IDENTIFICATION OF ASPARAGINE AS THE SUBSTANCE STIMULATING THE PRODUCTION OF BUTYL ALCOHOL BY CERTAIN BACTERIA[1]

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In a previous paper, Tatum, Peterson and Fred (1934) described a factor found in potato and potato extract which markedly stimulated the fermentation of starch and the production of butyl alcohol by certain butyric acid bacteria. The stimulant was found in many other plant materials and varied in its effect on different strains of the bacteria.

Since this publication the active principle has been isolated from potato and identified as *l*-asparagine. Both *l*-aspartic and *d*-glutamic acids have an equivalent stimulatory action, while other related compounds, ammonium malate and ammonium succinate, cause a definite but less effective stimulation.

EXPERIMENTAL

Isolation and identification of the active principle

As an index to the presence of the active principle during the isolation, culture 21 (McCoy, Fred, Peterson and Hastings (1930)), was used, since this culture gives a maximum and consistent response.

The fermentations were carried out in test-tubes containing 35 cc. of 4 per cent corn mash. The cultures were analyzed for butyl alcohol (Johnson (1932)), the amount of which is an accurate index of the degree of stimulation.

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Method of isolation. The method of isolation (fig. 1) involved treatment of the potato juice with lead acetate and ammonium hydroxide to remove carbohydrates, proteins, etc. The ammonia was removed by aeration from alkaline solution, and the active principle precipitated by mercuric acetate and sodium carbonate (Neuberg and Kerb (1912)). The mercury was removed from the precipitate with H_2S and the excess H_2S by aeration. Evaporation of the solution led to the crystallization of an active

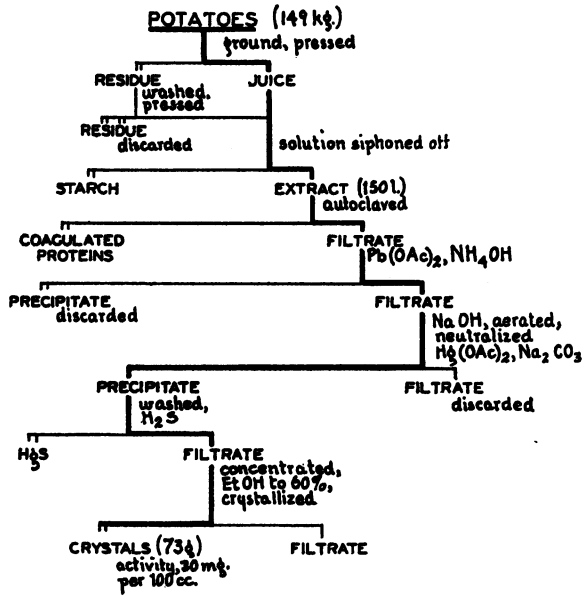

FIG. 1. METHOD OF ISOLATION OF ACTIVE SUBSTANCE

substance which was almost insoluble in 60 per cent alcohol. After washing the crystals with dilute alcohol to remove a gummy material, they were recrystallized from hot dilute alcohol. From 149 kgm. of potatoes 73 grams were obtained. Tests on each discarded fraction showed that the crystalline material alone possessed activity.

Identification. The crystals were proven to be *l*-asparagine by their water of crystallization, total and amid nitrogen content, and specific rotation and by the preparation and analysis of the benzoyl derivative.

A sample was dried over calcium chloride to constant weight, and the water of crystallization driven off at 105°C.

1.2477 grams ($CaCl_2$ dried) lost 0.1522 gram at 105°C.
Calculated for $C_4H_8N_2O_3 \cdot H_2O$ ..... H_2O 12.0
Found ..... H_2O 12.2

Total nitrogen by the Kjeldahl method was run on anhydrous samples (dried at 105°C.).

0.2087 gram gave 31.3 cc. 0.1 N NH_4OH
0.1953 gram gave 29.2 cc. 0.1 N NH_4OH
Calculated for $C_4H_8N_2O_3$ ..... N 21.19
Found ..... N 21.00, 20.94

A determination of amid nitrogen was run on the anhydrous material by measuring the ammonia evolved from boiling 1 N NaOH.

0.5228 gram gave 38.0 cc. 0.1 N NH_4OH
Calculated for $C_4H_8N_2O_3$ ..... N 10.59
Found ..... N 10.18

The specific rotation for a 0.4 per cent solution was $-9.7°$ in water and $+34.7°$ in 10 per cent HCl. The reported values are $-6.2°$ in water and $+37.3°$ in HCl (Mulliken (1918)).

The benzoyl derivative was prepared by dissolving the sample in sodium bicarbonate solution and adding benzoyl chloride with constant shaking (Fischer (1899)). The derivative was precipitated with acid, filtered and washed with alcohol and ether to remove benzoic acid and recrystallized.

0.02396 gram gave 0.04942 gram CO_2, 0.01025 gram H_2O
0.03253 gram gave 0.06710 gram CO_2, 0.01430 gram H_2O
1.0258 gram gave 43.7 cc. 0.1 N NH_2OH (amid nitrogen)
Calculated for $C_{11}H_{12}N_2O_4$ ..... C 55.9 H 5.08 Amid N 5.93
Found ..... C 56.2 H 4.78 Amid N 5.98
C 56.3 H 4.93

The benzoyl derivative had a melting point of 191°—192°C. The same melting point was found for the derivative of an authentic sample of asparagine, and for a mixture of the two preparations.

The properties of the stimulating factor as determined by biological activity during the preliminary investigations were

similar to those of asparagine, namely, solubility in water and insolubility in organic solvents; stability to autoclaving; destruction by ashing; ease of dialysis; non-adsorption; non-precipitation by lead acetate, tungstic acid or phosphotungstic acid; precipitation by Neuberg's reagent; destruction by nitrous acid.

The identification of aspartic acid. *l*-Aspartic acid was isolated by crystallization from the acid hydrolysate of the crystals. The benzoyl derivative was prepared as above and analyzed.

0.02648 gram gave 0.05498 gram CO_2, 0.01142 gram H_2O
0.03084 gram gave 0.06307 gram CO_2, 0.01313 gram H_2O
0.0416 gram gave 9.535 cc. 0.1 N NH_4OH (Kjeldahl)

Calculated for $C_{11}H_{11}NO_5$	C 55.6	H 4.6	N 5.9
Found	C 55.6	H 4.8	N 5.73
	C 55.8	H 4.7	

The insoluble copper salt of aspartic acid was prepared by adding a hot solution of copper acetate to a water solution of the isolated aspartic acid neutralized with sodium hydroxide. On cooling, the copper salt separated out. It was dried at 140°C. and analyzed for copper.

0.1111 gram gave 0.0447 gram CuO

Calculated for $C_4H_5NO_4Cu$	Cu 32.2
Found	Cu 32.7

The specific rotation of the free acid recovered from the copper salt was +22.9° for a 1.25 per cent solution in 10 per cent HCl. The reported values range from +23° to +25° (Mitchell and Hamilton (1929)).

Biological effects

Effect of l-asparagine on the production of butyl alcohol. The stimulating influence of the isolated asparagine is shown in figure 2. With increasing concentration of asparagine the butyl alcohol increased. All samples of commercial asparagine caused a stimulation identical with that brought about by the potato preparation. When the stimulative effect of crude potato extract is plotted against its asparagine content as determined by amid nitrogen, a similar curve is obtained (fig. 2). The slope of the curve is identical with that of pure asparagine and the maximum

is reached at the same concentration. The higher yield is probably due to the presence of aspartic acid or other related compounds which, as will be shown later, also have a stimulatory effect.

Effect of aspartic acid on the production of butyl alcohol. Aspartic acid had an effect quite similar to that of asparagine (fig. 3). Although the maximum yield of butyl alcohol is a little higher than with asparagine, the slope of the curve is not as steep. The

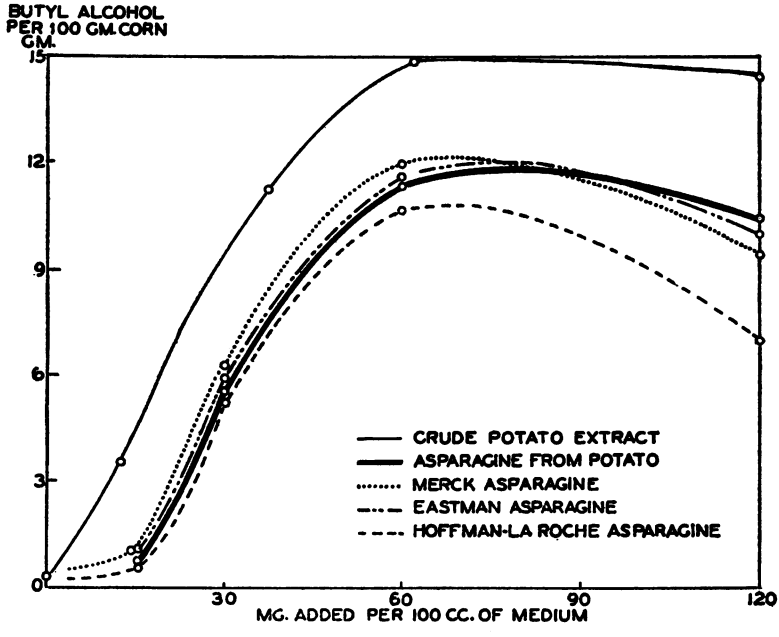

FIG. 2. EFFECT OF ASPARAGINE ON THE PRODUCTION OF BUTYL ALCOHOL

addition of ammonium sulfate in one-half molal quantity per mol aspartic acid overcame this lag without changing the maximum yield of butyl alcohol. The effect of ammonium salts alone was not at all comparable with that of asparagine or aspartic acid.

The activity of the asparagine samples was not due to an adsorbed impurity such as thallium (Richards (1932)), since repeated recrystallization had no effect on their activity. Aspartic acid, recovered from the copper salt, or from the ether-extracted

and recrystallized benzoyl derivative, had the same activity as the original acid. This indicated that the stimulant was aspartic acid itself and not an impurity since it is improbable that an impurity would remain through the formation of a derivative, recrystallization, ether extraction (in the case of the benzoate) and regeneration. The ether extraction was especially conclusive

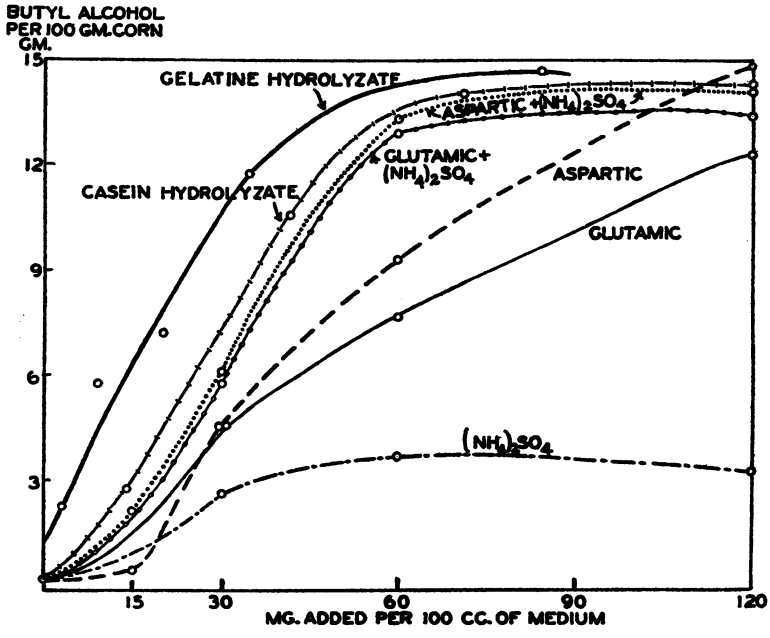

FIG. 3. THE EFFECT OF ASPARTIC AND GLUTAMIC ACID ON THE PRODUCTION OF BUTYL ALCOHOL

Glutamic and aspartic acid neutralized with NaOH before adding to fermentation. Ammonium sulfate added in the ratio of 0.5 mol per mol of amino acid.

since the stimulant as such could under no conditions be extracted with ether.

Effect of other amino acids. A number of other amino acids were tested for stimulatory action, *viz.*, glycine, alanine, leucine, cystine, glutamic acid and a mixture of mono-amino acids obtained from a butyl alcohol extract of casein hydrolyzate (Dakin (1918)). Of these, only glutamic acid had any effect. It increased the

butyl alcohol production just as did aspartic acid, the effect being similarly influenced by the addition of ammonium salts (fig. 3). The specific effect of dibasic amino acids was confirmed by the stimulation caused by casein and gelatin hydrolyzates (fig. 3). Here the production of butyl alcohol was plotted against the amount of dibasic amino acids in the added hydrolyzate. The figure for each protein was calculated from data given by Mitchell and Hamilton (1929). The curves are practically identical with that of aspartic or glutamic acid and ammonium sulfate.

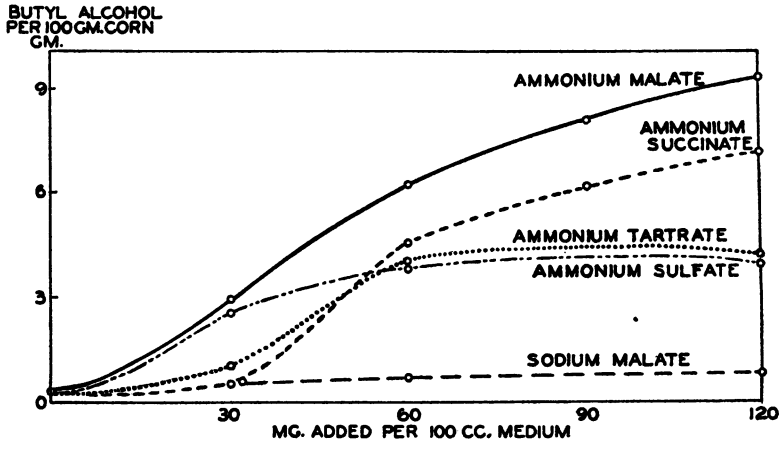

FIG. 4. EFFECT OF AMMONIUM MALATE, SUCCINATE, AND TARTRATE ON THE PRODUCTION OF BUTYL ALCOHOL

Acids neutralized with NH_4OH or NaOH before adding to fermentation

As a result of hydrolysis, there probably was a considerable amount of ammonium sulfate present to increase the stimulative action of the dibasic amino acids. Since casein contains 36.4 per cent dibasic amino acids (including β-OH glutamic acid) while gelatin contains only 9.2 per cent, the remarkable similarity of the curves when calculated on the dibasic acid content is very strong confirmatory evidence that all the dibasic amino acids, and only the dibasic acids, are concerned in the stimulation.

Substitution of related compounds for aspartic acid. Since the stimulative property is apparently confined to asparagine, and the dibasic amino acids, an attempt was made to substitute for

aspartic acid compounds related to it. The ammonium salts of malic, succinic and tartaric acids were used in amounts equivalent to the necessary aspartic acid (fig. 4). Ammonium malate, although not equal to aspartic acid, was the best substitute for it, as would be expected from its structure. Ammonium succinate was found to be fairly good, while ammonium tartrate had no effect beyond that attributable to the ammonium radical itself. This result is not unexpected because tartaric acid is utilized by very few microorganisms, while succinic acid is fairly available. Sodium malate and succinate had no effect on the fermentation probably because of the lack of available nitrogen in the medium.

DISCUSSION

The widespread occurrence of the stimulating substance as previously described is explained by the general distribution of asparagine in natural materials.

The poor fermentation of unsupplemented corn by the butyric acid bacteria as compared with the vigorous fermentation by *Clostridium acetobutylicum* is probably related to the former organism's lack of proteolytic power. *Cl. acetobutylicum* brings about so extensive a proteolysis of corn that glutamic acid can be isolated (Peterson, *et al.* (1924)). In contrast, the butyric acid bacteria bring about no increase in soluble nitrogen in corn either with or without the addition of aspartic acid. The aspartic and glutamic acids required for a vigorous fermentation may be furnished by adding either the pure compounds or a filtrate from a *Cl. acetobutylicum* corn fermentation, or by digesting the corn mash with an enzyme preparation (trypsin) before fermenting.[2]

The stimulative effect of dibasic amino acids and asparagine on the butyric acid bacteria is not surprising since stimulating of bacterial growth has often been associated with these substances. Asparagine has been widely used in growing the tubercle organism in synthetic media. The presence of asparagine may explain the results of Uyei (1930) who reported a growth stimulant for this organism in potato. He showed that the unknown substance

[2] The data on which these statements are based are omitted because they seem somewhat extraneous to the main purpose of this paper.

was insoluble in organic solvents, was not contained in a protein preparation from potato nor in the known carbohydrate constituents.

The rôle of asparagine and similar compounds in the butyric acid fermentation is not certain. In view of the relatively large amounts necessary (60 mgm. per 100 cc. of medium), it is possible that they are essential constituents of the cell protoplasm. The stimulatory effect therefore may be concerned with cell proliferation rather than with metabolism.

The ability of the butyric acid organisms to utilize ammonium malate fairly well and ammonium succinate to a lesser extent in place of asparagine or aspartic acid shows that the structural configuration of the carbon residue and its availability has an effect over and above the nitrogen content alone. This result is similar to that reported for the tubercle bacillus by Henley and Le Duc (1930). They found that ammonium malate was an efficient substitute for asparagine, and that ammonium succinate and tartrate were about one-half as efficient. This substitution might be compared with the substitutions of closely related derivatives for certain essential amino acids in animal nutrition, for example, the substitution of imidazole lactic acid for histidine (Cox and Rose (1926)), and the substitution of 3-indol-pyruvic acid for tryptophane (Berg, Rose and Marvel (1929)).

SUMMARY

The substance in potato extract which stimulates the fermentation of starch and the production of butyl alcohol by certain butyric acid bacteria has been isolated and identified as *l*-asparagine.

l-Aspartic acid and *d*-glutamic acid when supplemented with molal equivalents of ammonium sulfate brought about a stimulative effect equivalent to that of asparagine.

The optimum concentration of these substances was 60 mgm. per 100 cc. of 4 per cent corn-mash, although the effect was noticeable in a concentration of 20 mgm. per 100 cc.

Mono-amino acids and ammonium salts were ineffective in bringing about the stimulation.

Ammonium malate was a good and ammonium succinate a fairly

good substitute for aspartic acid but ammonium tartrate had no effect.

Certain strains of butyric acid bacteria apparently require a four or five carbon dicarboxylic amino acid or a substance which may be transformed into one, in order to ferment starch efficiently and vigorously.

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