PROBABLE IDENTITY OF THE GROWTH PROMOTING FACTOR FOR BUTYRIBACTERIUM RETTGERI WITH OTHER BIOLOGICALLY-ACTIVE SUBSTANCES[1]

LEO KLINE,[2] L. PINE, I. C. GUNSALUS, AND H. A. BARKER

Division of Plant Biochemistry, University of California, Berkeley, California and Department of Bacteriology, University of Illinois, Urbana, Illinois

Received for publication March 26, 1952

Kline and Barker (1950) reported the existence and some properties of an apparently new unidentified growth factor (B.R. factor) required by *Butyribacterium rettgeri* for growth on lactate as the fermentable carbon source. Certain similarities in properties were given as the basis for a postulated relationship of the B.R. factor to other unidentified growth or metabolically active substances. These related factors included: (a) the acetate replacing factor reported by Guirard, Snell, and Williams (1946) as stimulatory for the early growth of *Lactobacillus casei;* (b) the pyruvic oxidase factor reported by O'Kane and Gunsalus (1947, 1948) as necessary for activation of the apo-pyruvic oxidase of *Streptococcus faecalis;* and (c) a factor necessary for the growth of *Tetrahymena geleii* called protogen by Stokstad *et al.* (1949) who reported some of its properties. Stokstad *et al.* (1950) later reported that protogen was also necessary for the growth of an unidentified *Corynebacterium.*

Snell and Broquist (1949) presented evidence for the interchangeability of these three latter activities. They reported that concentrates, purified with respect to pyruvic oxidase activity and having activities of 1, 70, and 3,000 pyruvic oxidase units per mg, had relative acetate replacing activity for *L. casei* of 1, 90, and 3,300, respectively. They also showed that a protogen preparation purified 10,000-fold over the source material as indicated by *Tetrahymena* assay was highly active for *L. casei;* 3 mμg of this preparation were equivalent to the growth promoting activity of 400 μg of sodium acetate.

The purpose of this report is to present further evidence on the relation of the B.R. factor to the three activities previously related by Snell and Broquist (1949).

METHODS

The methods employed in this work were the same as those previously described (Kline and Barker, 1950).

EXPERIMENTAL RESULTS AND DISCUSSION

Activity of pyruvic oxidase and acetate replacing preparations for B. rettgeri. Three pyruvic oxidase preparations with graded levels of activity, obtained

[1] This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

[2] Present address: Western Regional Research Laboratory, Albany, California.

through the courtesy of Dr. I. C. Gunsalus, were assayed by us for their activity in promoting the growth of *B. rettgeri* on lactate. As shown in table 1, these preparations were found to have about the same relative activities by the two methods. The most active pyruvic oxidase preparation, 3P, promoted half maximum growth of *B. rettgeri* at a level of 0.014 μg per ml of culture medium. This pyruvic oxidase preparation was simultaneously paper-chromatographed adjacent to a second preparation purified by us on the basis of B.R. factor activity (no. 6, figure 1). The movable B.R. factor activity in both preparations appeared similarly homogenous as developed with chloroform, whereas development with benzene showed the same two and possibly three active components in each (figure 1). These results indicate either that the pyruvic oxidase and B.R. factor activities are identical or that the B.R. factor accompanied the pyruvic oxidase activity during purification of the latter.

TABLE 1

Activity of pyruvic oxidase preparations for promoting the growth of Butyribacterium rettgeri*

PREPARATION NO.	PYRUVIC OXIDASE ACTIVITY*	RELATIVE B.R. FACTOR ACTIVITY
E5	20	20
3CA	250	193
3P	3,000	1,800

* Pyruvic oxidase preparations and activity figures kindly furnished by Dr. I. C. Gunsalus, private communication, 1948. One pyruvic oxidase unit is equivalent to the manometric response produced by 1 mg of a standard yeast extract.

This second possibility is, however, greatly minimized by the result we wish to report now; namely, that a very highly purified preparation, designated α-lipoic acid by its discoverers (Reed *et al.*, 1951*a*) and known to be extremely potent for both acetate replacing activity for *Streptococcus lactis* and for pyruvic oxidase activity for *S. faecalis*, is also extremely active in promoting the growth of *B. rettgeri*.

The α-lipoic acid[3] which we used had a potency of 100,000 pyruvic oxidase factor units per mg and was estimated to be 40 per cent pure. We found that 0.096 mμg of this preparation per ml of culture medium gave half maximum growth of *B. rettgeri*. Therefore, one B.R. factor unit, the quantity causing half maximum growth in 10 ml of medium, is equivalent to 0.38 mμg of pure α-lipoic acid, or 1 mg α-lipoic acid = 2.6×10^6 B.R. factor units. On the basis of our results and the data given by Reed *et al.* (1951*a*) it appears that with pure α-lipoic acid 1 B.R. factor unit = 0.1 pyruvate oxidation factor unit = 5.8 acetate units.

A sample of β-lipoic methyl ester, having a potency of 60,000 pyruvic oxidase factor units per mg, also was tested for B.R. factor activity. One B.R. factor unit was contained in 3.54 mμg of this preparation. Since the purity of the

[3] The authors are indebted to Dr. L. J. Reed for the preparations of α-lipoic acid and β-lipoic methyl ester.

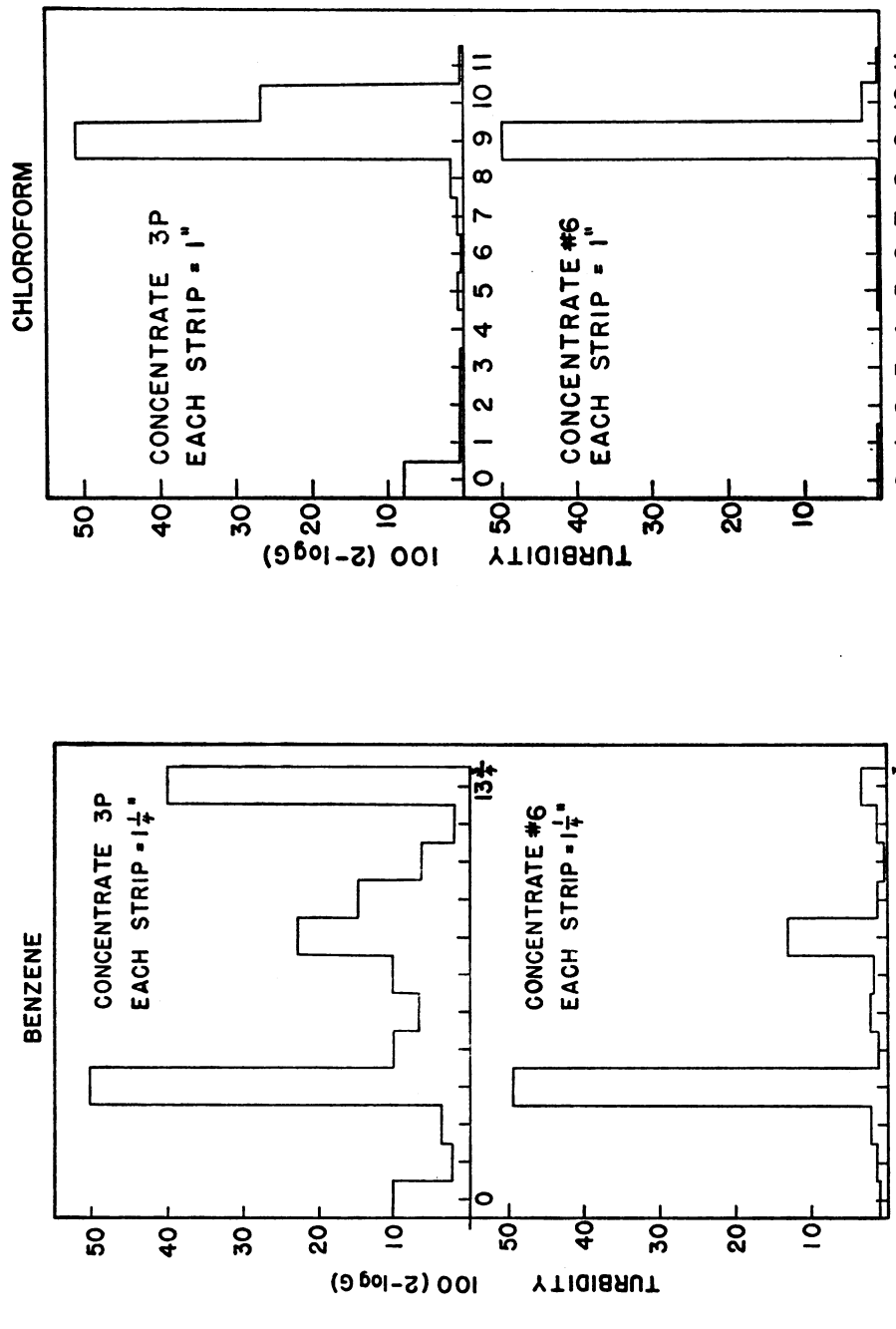

Figure 1. Filter paper chromotographs of the B.R. factor activity in a purified pyruvic oxidase preparation (no. 3P, 0.014 μg/ml = half maximum growth of *Bulgribacterium rettgeri*), and that in a purified B.R. factor preparation (no. 6, 0.37 μg/ml = half maximum growth). Developing solvents: chloroform and benzene, each saturated with water and containing 0.1 per cent acetic acid. Filter papers after drying were cut into strips of the size indicated. Each strip was immersed for 10 minutes in 10 ml of assay medium.

β-lipoic methyl ester sample is not known, the potency of the pure compound cannot be calculated.

We have not tested any purified protogen preparations for their B.R. factor activity. However, a recent note by Pierce *et al.* (1951) describes a crystalline S-benzylthiuronium salt of protogen-B which supported half maximum growth of *T. geleii* at a level of 0.3 mμg per ml of assay medium and which, significantly, was stated to have biological activity as the acetate replacing factor. Since this manuscript was submitted, Slater (1952) has published a report on the comparative biological activity of α-lipoic acid and protogen in the growth of *T. geleii*.

The interchangeability of these various highly purified or crystalline forms, considered together with the extremely potent activity of α- and β-lipoic acids for *B. rettgeri*, provides strong evidence for the probable identity of the B.R. factor with protogen, the pyruvic oxidase factor, and the acetate replacing factor.

Similar properties. Recent reports on the properties of the acetate replacing factor (Reed *et al.*, 1951*b*, 1951*c*), pyruvic oxidase (Reed *et al.*, 1951*a*, 1951*d*), and protogen (Pierce *et al.*, 1951) have disclosed marked similarities in properties to those previously reported for the B.R. factor (Kline and Barker, 1950). The B.R. factor was shown to have the properties of a monobasic acid with pK close to 5. Partition coefficients of some forms of this factor indicated that it was not a simple fatty acid. Since the activity was extractable with organic solvents from acidic solution (pH 1), it did not appear to contain a basic amino group. The highly purified or crystalline preparations of Reed and collaborators have also now been reported as acidic substances with pK values of 4.7 and 4.8 (Reed *et al.*, 1951*a*, 1951*d*). Their preparations are similarly extractable with organic solvents from strongly acid solutions (Reed *et al.*, 1951*c*). A highly purified form of protogen has also been reported to be an acidic oil (Pierce *et al.*, 1951).

As regards heat stability in acid and alkaline solutions, the B.R. factor again resembles the others. Autoclaving in 1 N acid or alkali for at least 30 minutes was the standard procedure previously reported by some of us (Kline and Barker, 1950) for liberating the active form or forms from inactive source materials. Also the activity in one particularly active yeast extract was not appreciably changed by such treatment. These results do not, of course, represent an absolute measure of stability but do serve to indicate a marked degree of stability in the crude form. Pyruvic oxidase, protogen, and acetate replacing factor have all been reported to exhibit relative stability in 1 N to 6 N acid or alkali in up to one hour at 120 C (O'Kane and Gunsalus, 1947; Stokstad *et al.*, 1949; Pierce *et al.*, 1951; Reed *et al.*, 1951*c*). A possible exception is the limited stability of purified concentrates of acetate replacing factor to autoclaving in 0.1 N to 1.0 N alkali.

Another property relating these various activities is their common occurrence in more than one active form distinguishable on the basis of partitioning behavior into immiscible organic solvents from aqueous solution. It was recognized in the case of the B.R. factor that solvent partition necessarily depended both upon the degree of liberation of the active moieties from inactive precursors and the extent to which these moieties were in the associated weak acid form. The

B.R. factor was shown (Kline and Barker, 1950) to occur in source materials both in an inactive form or forms which were not dissolved or partitioned into common organic solvents, and in an active form or forms, some of which were extractable from acidic aqueous solution by solvents, whereas others were not. At least three extractable forms in acid autoclaved sources were disclosed on chromatographic separation with benzene. An apparent lack of cognizance of the weak acid and multiform properties in early reports on the related three factors appear to be responsible for some contradictory data on their solvent properties. Thus pyruvic oxidase, originally reported to be nonextractable by ether (O'Kane and Gunsalus, 1947), is now reported (in the form of α-lipoic acid) to be very soluble in organic solvents and only sparingly soluble in water (Reed *et al.*, 1951*a*). Protogen similarly was reported to be nonextractable from aqueous solution at pH 3 by chloroform (Stokstad *et al.*, 1949), but in a very recent report (Pierce *et al.*, 1951) it is described as extractable with chloroform from a papain digest of liver after autoclaving in 3 N NaOH and acidifying. Protogen was originally reported to occur in at least two forms as judged by their separation on a Florosil column, and more recently several forms have been disclosed by the Lederle group using different solvent systems in the countercurrent method of Craig. Reed and collaborators (1951*c*) reported 5 distinct acetate replacing moieties in yeast extract by chromatographic separation with *n*-butanol and bioautographic identification. These workers also found that liver contained a microbiologically inactive form or forms of acetate replacing factor. It appears significant that both Reed and collaborators and the Lederle group have found that highly purified concentrates of acetate replacing factor and protogen, respectively, contain two principal components, the less polar of which is converted to the more polar during partition manipulation (Reed *et al.*, 1951*b*, 1951*d;* Pierce *et al.*, 1951).

General considerations. There is some evidence that certain forms of these apparently related factors may be active for one function and not for another. Thus we have found that two different batches of yeast extract which were equally active for pyruvic oxidase activity (private communication from Dr. I. C. Gunsalus, 1948) had relative activities for *B. rettgeri* of 50 to 1. After acid autoclaving solutions of these two extracts, they had approximately equal activities. This would suggest that a more conjugated form is utilizable in activating the apo-pyruvic oxidase system of *S. faecalis.*

We also find that cells of *B. rettgeri* grown on lactate in the presence of the growth promoting factor do not release any growth promoting activity when autoclaved in neutral solution but do release it when autoclaved in 1 N acid (Kline, 1950). This would suggest that the metabolically active form is more complex than the growth promoting form.

SUMMARY

Properties relating the growth promoting factor or factors for *Butyribacterium rettgeri* to the acetate replacing, pyruvic oxidase, and protogen group of activities are discussed.

Three pyruvic oxidase preparations in graded stages of activity were found to

have about the same relative activities for *B. rettgeri*. Chromatographic behavior of the B.R. factor activity contained in the most active of the three preparations was similar to that contained in a preparation purified on the basis of B.R. factor activity alone.

Highly purified preparations of acetate replacing factor (α- and β-lipoic acids) were extremely potent for *B. rettgeri;* 0.096 mμg of α-lipoic acid concentrate per ml of assay medium yielded half maximum growth.

Both the B.R. factor and crystalline acetate replacing factor have been reported to be weak acids with pK close to 5. Protogen is also reported to be an acidic substance.

Considerable heat stability at 120 C in 1 N to 6 N acid or alkali is exhibited by all four activities.

All occur in two or more active forms differentiated on the basis of varying partitioning behavior between aqueous solutions and immiscible organic solvents.

REFERENCES

GUIRARD, B. M., SNELL, E. E., AND WILLIAMS, R. J. 1946 The nutritional role of acetate for lactic bacteria. II. Fractionation of extracts of natural materials. Arch. Biochem., **9**, 381–386.

KLINE, L. 1950 Dissertation. University of California, Berkeley, California.

KLINE, L., AND BARKER, H. A. 1950 A new growth factor required by *Butyribacterium rettgeri*. J. Bact., **60**, 349–363.

O'KANE, D. J., AND GUNSALUS, I. C. 1947 Accessory factor requirement for pyruvate oxidation. J. Bact., **54**, 20–21.

O'KANE, D. J., AND GUNSALUS, I. C. 1948 Pyruvic acid metabolism. A factor required for oxidation by *S. faecalis*. J. Bact., **56**, 499–506.

PIERCE, J. V., MACCHI, M. E., HOFFMAN, C. E., FONG, C. T. O., STOKSTAD, E. L. R., JUKES, T. H., PATTERSON, E. L., BROCKMAN, J. A., AND DAY, F. P. 1951 Crystallization of a derivative of protogen-B. J. Am. Chem. Soc., **73**, 5919–5920.

REED, L. J., DEBUSK, B. G., GUNSALUS, I. C., AND HORNBERGER, C. S., JR. 1951*a* Crystalline alpha-lipoic acid: a catalytic agent associated with pyruvate dehydrogenase. Science, **114**, 93–94.

REED, L. J., DEBUSK, B. G., JOHNSTON, P. M., AND GETZENDANER, M. E. 1951*b* Acetate-replacing factors for lactic acid bacteria. I. Nature, extraction, and distribution. J. Biol. Chem., **192**, 851–858.

REED, L. J., GETZENDANER, M. E., DEBUSK, B. G., AND JOHNSTON, P. M. 1951*c* Acetate-replacing factors for lactic acid bacteria. II. Purification and properties. J. Biol. Chem., **192**, 859–865.

REED, L. J., DEBUSK, B. G., AND GUNSALUS, I. C. 1951*d* Chemical nature of alpha-lipoic acid. J. Am. Chem. Soc., **73**, 5920.

SLATER, J. V. 1952 Comparative biological activity of α-lipoic acid and protogen in the growth of *Tetrahymena*. Science, **115**, 376–377.

SNELL, E. E., AND BROQUIST, H. P. 1949 On the probable identity of several unidentified growth factors. Arch. Biochem., **23**, 326–328.

STOKSTAD, E. L. R., HOFFMANN, C. E., REGAN, M. A., FORDHAM, D., AND JUKES, T. H. 1949 Observations on an unknown growth factor essential for *T. geleii*. Arch. Biochem., **20**, 75–82.

STOKSTAD, E. L. R., HOFFMANN, C. E., AND BELT, M. 1950 Role of protogen in the nutrition of an unidentified *Corynebacterium*. Proc. Soc. Exptl. Biol. Med., **74**, 571–573.