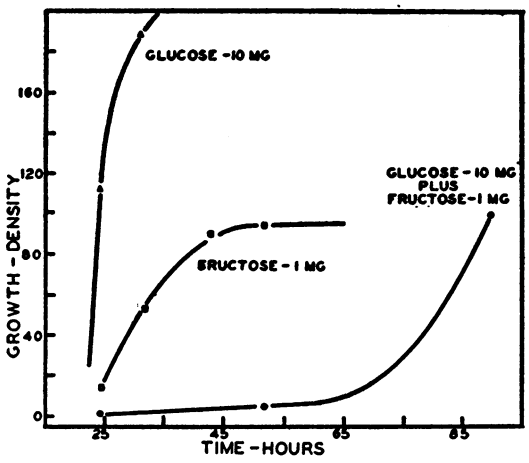

Figure 3. Inhibition of rate of growth of cells in minimal medium containing fructose and glucose. Concentrations shown are in mg per 5 ml of medium.

menced (figure 3). It is interesting to note the mutual interference between glucose and fructose, the cells responding to neither substrate for a long time period when both were added concurrently. If, however, fructose was added after four hours, it had no effect on growth with glucose as the initial carbon source.

2. *Respiration studies.* In order to ascertain the effects of fructose on the oxygen uptake of these organisms, resting cell studies were made on bacteria which had grown on minimal medium in the presence of either glycerol, glucose, or fructose plus an amino acid. These experiments showed that prior growth on glycerol resulted in a lag period before oxygen consumption reached a steady state in the presence of fructose. The addition of an amino acid did not alter this lag to any appreciable extent. Organisms which had been grown previously on fructose plus an amino acid exhibited no such lag period on fructose but did show this effect in the presence of glycerol. If glucose was used as the carbon source for growth, a long lag period occurred on exposure to glycerol and what might be termed a semilag period occurred in the presence of fructose. There was no lag period when organisms grown previously on any of the carbon sources were exposed to glucose.

A comparison was made between the oxidative ability of cells grown on a minimal medium plus glycerol and a complete glycerol medium. Results indicated no difference in their behavior toward the various substrates; therefore, cells grown on complete glycerol medium were used in the rest of these studies.

Experiments were done to determine the effects of glucose and fructose upon one another. Results expressed in figures 4 and 5 established the fact that they are acted upon independently. If glucose was tipped in after the lag period on fructose was over, the oxygen uptake increased to a value slightly higher than that on either substrate by itself (figure 4). If fructose and glucose were added concurrently, the lag period prior to fructose utilization was not shortened, nor was the oxygen uptake for the first thirty minutes significantly different from that in the control glucose vessel (figure 5).

3. *Enzymatic studies.* In order to further understand the mechanism of action of fructose on the metabolism of this organism, an investigation of some of its enzyme systems was undertaken. Cells were grown in liquid minimal medium containing either glycerol, glucose, or fructose (plus an amino acid) as carbon sources. Extracts were made as described under Methods and were tested for the ability to oxidize various carbon sources, particularly glucose-6-phosphate. Extracts from cells grown under any of the conditions noted were very active in reducing tri-

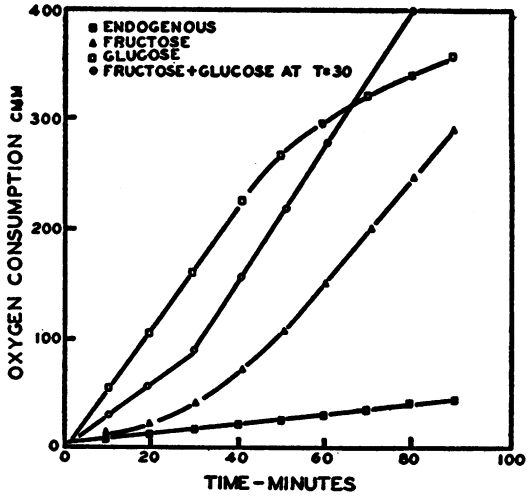

Figure 4. Oxygen consumption of cells in the presence of fructose with later addition of glucose. Glucose added at 30 min to cells which were in the presence of fructose prior to T = 0. Substrates present in following concentrations: glucose 3.2 μM; fructose 3.2 μM.

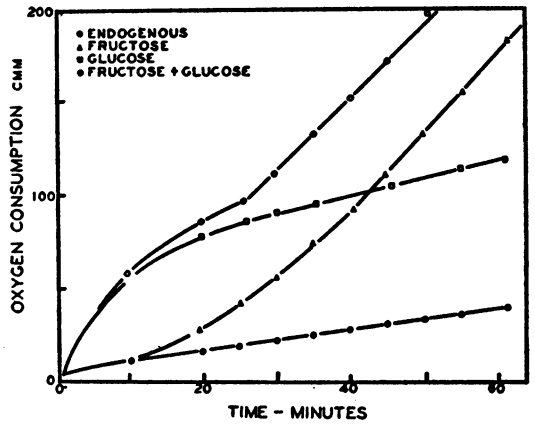

Figure 5. Oxygen consumption of cells in the presence of glucose and fructose. Substrates present in the following concentrations: fructose 1.6 μM; glucose 0.8 μM.

phosphopyridine nucleotide in the presence of glucose-6-phosphate.

Since the bacteria exhibited an active zwischenferment (glucose-6-phosphate dehydrogenase), it was possible to couple this system to any which would produce glucose-6-phosphate. Hexokinase could be tested by this means as could hexose isomerase. The addition of either fructose or glucose plus adenosinetriphosphate and Mg^{++} would permit triphosphopyridine nucleotide reduction to occur if hexokinase and hexose isomerase were present. These systems were found to be present in all extracts tested. Between 50 and 100 times as much fructose as glucose had to be added to the reaction mixture to permit maximum activity. It should be noted that the respiration experiments showed a slightly increased oxygen consumption in the presence of glucose and fructose over the consumption in the presence of either substrate by itself. These latter experiments suggest that there was more than one enzyme present. A more thorough investigation would be necessary to settle this point. Since extracts from cells grown on glucose, fructose, plus an amino acid, or glycerol contained the above named enzymes in comparable concentrations, we were satisfied that if any adaptation were occurring to fructose utilization, it was at an entirely different site. Other enzyme systems were studied in an attempt to obtain an over-all picture of the metabolic patterns in this organism. In these studies, extracts were made from cells grown on a liquid glycerol complete medium, and the existence of the Embden-Meyerhof scheme was tested through an investigation of individual enzyme systems. Aldolase was determined by two methods. The first, a spectrophotometric method, utilized the reduction of diphosphopyridine nucleotide as a criterion of the presence of the enzyme. Fructose diphosphate was incubated in the presence of the extract, diphosphopyridine nucleotide, triose phosphate dehydrogenase, and sodium arsenate in a pyrophosphate buffer at pH 8.6. No reduction of diphosphopyridine nucleotide ensued. A sample of aldolase prepared from rabbit muscle was active in this system. If, however, the Sibley-Lehninger (1948) method of detection of aldolase was used, definite production of triose phosphates was observed. Since this latter method involves a trapping reaction, in which hydrazine reacts with triose phosphates to form the hydrazones, it is possible that it is far superior to the spectrophotometric method.

Triose phosphate dehydrogenase was assayed by the same spectrophotometric method as was aldolase. In this case, however, aldolase was added to the reaction mixture instead of triose phosphate dehydrogenase. Cysteine also was added in order to maintain triose phosphate dehydrogenase activity. A rapid reduction of diphosphopyridine nucleotide ensued, demonstrating the presence of triose phosphate dehydrogenase.

Enzyme determinations beyond this point have met with no success. Phosphoglyceromutase and enolase were measured using a system which included 3-phosphoglyceric acid, lactic dehydrogenase, reduced diphosphopyridine nucleotide, pyruvate kinase, adenosine diphosphate, $MgCl_2$, and bacterial extract. We have never been able to detect an oxidation of reduced diphosphopyridine nucleotide under these conditions, suggesting that either phosphoglyceromutase or enolase is absent.

This organism can metabolize hexoses through the hexose monophosphate shunt as shown by the formation of Meijbaum (1939) positive compounds on incubation of glucose-6-phosphate, triphosphopyridine nucleotide, and Mg^{++}, with the bacterial extract in the presence of a system for regenerating triphosphopyridine nucleotide. The system used in this case was pyruvate plus lactic dehydrogenase (Horecker and Smyrniotis, 1951).

DISCUSSION

Since there seems to be no real specificity in the amino acid requirement for growth on fructose, it remains a very difficult problem to determine the mechanism of action of these compounds. An interesting consideration, however, and one which deserves to be stated again is the fact that the most effective amino acids appear to be glutamic acid, histidine, methionine, and cysteine. Farghaly (1950), studying the growth of *A. fischeri* in carbon dioxide free air, found that these same amino acids were the most effective in initiating growth under conditions of aeration which inhibited growth in the minimal medium. It is premature, of course, to suggest the possibility that the mode of action of these compounds may be the same in both cases, but it is interesting to note that in both experiments it is the amino nitrogen which is required since the keto analogues of the amino acids were not active.

The proposal, that amino nitrogen is the limiting factor and that fructose or compounds formed from fructose inhibit growth through reaction with free amino nitrogen in the cell, is supported by much indirect evidence. The general importance of amino acids to the metabolism of this organism, as well as other luminescent bacteria, is well documented (Doudoroff, 1942*a*, *b*; McElroy and Farghaly, 1948; Farghaly, 1950). Unpublished experiments of McElroy may provide a possible explanation for unusual amino acid requirements under some conditions. If *A. fischeri* was incubated at a low temperature (5 to 10 C) in a minimal medium without a carbon source for several hours and then streaked on minimal medium agar plates containing a carbon source, no growth occurred. Addition of amino acids to these plates resulted in the appearance of colonies. Increasing the number of amino acids present caused an increase in the number of colonies. In some cases, fifteen amino acids were added before all colonies appeared. Once growth had started, however, these colonies behaved as normal wild type organisms, in that they would grow when inoculated into a minimal medium without amino acids. The results indicated that the amino acids were lost rapidly from the cells and that the latter subsequently had lost the ability to synthesize these compounds on the minimal medium. The results of the present investigation suggest that fructose in some way rapidly depletes the amino acid pool in an analogous manner. A loss of amino acids into the medium or their removal by other means presumably could lead to a loss in transaminating mechanisms. This might be likened to the disappearance of adaptive enzymes in the absence of substrate as described by Spiegelman and Reiner (1947). For this reason, large numbers of amino acids might be required by certain cells before growth would occur, as was observed in the unpublished experiments mentioned above. Similarly, if fructose was tying up free amino acids in the cell, the above situation might result. Investigation of the transaminases of these bacteria might help to answer some of the questions raised by this study.

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SUMMARY

Enzymatic studies on *Achromobacter fischeri* have demonstrated the presence of hexokinase, phosphoglucomutase, phosphohexoseisomerase, aldolase, triose phosphate dehydrogenase, and glucose-6-phosphate dehydrogenase. Enzymes not specifically identified, but which appear to be present, are those involved in the formation of pentoses from 6-phosphogluconate.

Respiration studies on *A. fischeri* show an apparent adaptation to fructose oxidation by cells grown on glycerol minimal medium. An adaptation to glycerol oxidation by cells grown on minimal medium containing either glucose or fructose plus an amino acid also is noted. Cells grown on either glucose, glycerol, or fructose plus amino acid medium exhibit no lag in glucose oxidation.

An inhibition of growth when high concentrations of fructose are used as a carbon source has been found in *A. fischeri*. This inhibition can be reversed by addition of small amounts of single amino acids to the medium within four hours after inoculation with the bacteria.

Of eighteen amino acids tested, only two, serine and alanine, do not reverse this inhibition.

The effectiveness of the amino acids in promot-

ing this phenomenon varies considerably. Glutamic acid, methionine, histidine, and cysteine are most active in low concentrations.

Increasing the concentration of amino acid incorporated into the medium increases the rate of growth on fructose.

Fructose does not act as a bactericidal agent since cells incubated in a fructose medium, then plated onto complete, exhibit no decrease in numbers.

Fructose is found to inhibit utilization of glucose for growth. This, too, is a time dependent phenomenon. If fructose is added more than four hours after inoculation of the bacteria into the glucose medium, there is no lag in growth.

The possibility of a tie-in between fructose inhibition and a reduction in the free amino acid pool is discussed.

REFERENCES

Cori, G. T., Slein, M. W., and Cori, C. F. 1948 Crystalline d-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle. J. Biol. Chem., **173**, 605–618.

Doudoroff, M. 1942*a* Studies on luminous bacteria. I. Nutritional requirements of some species, with special reference to methionine. J. Bact., **44**, 451–459.

Doudoroff, M. 1942*b* Studies on the luminous bacteria. II. Some observations on the anaerobic metabolism of facultatively anaerobic species. J. Bact., **44**, 461–467.

Farghaly, A. H. 1950 Factors influencing the growth and light production of luminous bacteria. J. Cellular Comp. Physiol., **36**, 165.

Horecker, B. L., and Smyrniotis, P. Z. 1951 Phosphogluconic acid dehydrogenase from yeast. J. Biol. Chem., **193**, 371–381.

Johnson, F. H. 1936 The aerobic oxidation of carbohydrates by luminous bacteria, and the inhibition of oxidation by certain sugars. J. Cellular Comp. Physiol., **8**, 439–463.

McElroy, W. D., and Farghaly, A. H. 1948 Biochemical mutants affecting growth and light production in luminous bacteria. Arch. Biochem., **17**, 379–390.

Meijbaum, W. 1939 Über die bestimmung kleiner pentosemengen, insbesondere in derivaten der adenylsaure. Z. physiol. Chem. (Hoppe-Seyler's), **258**, 117–121.

Pullman, M. E., Colowick, S. P., and Kaplan, N. O. 1952 Comparison of diphosphopyridine nucleotide with its deaminated derivative in various enzyme systems. J. Biol. Chem., **194**, 593–602.

Sibley, J. A., and Lehninger, A. L. 1948 Determination of aldolase in animal tissues. J. Biol. Chem., **177**, 859–872.

Spiegelman, S., and Reiner, J. M. 1947 The formation and stabilization of an adaptive enzyme in the absence of its substrate. J. Gen. Physiol., **31**, 175–193.

Straub, F. B. 1940 Crystalline lactic dehydrogenase from heart muscle. Biochem. J. (London), **34**, 483–486.

Sumner, J. B. 1944 A colorimetric determination of phosphorus. Science, **100**, 413.