PANTOTHENIC ACID SYNTHESIS BY SMOOTH BRUCELLA ABORTUS

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The relationship between formation of exogenous alanine and the establishment of nonsmooth variants in originally smooth cultures of *Brucella abortus* strains has been clearly demonstrated (Goodlow *et al.*, 1950). Furthermore, the addition of D-alanine to smooth cultures in synthetic medium (Gerhardt and Wilson, 1948) results in earlier establishment of nonsmooth cells (Goodlow *et al.*, 1951). Subsequently, it was found that an increase in concentration of pantothenic acid in Gerhardt-Wilson synthetic medium not only delayed and nearly eliminated the population changes of smooth *B. abortus* cultures but also antagonized the action of added D-alanine in selectively favoring nonsmooth types (Mika *et al.*, 1951). The apparent interrelationships between population changes, alanine accumulation, and pantothenic acid supply prompted an investigation of the mechanism of pantothenate synthesis by the smooth type of *B. abortus*, strain 19, in an effort to determine a basic link connecting these factors.

The data of this investigation describe conditions and substrates required for the synthesis of pantothenate by smooth *B. abortus*, strain 19. Both valine and α-ketoisovaleric acid are precursors for pantoate; and L-asparagine or L-aspartate yields β-alanine in this organism. The importance of stereoisomerism in the stimulation or inhibition by amino acids of various steps in pantothenate synthesis is emphasized. These results are in general agreement with those of other studies concerning synthesis of pantothenic acid and its component parts (Maas and Vogel, 1953; Lansford and Shive, 1952). Also, it has been shown that pH, substrate concentrations, and age of cells are significant factors influencing the ability of resting cells to carry out metabolic reactions terminating in synthesis of pantothenic acid. The discovery of these relationships has contributed to an understanding of selective factors promoting population changes of smooth cultures of *B. abortus*.

MATERIALS AND METHODS

Cells were grown on Albimi agar for 24–72 hours at 37 C and harvested as has been previously described (Altenbern and Housewright, 1951). The cell crop was washed twice with 0.1 M phosphate buffer, pH 7.4, and finally resuspended in buffer of the desired pH. All substrates were dissolved in buffer of the proper pH and stored under refrigeration until used.

Pantothenic acid synthesis was determined by adding 5 ml of cell suspension and substrates to a 125 ml flask and diluting with buffer to a total volume of 20 ml. The flasks were then shaken on a reciprocating shaker at 37 C. Samples were withdrawn after 1, 2, 4, and 6 hours of incubation, centrifuged to remove cells, and the supernatants were assayed for pantothenic acid.

Assay for pantothenate was conducted with *Lactobacillus arabinosus*, strain 17-5, using pantothenate assay medium (Difco), dehydrated. Stock cultures of *L. arabinosus* were maintained in stabs in micro-inoculum culture agar (Difco). The inoculum was grown for 18 hours at 37 C in micro-inoculum broth (Difco), the cells were centrifuged down, washed twice in sterile saline, and finally diluted 1–100 in sterile saline for the inoculum, 0.1 ml of which was added to each assay tube. The assays were read at 515 mμ in a Coleman spectrophotometer after 18–24 hours of incubation at 37 C. The general procedure was that of Skeggs and Wright (1944).

RESULTS

Both the smooth and rough types of *B. abortus*, strain 19, possess an enzyme coupling pantoyl lactone and β-alanine to produce pantothenic acid. There are, however, two distinct rates of pantothenate synthesis indicative of an adaptive lag, possibly adaptation to hydrolysis of the pantoyl lactone. Preincubation of cells with pantoyl lactone plus glucose for 2–4 hours eliminates this lag and allows an initial rapid rate of pantothenate production from pantoyl

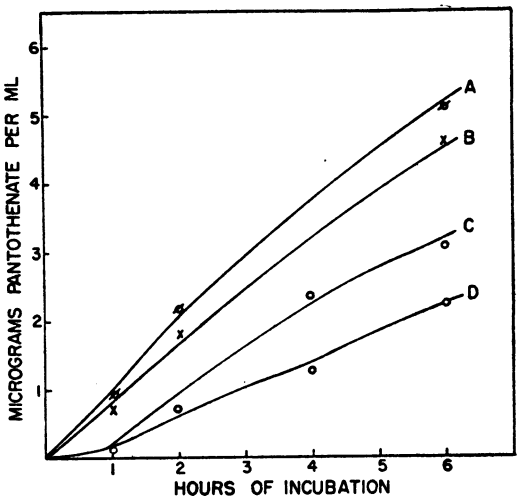

Figure 1. Adaptation of smooth *Brucella abortus* to synthesis of pantothenate from pantoyl lactone by preliminary incubation with pantoyl lactone. Five ml of cell suspension plus substrates diluted to 20 ml with 0.1 M phosphate buffer, pH 7.4. Incubated on shaker at 37 C and sampled at times indicated. All flasks contained 0.1 M glucose. Adapted cells were incubated with 0.01 M pantoyl lactone for 4 hours at 37 C, recovered by centrifugation, and resuspended in same volume of pH 7.4 buffer. Curve A, adapted cells with 0.01 M pantoyl lactone plus 0.01 M β-alanine; curve B, adapted cells with 0.01 M pantoyl lactone; curve C, unadapted cells with 0.01 M pantoyl lactone plus 0.01 M β-alanine; curve D, unadapted cells with 0.01 M pantoyl lactone. All molarities are final concentrations.

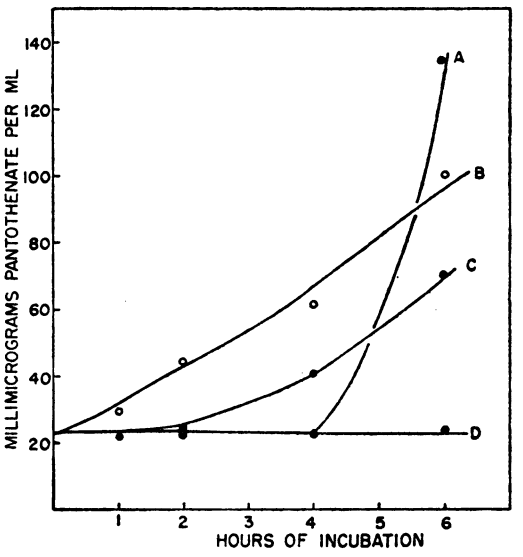

Figure 2. Synthesis of pantothenic acid from L-valine by smooth *Brucella abortus*. Five ml of cell suspension plus substrates diluted to 20 ml with 0.1 M phosphate buffer, pH 7.4. Incubated on shaker at 37 C and sampled at times indicated. Complete system contained 0.01 M L-valine, 0.01 M β-alanine and 0.1 M glucose as final concentration. Curve A, minus glucose; curve B, complete system; curve C, minus β-alanine; curve D, minus valine. Base line of 23 mμg pantothenate per ml present in cell suspension in complete absence of substrate. The long lag and late synthesis of pantothenate in the absence of glucose were not investigated further.

lactone plus β-alanine. Rough cells also exhibit this phenomenon of adaptation. These cells possess considerable amounts of endogenous β-alanine, and the addition of β-alanine results in only a moderately increased rate of pantothenate production (figure 1). This coupling reaction requires the concomitant oxidation of glucose as an energy source. Smooth and rough types of the organism display, qualitatively, the same reactions although there are apparently quantitative differences, synthesis of pantothenate being more rapid with smooth cells than with rough cells.

In the absence of pantoyl lactone there is no synthesis of pantothenate, however, good synthesis of pantothenate is obtained using valine or α-ketoisovalerate as a precursor for pantoic acid. Although this rate of production is less than the rate of synthesis by cells adapted to pantoyl lactone, there is no lag and the initial synthesis rate remains the same. Pyridoxal phosphate (20 μg per ml), folic acid (10 μg per ml), methanol, sodium formate, or formaldehyde (0.001–0.01 M) was added and was ineffective, with the exception of formaldehyde which was markedly inhibitory at 0.01 M concentration. Elimination of glucose resulted in a long lag, and there was little or no synthesis of pantothenic acid in the absence of valine, demonstrating the inability of these cells to synthesize or to mobilize endogenous sources of valine or ketovaline (figure 2). D-Valine, alone or in combination with L-valine, also served as an efficient precursor for pantothenate (table 1). The fact that the rate of pantothenate synthesis from

TABLE 1

Synthesis of pantothenic acid from L- or D-valine plus β-alanine by smooth Brucella abortus

Substrate	Mμg of Pantothenate Per Ml After:		
	2 hr	4 hr	6 hr
D-Valine (0.01 M) + basal substrate	12	72	116
L-Valine (0.01 M) + basal substrate	11	71	163
L-Valine (0.01 M) + D-valine (0.01 M) + basal substrate	16	245	432
Basal substrate alone	9	9	9

5 ml of cell suspension plus substrates diluted to 20 ml with 0.1 M phosphate buffer, pH 7.4. Incubated on shaker at 37 C and sampled at times indicated. Basal substrate consisted of β-alanine (0.01 M) and glucose (0.1 M). In all tables, molarities represent final concentrations.

valine was equal to the rate from ketovaline but was much less than the rate from pantoyl lactone indicated that the rate limiting reaction is probably in the transformylation step, converting ketovaline to ketopantoate. Attempts to increase the synthesis rate of fresh cells by the addition of folic acid plus C_1 sources were unsuccessful.

By employing cells which had been aerated on a shaker for 72 hours at 37 C in the absence of substrate or for 48 hours in the presence of ketovaline plus β-alanine to reduce the endogenous C_1 supply, it was possible to demonstrate that DL-serine increases the rate of pantothenate synthesis from L-valine or ketovaline. Neither glycine alone nor glycine plus formaldehyde or formate was able to stimulate pantothenate production to a measurable degree. Different batches of cells varied considerably in their endogenous supply of both β-alanine and C_1 donors. At pH 7.4, it was apparently essential that endogenous C_1 supply was nearly exhausted before a significant effect of DL-serine could be observed. However, at pH 5.4, L-serine greatly enhanced the rate of pantothenic acid synthesis from L-valine plus β-alanine, and D-serine effectively antagonized the stimulatory action of L-serine (table 2).

Compounds analogous to valine inhibited synthesis of pantothenic acid from L-valine plus β-alanine. Both L-leucine and L-isoleucine decreased the production of pantothenate from these substrates, and the inhibitions produced were additive. Analysis of this phenomenon on a quantitative basis revealed that L-leucine was more inhibitory than L-isoleucine (table 3). Since leucine is more closely analogous to valine by virtue of a common terminal isopropyl group, the greater inhibition by L-leucine was anticipated. It is probable that interference occurs at the transformylation step converting ketovaline to ketopantoate inasmuch as neither leucine nor isoleucine inhibited coupling of pantoyl lactone and β-alanine. Valine, leucine, and isoleucine can be converted to the corresponding keto acids by transamination (Altenbern and Housewright, 1953) and perhaps by direct oxidation.

The effect of pH on pantothenate synthesis from pantoyl lactone was also determined. It

TABLE 2

Stimulation by serine of pantothenic acid synthesis from L-valine plus β-alanine by smooth Brucella abortus

	Mμg of Pantothenate Per Ml After 6 Hours of Incubation	
	Depleted cells at pH 7.4	Fresh cells at pH 5.4
Basal substrate	78	105
Basal + 0.01 M DL-serine	200	—
Basal + 0.01 M glycine	81	—
Basal + 0.01 M glycine + 0.001 M formaldehyde	68	—
Basal + 0.005 M L-serine	—	195
Basal + 0.010 M L-serine	—	765
Basal + 0.015 M L-serine	—	443
Basal + 0.015 M L-serine + 0.005 M D-serine	—	325
Basal + 0.015 M L-serine + 0.010 M D-serine	—	274
Basal + 0.015 M L-serine + 0.015 M D-serine	—	137

5 ml of cells plus substrate solutions diluted to 20 ml with 0.1 M phosphate buffer and shaken at 37 C in 125 ml flasks and sampled at various times. Basal substrate consisted of L-valine (0.01 M), β-alanine (0.01 M), and glucose (0.1 M). Cells grown for 72 hours and depleted by shaking at 37 C for 48 hours at 37 C in the presence of 0.01 M ketovaline plus 0.01 M β-alanine showed essentially the same results.

TABLE 3

Leucine and isoleucine inhibition of pantothenate synthesis from valine plus β-alanine by smooth Brucella abortus

	Mμg of Pantothenate Per Ml After 6 Hours
Basal substrate	225
Basal substrate + 0.0025 M L-leucine	20
Basal substrate + 0.005 M L-leucine	16
Basal substrate + 0.01 M L-leucine	0
Basal substrate + 0.0025 M L-isoleucine	105
Basal substrate + 0.005 M L-isoleucine	20
Basal substrate + 0.01 M L-isoleucine	20

5 ml of cell suspension plus substrate solutions diluted to 20 ml with 0.1 M phosphate buffer, pH 7.4. Incubated on shaker at 37 C in 125 ml flasks and sampled at various times. Basal substrate consisted of L-valine (0.01 M), β-alanine (0.01 M), and glucose (0.1 M).

was found that acid pH values substantially reduced the production of this vitamin from pantoyl lactone alone; however, the rate of pantothenate synthesis from pantoyl lactone plus β-alanine was unaffected by pH changes. It is concluded that acid pH values diminished endogenous β-alanine production (table 4).

Synthesis of β-alanine was likewise investigated. Attempts to show that L-asparagine and

TABLE 4

The effect of pH on synthesis of pantothenic acid from pantoyl lactone with and without β-alanine by smooth Brucella abortus

Substrate	Mμg of Pantothenate Per Ml After 4 Hours of Incubation			
	pH 5.4	pH 6.4	pH 7.4	pH 8.0
Pantoyl lactone	335	1,200	910	1,740
Pantoyl lactone + β-alanine	9,000	9,500	10,000	10,000

5 ml of cells suspension plus substrate solutions diluted to 20 ml with proper 0.067 M phosphate buffer. Incubated on shaker at 37 C and sampled at various times. All substrates dissolved in appropriate buffer. Density of cell suspension same at each pH. Pantoyl lactone and β-alanine used in 0.01 M final concentration.

L-aspartic acid are precursors of β-alanine led to a complex over-all picture. Initially, age of the cells appeared to be of great importance in demonstrating β-alanine synthesis from asparagine or aspartate. Cells harvested after 24 hours growth and then depleted in endogenous β-alanine at pH 7.4 by 48 hour shaking in the presence of pantoyl lactone were able to use L-asparagine or L-aspartate for β-alanine synthesis. However, 48 hour cells subjected to the same depletion procedure used small amounts of L-asparagine for β-alanine production but were inhibited by larger amounts whereas all concentrations of L-aspartate were equivalent and only feebly stimulated pantothenate synthesis. Cells grown for 72 hours and similarly

TABLE 5

Production of β-alanine from L-asparagine or L-aspartic acid by smooth Brucella abortus under various conditions

Substrate	Mμg of Pantothenate Per Ml After 6 Hours of Incubation			
	Depleted 24 hr cells at pH 7.4	Depleted 48 hr cells at pH 7.4	Depleted 72 hr cells at pH 7.4	Fresh 72 hr cells at pH 5.4
Pantoyl lactone (PL)	2,200	820	975	190
PL + β-alanine	2,100	1,710	2,250	7,250
PL + 0.001 M L-asparagine	2,200	1,345	890	310
PL + 0.005 M L-asparagine	2,925	1,550	490	1,035
PL + 0.010 M L-asparagine	3,075	1,220	325	2,020
PL + 0.015 M L-asparagine	3,650	955	—	1,860
PL + 0.02 M L-asparagine	3,650	700	0	2,010
PL + 0.001 M L-aspartic acid	2,300	1,180	1,050	210
PL + 0.005 M L-aspartic acid	2,300	1,000	1,080	317
PL + 0.01 M L-aspartic acid	2,925	1,050	1,470	1,115
PL + 0.015 M L-aspartic acid	3,000	935	—	765
PL + 0.02 M L-aspartic acid	3,150	1,030	—	815

5 ml of cells plus substrates diluted to 20 ml with 0.1 M buffer of proper pH. Incubated at 37 C on shaker and sampled at various times. Pantoyl lactone and β-alanine used in 0.005 M concentrations. Glucose (0.1 M) present in all flasks.

treated were inhibited by L-asparagine and were stimulated only by high concentrations of L-aspartate. Undepleted 72 hour cells suspended in pH 5.4 buffer were stimulated by both L-asparagine and L-aspartate (table 5). Regardless of these complexities, the data do show that either L-asparagine or L-aspartic acid can, under proper conditions, increase pantothenic acid synthesis by furnishing additional β-alanine.

Since in Gerhardt-Wilson synthetic medium, DL-asparagine is the sole nitrogen source, the effect of D-asparagine on pantothenate synthesis from pantoyl lactone plus L-asparagine was determined. Employing fresh 72 hour cells at pH 5.4, we found that D-asparagine (0.005–0.01 M) antagonized β-alanine synthesis from L-asparagine (0.01 M) by 60 to 65 per cent. This inhibition could not be demonstrated using β-alanine-depleted 24 hour cells at pH 7.4. Neither L-nor D-alanine at 0.005 M concentration inhibited pantothenic acid synthesis from pantoyl lactone plus β-alanine or L-asparagine at pH 7.4 or pH 5.4 using 72 hour cells.

It has been reported that degradation of uracil via dihydrouracil leads to β-alanine in animal tissues (Fink *et al.*, 1953). Various inhibitors of uracil metabolism were tested for their ability to reduce pantothenic acid synthesis from pantoyl lactone. Thiouracil, sodium barbital, and barbituric acid were without effect, but high concentrations of benzimidazole (0.005–0.01 M) were inhibitory. The inhibition was not, however, overcome by an equivalent amount of uracil, and its significance remains undetermined.

DISCUSSION

The results recorded here are in essential agreement with those from other investigations on the synthesis of pantothenic acid (Maas and Vogel, 1953; Lansford and Shive, 1952). The route of pantoic acid synthesis by this organism is probably similar to the mechanism proposed in other organisms where valine is converted to ketovaline, thence to ketopantoate, and finally to pantoate. Experiments with sonic extracts designed to reveal the stepwise synthesis both of pantoic acid and of β-alanine are now in progress.

Stimulation of pantothenate synthesis by asparagine or aspartic acid and inhibition of synthesis by the same compounds under varying conditions may resolve some seeming anomalies of other investigations. It has been reported (Billen and Lichstein, 1949) and theoretically accepted that β-alanine may arise by decarboxylation of aspartic acid. Contrariwise, Maas and Davis (1950) have demonstrated that L-aspartic acid inhibits pantothenic acid synthesis in *Escherichia coli* although stimulation of synthesis by aspartate would be expected. Our results show that both situations may be observed with one organism and that pH, substrate concentration, and age of cells play determinative roles in the ability of asparagine or aspartate either to stimulate or to inhibit synthesis of pantothenate.

All evidence indicated that transformylation is a sluggish reaction in smooth *B. abortus*, strain 19. At pH 7.4, DL-serine definitely but moderately enhanced the rate of pantothenic acid production from L-valine or ketovaline plus β-alanine where endogenous C_1 sources were nearly depleted. At pH 5.4, L-serine stimulation of pantothenate synthesis from L-valine plus β-alanine could be clearly demonstrated. Under these conditions, D-serine strongly antagonized the action of L-serine. Reports that D-serine is inhibitory to pantothenate synthesis in *E. coli* (Maas and Davis, 1950) may also indicate that transformylation from L-serine to ketovaline is antagonized by D-serine. Furthermore glycine reversal of D-serine inhibition in *E. coli* could reflect increased L-serine synthesis thereby effectively reversing D-serine inhibition of the transformylation step. Apparently C_1 transfer in *B. abortus*, strain 19, is so sluggish that only serine has a demonstrable effect.

It is known that D-asparagine has a detrimental effect on metabolism and growth of *B. abortus*. Smooth strain 19 growing in Gerhardt-Wilson medium employing L-asparagine as the nitrogen source synthesizes pantothenate and excretes the vitamin into the medium. Addition of equimolar D-asparagine to the medium at any time stops the synthesis and accumulation of pantothenate. On the other hand, the rough type growing in Gerhardt-Wilson medium containing L-asparagine takes up pantothenate from the medium (Altenbern, 1952, *unpublished data*). Goodlow *et al.* (1952) have shown that the configuration of asparagine controls population changes of *B. abortus* cultures. They have demonstrated that little if any population change takes place when smooth types are grown in Gerhardt-Wilson medium containing L-asparagine, whereas DL-asparagine results in considerable population change and D-asparagine yields an even higher percentage of nonsmooth types.

Unpublished studies on sonic extracts of strain 19 cells have revealed that the efficiency of the coupling reaction leading to pantothenate is very low in smooth cells. Thus, it is not surprising that the addition of high concentrations of pantothenate can stimulate the growth of smooth cells. The correlation between the study of population changes related to asparagine configuration and the production of pantothenate as a function of asparagine isomers suggests that smooth cells may depend primarily on their own synthetic ability to satisfy their need for pantothenic acid. In contrast, rough cells show a greater ability to utilize preformed, extracellular pantothenate. Thus, in a medium containing proper concentration of D-asparagine, smooth cells, in which synthesis is the primary source of required pantothenic acid, would be more inhibited than rough cells which can satisfy their need for pantothenate acid from extracellular sources (medium). Experiments on growing cells have demonstrated that the addition of high levels of pantothenate can reverse inhibitory effects against smooth cells (Mika *et al.*, 1951). It can be suggested that under these conditions, i.e., whenever extracellular pantothenate concentration exceeds a threshold value, smooth cells also become capable of utilizing preformed pantothenate and thus are able to attain a higher selective advantage. Such considerations fit well with all data so far collected from population studies, resting cell experiments, and studies on sonic extracts. They suggest that D-asparagine, by differential inhibiting effects upon smooth and rough cells, may represent a major selective factor by interfering with pantothenate (β-alanine) synthesis in smooth cells, thereby selectively favoring nonsmooth types. However, so far D-asparagine interference with pantothenate synthesis from pantoyl lactone plus L-asparagine by smooth resting cells can be shown only at pH 5.4 and not at pH 7.4.

Although D-alanine selectively favors nonsmooth types (Goodlow *et al.*, 1951), unequivocal evidence that D-alanine is the natural metabolic substance influencing population changes has not been obtained. Neither L- nor D-alanine inhibited pantothenic acid synthesis under any conditions of pH or substrate concentration in resting cells and appeared to have no influence on the synthesis of this vitamin from pantoate and β-alanine. The precise nature of the relationship between alanine accumulation and selection of nonsmooth types is under investigation at present.

The results recorded here reveal the importance of permeability in demonstrating metabolic reactions in whole cell suspensions of these organisms. Previous studies (Altenbern and Housewright, 1952; Gerhardt *et al.*, 1953) have also demonstrated permeability barriers in brucellae and have shown that various manipulations (pH adjustment, cell-free extracts) eliminate this obstacle. However, the fact remains that *brucellae* have a pH optimum for growth of 6.8 to 7.2, conditions under which some of the reactions and inhibitions described in this report cannot be demonstrated using resting cell suspensions. Results from growth experiments discussed above indicate that such reactions and inhibitions do occur during growth but at a slow rate, and, therefore, it remains possible that D-asparagine may be a prime selective agent in smooth cultures of brucellae in liquid Gerhardt-Wilson medium.

Previous studies concerning oxidation of amino acids by various brucella strains have revealed that, of the D-isomers, only D-alanine is detectably oxidized (Gerhardt *et al.*, 1950). The data of the present study show that both isomers of valine are oxidized as judged by the synthesis of pantoate and it is possible that other D-amino acids are oxidized at a rate not demonstrable by manometric techniques.

SUMMARY

Smooth cells of *Brucella abortus*, strain 19, possess an enzyme which couples pantoyl lactone and β-alanine to produce pantothenic acid. Resting cells exhibit a one to two hour lag in pantothenate synthesis from these substrates, but the lag can be eliminated by preincubation with pantoyl lactone. In the absence of pantoyl lactone, no pantothenate is produced; however, L- or D-valine and α-ketoisovaleric acid (ketovaline) serve as efficient precursors for the pantoate portion of the pantothenate molecule. L-Serine stimulated production of pantothenate from L-valine plus β-alanine, and this stimulation was antagonized by D-serine. Both L-leucine and L-isoleucine were potent inhibitors of pantothenate synthesis from L-valine.

β-Alanine is present endogenously in nearly maximal amounts but by depletion procedures or by adjustment of pH to 5.4, it has been possible to show that β-alanine can be produced from

L-asparagine or L-aspartic acid and that D-asparagine interferes with this process. Age of cells and concentration of substrate were shown to be important factors in the demonstration of β-alanine production from L-asparagine or L-aspartic acid. Acid pH conditions reduce endogenous β-alanine supply but do not affect the rate of coupling pantoyl lactone and β-alanine.

These metabolic reactions have been discussed in connection with studies concerning population changes of cultures of smooth brucellae and the nature of the selective agents responsible for such population changes.

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