'Iron Transport' Compounds as Growth Stimulators for *Microbacterium* sp.

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SUMMARY: An organism, resembling *Microbacterium lacticum*, was found to be stimulated by liver or yeast extract in a medium composed of glucose, amino acids, vitamins, adenine, guanine, uracil, ammonium formate, and mineral salts. These complex materials could be replaced by comparable concentrations of porphyrin-containing compounds and by extremely low concentrations of ferrichrome, coprogen, or terregens factor, which are thought to act as intracellular 'iron transport factors'.

In 1952 the discoveries of three new organic compounds of biological interest were announced. These were coprogen (Hesseltine *et al.* 1952), ferrichrome (Neilands, 1952), and terregens factor (Lochhead, Burton & Thexton, 1952). Coprogen, an essential growth factor for the fungus *Pilobolus*, is produced by various bacteria (including actinomycetes) and fungi (Hesseltine *et al.* 1953). Ferrichrome was isolated from *Ustilago sphaerogena* and can replace coprogen in the nutrition of *Pilobolus*. Terregens factor is produced by *Arthrobacter pascens* and promotes the growth of *A. terregens*, a requirement which can also be met by coprogen and ferrichrome (Burton, Sowden & Lochhead, 1954). Neilands (1957), in his excellent review on microbial iron metabolism, has proposed a unified hypothesis to account for the biological activity of these three different chemical entities. According to Neilands, these growth factors may act as coenzymes for the intracellular transfer of iron in micro-organisms.

The importance of the 'iron transport' compounds in microbial metabolism would be considerably enhanced if their growth-promoting characteristics could be demonstrated in other microbes. Thus, we wish to describe experiments which show that these compounds are potent growth stimulators for an organism resembling *Microbacterium lacticum*.

METHODS

Identification of organism

The culture was isolated as a laboratory contaminant. For its identification, the following methods were used according to the Society of American Bacteriologists, Committee on Bacteriological Technic (1944): fermentations, indole production (Gnezda test), gelatin liquefaction, starch hydrolysis, and action on litmus milk. The procedures recommended by Smith, Gordon & Clark (1952) were used for the detection of gelatin hydrolysis, production of urease, production of nitrite from nitrate, production of acetylmethylcarbinol

and casein hydrolysis. Catalase was detected by gas evolution upon addition of 3% (v/v) H_2O_2 to a slant culture. Heat resistance was determined by harvesting cells grown in a 10 ml. liquid culture containing basal medium + 0.04% injectable liver (Armour Laboratories, Kankakee, Illinois) for 2 days, adding 10 ml. sterile skim milk and dividing into several pre-heated tubes. The tubes were immersed in a water bath at 72°. At various times up to 60 min., tubes were withdrawn, cooled, and 0.1 ml. was spread on plates of basal medium containing liver + agar. The plates were incubated at 37° and examined after 3 days.

Acid production was determined by titration of liquid cultures with 0.01N-NaOH using bromthymol blue. Lactic acid was estimated by the method of Barker & Summerson (1941).

Media

The basal medium used for the nutritional studies is shown in Table 1. The culture was preserved in lyophile tubes. Upon opening a tube, the cells were diluted in 0.02M- K_2HPO_4 and spread on a large number of slants of basal

Table 1. *Composition of basal medium**

Component	Amount per litre (g.)	Component	Amount per litre (mg.)	Component	Amount per litre ($\mu\text{g.}$)
Glucose (autoclaved separately)	10	L-Histidine.HCl.H ₂ O	12	Biotin	10
	(mg.)	DL-Tryptophan	12	Folic acid	10
Ammonium formate	100	Glycine	2.5	Lipoic acid	10
L-Glutamic acid	260	L-Cystine	2	Leucovorin	10
L-Glutamine	120	L-Hydroxyproline	1	B ₁₂	2
DL-Valine	79	Adenine.SO ₄	10		(g.)
DL-Aspartic acid	60	Guanine.HCl	10	Na ₂ SO ₄	2
DL-Leucine	48	Uracil	10		(mg.)
DL-Isoleucine	48	Inositol	2	KCl	760
DL-Serine	48	Calcium pantothenate	1	Na ₂ HPO ₄	660
L-Proline	44	Riboflavin	1	MgCl ₂ .6H ₂ O	420
DL-Threonine	40	Pyridoxine	1	NaCl	150
DL-Phenylalanine	39	Pyridoxamine	1	FeCl ₃ .6H ₂ O	25
DL-Methionine	33	Pyridoxal	1	MnCl ₂ .4H ₂ O	18
L-Tyrosine	33	Nicotinic acid	1	ZnCl ₂	10
L-Lysine.HCl.H ₂ O	32	Thiamin	1		
L-Asparagine	20		($\mu\text{g.}$)		
DL-Alanine	19	PABA	100		
L-Arginine.HCl	19	Pantetheine	100		

* pH adjusted to 7.5 with NaOH.

medium containing 2.5% (w/v) Bacto-agar and 0.04% (w/v) injectable liver. These slants were stored at 5° and used when needed. When new slants were required they were prepared from another lyophile tube.

For nutritional studies, the basal medium was prepared in double strength. Five ml. were added to each 20 by 175 mm. colorimeter tube followed by a calculated amount of distilled water. Small glass beakers (10 ml.) were used as closures for the tubes. The tubes were autoclaved at 120° for 15 min. and

cooled to room temperature. One ml. of 10 % (w/v) autoclaved glucose was then added followed by the sterile additives. These were autoclaved except in the case of heat labile materials where sintered glass filters were employed. Inoculum was prepared by suspending the slant growth in 0.02M- K_2HPO_4 and diluting to an optical density (o.d.) of 0.05, using the Lumetron colorimeter (model 402-E) fitted with a 660 m μ filter. One drop (0.05 ml.) of such a suspension was used to inoculate each tube. Incubation was carried out at 37° on a shaking machine imparting a rotary motion of 220 r.p.m. Duplicate tubes were used throughout the study.

Estimation of growth

Growth was measured after approximately 30 hr. with the Lumetron colorimeter. Uninoculated medium was used to set the instrument to an optical density of zero. When the o.d. was greater than 0.3, the tube contents were diluted with water and the o.d. was re-read. The o.d. figures given represent the product of the observed o.d. and the dilution factor.

RESULTS

Identification studies

Microscopic examination of growth from refrigerated slants showed clusters of Gram-positive 'cocci' similar to those of *Micrococcus*. A fresh slant was inoculated with such growth and examined at various ages. At 6 hr. the 'cocci' had developed into Gram-positive rods which were still present at 17 hr. By 40 hr. the culture was completely in the 'coccoid' stage again. Examination with the electron microscope revealed that the 'cocci' were really coccobacilli which elongated during early growth.

The organism was non-acid fast, non-sporeforming, and non-motile. Slant growth was yellow and glistening. The aerobic nature of the organisms was shown by its much more rapid growth under shaking conditions than in static culture.

Fermentation tests revealed that acid was produced from glucose, sucrose, maltose, galactose, fructose, mannose and mannitol. Lactose, xylose, rhamnose, sorbitol, glycerol and arabinose were not fermented but supported growth. No gas was produced.

The culture was catalase positive, but failed to produce urease, indole or acetylmethylcarbinol. Casein was not hydrolysed and there was no action on litmus milk. Nitrite was not produced from nitrate. Although gelatin was not liquefied, it was hydrolysed in the plate test. Starch was hydrolysed, but its fermentation was extremely weak. Under the conditions described above, the culture was not sterilized by 72° for 1 hr.

Acid production was quite weak under the static conditions used for the fermentation tests. In basal medium containing injectable liver, the culture produced 0.04 % acidity, calculated as lactic acid. About half of this was actually due to lactic acid according to the Barker-Summerson assay. On the other hand, under shaking conditions, there was much better growth and

maximum acid production was 0.09–0.16 %. However, over 95 % of the acidity produced with aeration was due to acids other than lactic.

The data obtained indicate that the culture is a member of the genus *Microbacterium*. When this genus was examined by Doetsch & Pelczar (1948), they found that all the isolates were Gram-positive, non-sporeforming, non-acid fast, non-motile short rods which appear coccobacillary at times. Furthermore, all produced acid but not gas from glucose, fructose, and mannose but not from rhamnose and sorbitol, were catalase positive, and failed to liquefy gelatin, digest casein or form indole or acetylmethyl carbinol. In addition, our culture possesses high heat resistance and carries out a weak lactic acid fermentation, both characters usually being associated with *Microbacterium*.

The remainder of the fermentation results of the present study do not correlate exactly with any of the 18 isolates (representing three species) studied by Doetsch & Pelczar. However, these workers present data which show a considerable overlap of sugar fermentations among the species and quite a variation within species. Examination of the recent classification of the genus by Doetsch in the seventh edition of *Bergey's Manual* (1957) indicates the present culture resembles *Microbacterium lacticum* but differs from the standard description in its inability to ferment lactose.

Nutrition studies

When *Microbacterium* sp. was inoculated into the basal medium its growth was stimulated by addition of injectable liver and other liver preparations. Growth-promoting activity was not found in ribonucleic acid or deoxyribonucleic acid; however, yeast extract (Difco Laboratories Inc., Detroit, Michigan) and 'vitamin-free' enzyme-hydrolysed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) were active. The possibility of a peptide requirement was explored by adding high levels (1–3 mg./ml.) of single amino acids to the basal medium (Peters, Prescott & Snell, 1953; Demain & Hendlin, 1958) but none of these supplements replaced liver.

A survey of approximately 200 compounds including purines, pyrimidines, nucleosides, nucleotides, organic acids, carbohydrates, intermediates of nitrogen and carbohydrate metabolism, sterols, amines and miscellaneous biochemicals revealed only one compound capable of replacing liver, i.e. cytochrome *c*. As shown in Fig. 1, cytochrome *c* was approximately as active as injectable liver. A further search for active materials was conducted with other porphyrin-containing compounds. The following compounds stimulated growth at levels below 50 µg./ml., but none was more active than cytochrome *c*: haemoglobin, haemin and protoporphyrin. Haematoporphyrin was inactive at 50 µg./ml. The porphyrin precursor, δ -amino laevulinic acid, showed activity but again less than the cytochrome.

None of the above compounds was active enough to account for the liver response. The stimulation by haemin, however, provided a clue for further experimentation. It was recalled that in the nutrition of *Pilobolus* (Hesseltine *et al.* 1952) and *Arthrobacter terregens* (Burton *et al.* 1954) the relatively large haemin requirement could be replaced by low concentrations of coprogen and

terregens factor, respectively. As shown in Fig. 2, coprogen was much more active than liver. Similar results were obtained with terregens factor and ferrichrome. The half-maximal figures for ferrichrome, coprogen and terregens factor concentrate were 1, 12 and 230 $\mu\text{g./ml.}$ respectively. In view of the observation by Burton *et al.* (1954) that the iron-chelating compound, aspergillie acid, was active for *Arthrobacter*, we tested this compound and found half-maximal activity at 60 $\mu\text{g./ml.}$ Furthermore, *N*-D-glucosylglycine ethyl ester was similarly active. This compound was tested when it was reported that fructose-phenylalanine was a potent chelator of the ferric

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Activity of cytochrome *c* (●) and injectable liver (○) for growth of *Microbacterium* sp.

Fig. 2. Activity of millimicrogrammes of coprogen (●) and microgrammes of injectable liver (○) for growth of *Microbacterium* sp.

Fig. 3. Growth curves of *Microbacterium* sp. in the basal medium in the absence (●) and presence (○) of 100 $\mu\text{g./ml.}$ of ferrichrome.

ion (Neilands, 1957). Ethylenediamine tetraacetate and citrate showed no activity in our tests, indicating that the requirement is rather specific.

The nature of the growth stimulation was determined by comparing the growth curves of *Microbacterium* sp. in the presence and absence of ferrichrome. Figure 3 shows that the lag phase is shortened and that there is no marked effect on the rate or extent of growth.

DISCUSSION

The present study extends to the genus *Microbacterium* the nutritional requirement for ‘iron transport factors’. Proof of the role of ferrichrome, coprogen, and terregens factor as coenzymes of iron metabolism, as suggested by Neilands (1957), must await refined biochemical studies; however, their importance increases as the list of organisms requiring such factors is enlarged.

The specificity of the requirement for our strain was shown by the lack of activity of citrate and ethylenediamine tetraacetate. Furthermore, the large number of compounds which were tested and found to be inactive, some of which were known chelating agents, speaks against a general requirement for chelating compounds. The specificity in the present work appears to be narrower, for example, than that observed for the autoclaved glucose-phosphate requirements in *Lactobacillus fermenti* (Ramsey & Lankford, 1956) and *Bacillus globigii* (Lankford, Kustoff & Sergeant, 1957). The inactivity of glucosyl-glycine with the latter organism also indicates that the glucose-phosphate effect is distinct from the ‘iron transport factor’ stimulation.

The activity of glucosyl-glycine in the present study suggests that ‘iron transport factors’ may also be required by *Lactobacillus gayoni*. Rogers, King & Cheldelin (1953) found that this compound could replace liver or yeast, which in turn replaced the stimulation obtained by autoclaving the components of the medium together. It is also of interest that glucosyl-glycine could replace the autoclaved glucose stimulation in *Propionibacterium freundenreichii* although high concentrations were required (Field & Lichstein, 1957). Similarly, Neilands (1957) has reported that autoclaved glucose-glutamate is an essential requirement for a strain of *Micrococcus lysodeikticus*. This requirement can be replaced by several compounds including fructose-phenylalanine and ferrichrome but not by ethylenediamine tetraacetic acid. Evidence is thus accumulating which indicates a connexion between compounds formed upon heating sugars with amino acids and ‘iron transport factors’. The potentialities even become more interesting when one considers the data of Borsook, Abrams & Lowy (1955) concerning the role of ketose-amino acids in stimulation of amino acid incorporation into protein of rabbit reticulocytes. Fructose-L-glutamic acid, fructose-L-alanine and fructose-glycine were isolated from liver and could replace liver extract in the amino acid incorporation assay when low concentrations of iron were present. More recently, Kruh & Borsook (1956) demonstrated that fructose-amino acids were capable of stimulating haem synthesis and amino acid incorporation into globin.

The replacement of haemin by extremely low concentrations of 'iron transport factors' as observed with *Microbacterium* sp. was previously demonstrated with *Pilobolus* and *Arthrobacter terregens*. This repeatedly observed phenomenon led us to wonder whether the haemin requirements of other organisms, e.g. *Haemophilus influenzae* (Granick & Gilder, 1946) and isoniazid-resistant strains of *Mycobacterium tuberculosis* (Fisher, 1954) might also be met by these compounds. Recent experiments (Demain, unpublished) have eliminated the haemin-dependent *Escherichia coli* H7 (Beljanski, 1955) from consideration since coprogen, ferrichrome and terregens factor failed to replace haemin. Nevertheless, we feel that a requirement for haemin or cytochrome *c* should be investigated further before it is concluded that the primary defect is a deficient porphyrin-synthesizing system. It is quite probable that the porphyrin requirement is merely a result of a deficient intracellular ability to transfer or store low concentrations of iron.

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