XVIII. COLOUR CHANGES PRODUCED BY TWO GROUPS OF BACTERIA UPON CASEINOGEN AND CERTAIN AMINO-ACIDS.

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The reactions which are described in this paper were undertaken in the course of work which was carried out in an endeavour to discover the causes of discolouration in Stilton cheese. This discolouration which varies from pink to black through many shades of yellow and brown is very widespread, and is a source of serious loss to some Stilton cheese manufacturers. The particular line of work adopted was based on the facts that Beijerinck [1911] had shown that some organisms were capable of producing discolouration in a medium which he had prepared and which contained tyrosine, and that Golding [1912] had, more recently, shown that certain organisms which he had isolated from discoloured cheeses were also capable of producing discolouration on Beijerinck's medium. Sixty-two discoloured Stilton cheeses were procured from a factory, and organisms were isolated from them.

In the very early days of the work the material was plated upon Beijerinck's medium. Although some of the colonies gave well-marked discolouration it was found very difficult to recover the organisms from these colonies. It was thought, therefore, that Beijerinck's medium was too severe, and that there was at least a possibility that colour producing organisms existing in cheeses were being lost. The discoloured cheeses were therefore plated upon + 10, neutral and - 5 lemco agar, Beijerinck's medium and a cheese medium obtained by mixing 20 % cheese and 2 % agar, made neutral and - 5 to Eyre's standard. In this way it was hoped that the organisms of more vigorous growth which could act upon tyrosine would be found upon the Beijerinck plates but the others which were either of less vigorous growth or

required other fractions than tyrosine would be found either upon the agar or cheese media. The reaction was varied because it had appeared that this affected the capacity for colour production. All those colonies which showed colour change on any of these media, + 10, neutral and -- 5 lemco agar, -- 1 and -- 3 Beijerinck's medium, or neutral and -- 5 cheese agar, were reisolated and when pure strains had been obtained were once more tested upon the media mentioned before further work was undertaken.

Bacteriological examination of the pure strains which showed discolouration was then made, with the result that organisms belonging to many different groups were isolated, including *B. coli*, *B. proteus*, *Streptotricheae*, Moulds etc. The present paper deals with strains belonging to only two of these numerous groups, namely *B. proteus* and a group of bacilli (called Gram-negative alkaline), which gave a negative reaction with Gram's stain and produced an alkaline reaction on all the fourteen sugars, alcohols and glucosides into which they were inoculated.

GROUP 1. *B. proteus vulgaris*.

Thirty-six members of this group were isolated from seven cheeses. Twenty-four strains from these seven were examined bacteriologically and were all found to correspond one with another. From among these, ten from seven cheeses were arbitrarily selected, and were used for further inoculations. Seven strains belonging to this group were isolated from two out of thirty-six samples of milk. These all gave identical reactions. One strain from each sample was taken for further experiment. All the members of this group gave the following reactions. Upon agar plates a rapidly spreading growth occurred. When examined under the microscope their length was seen to vary from 1 to 3μ and a tendency to chain formation was observed. Sometimes very long forms up to 14μ without any apparent division into cells were present. They were Gram-negative and motile, liquefied gelatin, produced indole from peptone water, gave a negative Voges-Proskauer reaction, and produced acid and gas in glucose, saccharose and galactose. The production of acid and gas in salicin, glycerol and dextrin was irregular, while there was no gas production in lactose, mannitol, sorbitol, dulcitol, adonitol, inositol, inulin or raffinose. In litmus milk a soft bleached clot and whey, a bad smell and an alkaline reaction were produced. It was felt that the consistency of these reactions justified the assumption that all these strains were members of one group of organisms and that this group was *B. proteus vulgaris*. Since these organisms produced a reddish brown

colour upon neutral lemco agar and upon the cheese medium which had been made, it was felt that a study ought to be made of their reactions upon caseinogen and its decomposition products. The inoculations were made upon the following media. Caseinogen water, 0.2 % suspension of Griffin's "extra pure casein" in distilled water, the solid being distributed as evenly as possible by continuous shaking during the process of filling out. Tyrosine, tryptophan, alanine, cystine and histidine hydrochloride, 0.1 % solutions in distilled water. Histidine water 0.1 %, the hydrochloric acid being neutralised by the calculated quantity of sodium carbonate, and neutral alanine water 0.1 % in which the carboxyl group was neutralised by the calculated quantity of sodium carbonate. The agars were in all cases made like Beijerinck's medium but contained tyrosine, tryptophan, alanine, cystine and histidine hydrochloride, and in every case were made — 1 and — 3 to Eyre's standard.

The inoculations were all made by transference of a loopful of the culture from neutral lemco agar media. It was thought possible that if colour changes occurred they might be due to the action of the organisms upon traces of agar transferred with the culture. Control experiments in distilled water and saline did not lead to the belief that any serious source of error was introduced in this way. The further possibility that the transference of particles of the agar medium along with the cultures to the amino-acids may have stimulated their growth [Bainbridge, 1911], and thus enabled the organisms to produce the colour changes which were seen, has not been guarded against. All tubes after inoculation were incubated at 30° for one week, then kept in the dark at laboratory temperature. They were examined at the end of 48 hours, 7 days, 14 days, 1, 2 and 3 months and the colour changes produced were recorded. No attempt was made to demonstrate the presence or absence of growth in the tubes.

Tyrosine has long been known to be present in old cheeses, and, with the very kind assistance of Professor F. G. Hopkins, Cambridge, the presence of tryptophan and histidine (or its base) in old Stilton cheeses had been demonstrated. Cystine and alanine are straight chain compounds and were chosen for this work because it had been suggested that it would be impossible to obtain any colouration upon aliphatic substances.

Some striking results were obtained which are shown in Table I.

The results obtained were, on the whole, very consistent for the members of this group and were most striking on tryptophan, in which an amber colour was invariably produced. Upon histidine hydrochloride water there was no visible reaction, but in the neutralised medium a light pinkish brown

colour occurred. Upon tyrosine water there was practically no change, but upon tyrosine agar a discolouration was seen varying from very slight to claret. Upon the other media and the controls no discolouration was produced.

TABLE 1.

No.	Strain	Histidine hydrochloride water	Histidine water	-1 Histidine agar	-3 Histidine agar	Cystine water	-1 Cystine agar	-3 Cystine agar	Tryptophan water	-1 Tryptophan agar	-3 Tryptophan agar
1	C ₂₀ ² B ₂	-	L. P. B.	v. v. sl.	v. sl.	-	-	-	Amber	L. Amber	L. Amber
2	C ₂₂ ² B	-	L. P. B.	v. sl.	v. sl.	-	-	-	"	"	"
3	C ₂₂ ⁹ B	-	-	v. L. P.	v. L. P.	-	-	-	"	Amber	Amber
4	C ₂₆ ⁴ A ⁷	-	L. P. B.	v. L. P.	v. L. P.	-	-	-	"	"	"
5	C ₂₆ ⁴ A ⁸	-	L. P. B.	v. L. P.	v. L. P.	-	-	-	"	"	"
6	C ₂₆ ⁴ B ₁	-	L. P. B.	v. L. P.	v. L. P.	-	-	-	"	"	"
7	C ₂₈ ⁵ A	-	L. P. B.	v. L. P.	v. L. P.	-	-	-	"	"	"
8	C ₂₈ ⁹ A	-	L. P. B.	-	v. sl.	-	-	-	"	"	"
9	C ₂₈ ³	-	L. P. B.	+sl.	+sl.	-	-	-	"	"	"
10	C ₅₈ ¹ A	-	v. L. P. B.	v. v. sl.	v. v. sl.	-	-	-	"	"	"
11	M ₂₀ ¹ C	-	L. P. B.	v. L. P.	v. L. P.	-	-	-	"	"	"
12	M ₂₆ ¹ C	-	L. P. B.	v. L. P.	v. L. P.	-	-	-	"	"	"

No.	Strain	Alanine water	Neutral alanine water	-1 Alanine agar	-3 Alanine agar	Caseinogen water	Tyrosine water	-1 Tyrosine agar	-3 Tyrosine agar	Sterile water	Sterile saline
1	C ₂₀ ² B ₂	-	-	-	-	-	v. sl.	+sl.	+sl.	-	-
2	C ₂₂ ² B	-	-	-	-	-	v. v. sl.	Claret	Claret	-	-
3	C ₂₂ ⁹ B	v. v. sl.	-	-	-	-	-	+sl.	+sl.	-	-
4	C ₂₆ ⁴ A ⁷	? +	-	-	-	-	-	+sl.	v. sl.	-	-
5	C ₂₆ ⁴ A ⁸	? +	-	-	-	-	-	+sl.	v. sl.	-	-
6	C ₂₆ ⁴ B ₁	-	-	-	-	-	-	+sl.	+sl.	-	-
7	C ₂₈ ⁵ A	-	-	-	-	-	-	+sl.	+sl.	-	-
8	C ₂₈ ⁹ A	-	-	-	-	-	-	+sl.	+sl.	-	-
9	C ₂₈ ³	-	-	-	-	-	v. sl.	+sl.	+sl.	-	-
10	C ₅₈ ¹ A	-	-	-	-	-	+sl.	+sl.	+sl.	-	-
11	M ₂₀ ¹ C	-	-	-	-	-	? +	+sl.	+sl.	-	-
12	M ₂₆ ¹ C	-	-	-	-	-	-	sl. Claret	sl. Claret	-	-

L. P. B. = Light Pinkish Brown. v. L. P. = Very Light Pink. sl. = slight.

GROUP 2. *Gram-negative, alkaline Bacilli.*

Eleven strains of this organism were isolated from four of the discoloured cheeses which had been obtained from the factory. Seven from four cheeses were studied in detail; twenty-six strains were isolated from twenty-one samples of milk which were being used in the factory. Seventeen from ten samples were examined in detail, and five from five samples were selected arbitrarily for further studies of colour changes.

These organisms all showed the following characteristics; a greenish yellow growth on neutralised potato and complete absence of growth on

lemco agar under anaerobic conditions. In litmus milk after fourteen days' incubation at 30° a flesh-coloured clot, whey, a brown ring at the surface and an alkaline reaction were found. When examined under the microscope they appeared as somewhat exaggerated *B. coli*; they were Gram-negative, motile, gave the indole reaction with peptone water, and a negative Voges-Proskauer reaction; they liquefied lemco gelatin with the production of a brownish colour when the medium was neutralised. Upon glucose, lactose, saccharose, galactose, raffinose, dulcitol, inositol, sorbitol, mannitol, adonitol, inulin, salicin, dextrin and glycerol they gave a consistently alkaline reaction. Fifty-four samples of water taken from the farms which were supplying milk to the factory were examined. Of these, eighteen showed organisms belonging to this group. Seventeen strains from seventeen samples have been further examined. Six of these strains, W₂^E, W₁₇^{1A}, W₁₉^{1B}, W₂₆², W₃₂^{3B,A} and W₅₀^{1A}, selected arbitrarily, showed all those reactions which have been already described for the cheese and milk strains. The remainder, W₁₄^{1A}, W₂₁¹, W₂₅^{3A}, W₂₇², W₂₈¹, W₃₀^{2B}, W₃₁^{1A}, W₃₆^{1C}, W₄₂^{3B}, W₄₅^{1A}, and W₅₂^{3A} were not tested upon galactose, raffinose, inositol, sorbitol, mannitol, adonitol, inulin, salicin, dextrin or glycerol, because Kahlbaum's preparations were being used and it was necessary to conserve them. In all other respects their reactions were identical with those which have been described for the other strains.

The twenty-nine strains which were selected comprised seven from four cheeses, five from five samples of milk and seventeen from seventeen samples of water. The colour changes resulting from the action of these organisms upon various media which have been already described under Group 1 are shown in Table II.

The results show that, when inoculated into histidine hydrochloride water, the reactions were either definitely negative or very doubtfully positive, and on only one occasion was a very light brown colour produced. When the hydrochloric acid of the histidine hydrochloride was neutralised, the colour was slightly more marked, though even then the maximum colour recorded is slight brown. Upon the histidine agar media the results were, for all practical purposes, negative. Negative results were also obtained with all the cystine media.

Upon the tryptophan water and agar media the results were very consistent, being much less marked upon the agar than upon the tryptophan water, upon which they showed a colour varying from slight yellow to a definite canary. Upon alanine again, the results on the agar media were

TABLE II.

No.	Strain	Histidine hydro- chloride water	Histidine water	-1 Histi- dine agar	-3 Histi- dine agar	Cys- tine water	-1 Cystine agar	-3 Cystine agar	Trypto- phan water	-1 Trypto- phan agar	-3 Trypto- phan agar	Alanine water
1	C ₂₃ 4B	-	v. sl. Y.	-	-	-	-	-	sl. Y.	+ v. sl.	+ v. sl.	sl. Y.
2	C ₂₃ ⁹	-	v. sl. Y.	-	-	-	-	-	sl. Y.	+ v. sl.	+ v. sl.	v. sl. Y.
3	C ₂₆ 5C ₁	? +	sl. Y. B.	-	-	-	-	-	sl. Y.	+ v. sl.	+ v. sl.	sl. Y.
4	C ₂₆ 5C ₂	? +	+ sl.	-	-	-	-	-	sl. Y.	+ v. sl.	+ v. sl.	v. sl. Y.
5	C ₂₇ 1A	+ sl.	+ sl.	-	-	-	-	-	Canary	+ sl.	+ sl.	v. sl. Y.
6	C ₂₇ 5C ₄	? +	sl. B.	-	-	-	-	-	"	+ sl.	+ sl.	v. sl. Y.
7	C ₂₇ 9A	-	? +	-	-	-	-	-	"	+ v. sl.	+ v. sl.	v. sl. Y.
8	M ₃ ² A	? +	+ sl.	-	-	-	-	-	"	+ sl.	+ sl.	sl. Y.
9	M ₆ ¹	-	+ sl.	-	-	-	-	-	"	+ sl.	+ sl.	v. sl. Y.
10	M ₂₆ ¹ A	-	+ v. sl.	-	-	-	-	-	v. sl. Y.	+ v. sl.	+ v. sl.	sl. Y.
11	M ₂₆ ³ A	? +	+ sl.	-	-	-	-	-	Canary	+ v. sl.	+ v. sl.	sl. Y.
12	M ₂₆ ³ A	+ v. sl.	+ v. sl.	-	-	-	-	-	"	+ sl.	+ sl.	sl. Y.
13	W ₃ E	-	sl. B.	-	-	-	? +	? +	"	+ v. sl.	+ v. sl.	sl. Y.
14	W ₁₄ ¹ A ₁	-	+ sl.	-	-	-	-	-	L. Canary	+ v. sl.	+ sl.	sl. Y.
15	W ₁₄ ¹ A	-	? +	-	-	-	-	-	Canary	+ sl.	+ sl.	sl. Y.
16	W ₁₄ ¹ B	? +	? +	-	-	-	-	-	L. Canary	+ sl.	+ sl.	sl. Y.
17	W ₂₁ ¹	-	+ sl.	-	-	-	-	-	"	+ v. sl.	+ v. sl.	+ v. sl.
18	W ₂₆ ³ A	v. L. B.	+ v. sl.	-	? +	-	-	-	"	+ sl.	+ sl.	? +
19	W ₂₆ ²	? +	? +	-	-	-	? +	? +	Canary	+ sl.	+ sl.	sl. Y.
20	W ₂₇ ²	-	+ sl.	-	-	-	-	-	"	+ sl.	+ sl.	sl. Y.
21	W ₂₉ ¹	-	L. B.	-	-	-	-	-	L. Canary	+ v. sl.	+ v. sl.	sl. Y.
22	W ₃₀ ² B	? +	v. L. B.	-	-	-	-	-	"	+ v. sl.	+ v. sl.	? +
23	W ₃₁ ¹ A	-	? +	-	-	-	-	-	"	+ sl.	+ sl.	? +
24	W ₃₁ ³ B ₁ A	+ v. v. sl.	+ v. sl.	-	-	-	-	-	"	+ sl.	+ sl.	sl. Y.
25	W ₃₆ ¹ C	-	sl. B.	-	-	-	-	-	"	+ sl.	+ sl.	? +
26	W ₄₂ ³ B	-	v. L. B.	-	-	-	-	-	"	+ sl.	+ sl.	sl. Y.
27	W ₄₅ ¹ A ₂	-	+ sl.	-	-	-	-	-	"	+ sl.	+ sl.	+ v. sl.
28	W ₅₀ ¹ A ₁	? +	+ sl.	-	-	-	-	-	Canary	+ sl.	+ sl.	+ v. sl.
29	W ₅₂ ³ A	-	+ sl.	-	-	-	-	-	"	+ sl.	+ sl.	+ v. sl.

No.	Strain	Neutral alanine water	-1 Alanine agar	-3 Alanine agar	Caseinogen water	Tyrosine water	-1 Tyrosine agar	-3 Tyrosine agar	Sterile water	Sterile saline
1	C ₂₃ 4B	sl. Y.	+ v. v. sl.	+ v. v. sl.	Brown	Bl.	Bl.	Bl.	-	-
2	C ₂₃ ⁹	sl. Y.	? +	"	"	B.—Bl.	Bl.	Bl.	-	-
3	C ₂₆ 5C ₁	sl. Y.	+ v. v. sl.	"	"	B.—Bl.	Bl.	Bl.	? +	? +
4	C ₂₆ 5C ₂	sl. Y.	"	"	"	B.—Bl.	Bl.	Bl.	-	-
5	C ₂₇ 1A	sl. Y.	"	"	"	B.—Bl.	Bl.	Bl.	? +	-
6	C ₂₇ 5C ₄	+ sl.	"	"	"	B.—Bl.	Bl.	Bl.	-	-
7	C ₂₇ 9A	sl. Y.	"	"	"	B.—Bl.	Bl.	Bl.	-	-
8	M ₃ ² A	sl. Y.	"	"	"	B.—Bl.	Bl.	Bl.	-	-
9	M ₆ ¹	v. sl. Y.	"	"	"	D. B.	Bl.	Bl.	-	-
10	M ₂₆ ¹ A	+ v. sl.	"	"	L. Brown	B.—Bl.	Bl.	Bl.	-	-
11	M ₂₆ ³ A	sl. Y.	"	"	Brown	B.—Bl.	Bl.	Bl.	-	-
12	M ₂₆ ³ A	sl. Y.	"	"	"	B.—Bl.	Bl.	Bl.	-	-
13	W ₃ E	sl. Y.	"	"	v. L. Brown	B.	Bl.	Bl.	-	-
14	W ₁₄ ¹ A ₁	sl. Y.	"	"	Brown	B.—Bl.	B.—Bl.	B.—Bl.	-	-
15	W ₁₄ ¹ A	v. sl. Y.	"	"	L. Brown	D. B.	Bl.	Bl.	-	-
16	W ₁₄ ¹ B	v. sl. Y.	"	"	"	B.—Bl.	Bl.	Bl.	-	-
17	W ₂₁ ¹	v. sl. Y.	"	"	"	B.—Bl.	B.	Bl.	-	-
18	W ₂₆ ³ A	sl. Y.	"	"	Brown	B.—Bl.	B.—Bl.	Bl.	-	-
19	W ₂₆ ²	sl. Y.	"	"	L. Brown	B.—Bl.	Bl.	Bl.	-	-
20	W ₂₇ ²	sl. Y.	"	"	Brown	D. B.	B.	B.—Bl.	-	-
21	W ₂₉ ¹	sl. Y.	"	"	"	B.—Bl.	B.—Bl.	Bl.	-	-
22	W ₃₀ ² B	sl. Y.	"	"	"	B.—Bl.	B.	B.—Bl.	-	-
23	W ₃₁ ¹ A	sl. Y.	"	"	"	B.—Bl.	L. B.	Bl.	-	-
24	W ₃₁ ³ B ₁ A	+ v. sl.	"	"	"	B.—Bl.	B.—Bl.	Bl.	? +	-
25	W ₃₆ ¹ C	? +	"	"	"	B.—Bl.	B.—Bl.	B.—Bl.	-	-
26	W ₄₂ ³ B	sl. Y.	"	"	"	B.—Bl.	B.—Bl.	Bl.	-	-
27	W ₄₅ ¹ A ₂	+ v. sl.	"	"	L. Brown	B.—Bl.	Bl.	Bl.	-	-
28	W ₅₀ ¹ A ₁	+ v. sl.	"	"	Brown	B.—Bl.	Bl.	B.—Bl.	? +	-
29	W ₅₂ ³ A	v. sl. Y.	"	"	"	B.—Bl.	Bl.	B.	-	-

L. = Light. D. = Dark. sl. = Slight. Y. = Yellow. B. = Brown. Bl. = Black.

less marked than upon the water. The results upon alanine water and upon neutralised alanine water did not appear to differ greatly, the maximum effect obtained being a slight yellowish colour. Upon caseinogen water, these organisms again produced a definite brownish colour, which differentiated them completely from the *proteus* group, the members of which failed entirely to produce any change upon this medium. Upon the tyrosine media the colours varied from brown to black. The control tubes made by the inoculation of sterile saline and sterile water were, as a rule, quite negative, but occasionally showed a very doubtful colour change.

The maximum and minimum reactions for each group are shown in Table III.

TABLE III.

No.	Strain	Histidine hydrochloride water	Histidine water	-1 Histidine agar	-3 Histidine agar	Cystine water	-1 Cystine agar	-3 Cystine agar	Tryptophan water	-1 Tryptophan agar	-3 Tryptophan agar
1	<i>B. proteus vulgaris</i>	- to v. sl.	v. L. Y. to P. B.	- to L. P.	v. v. sl. to L. P.	- to +sl.	- to ?+	- to ?+	L. Amber to D. Amber	L. Amber to D. Amber	L. Amber to D. Amber
2	Gram-neg. alkaline	- to ?+	?+ to L. B.	- to v. sl.	- to +sl.	- to v. sl.	- to v. sl.	- to v. sl.	v. sl. Y. to Canary	v. sl. to +sl.	v. v. sl. to +sl.

No.	Strain	Alanine water	Neutral alanine water	-1 Alanine agar	-3 Alanine agar	Caseinogen water	Tyrosine water	-1 Tyrosine agar	-3 Tyrosine agar	Sterile water	Sterile saline
1	<i>B. proteus vulgaris</i>	- to v. v. s.	-	-	-	-	- to L. B.	+sl. to Claret	v. sl. to Claret	-	-
2	Gram-neg. alkaline	v. sl. to sl. Y.	- to sl. Y.	?+ to v. v. sl.	v. v. sl.	v. sl. to D. B.	B. to Bl.	L. B. to Bl.	B. to Bl.	- to ?+	- to ?+

B. = Brown. Bl. = Black. D. = Dark. L. = Light. P. = Pink. Y. = Yellow. sl. = Slight.

The most characteristic differences between the members of these groups under the particular conditions of experiment, are the production of a well-marked amber colour upon tryptophan and absence of reaction in caseinogen water by members of the *proteus* group, with the production of a colour which was never more than canary yellow upon tryptophan and of a definite brown colour in caseinogen water by members of the Gram-negative, alkaline group.

SUMMARY.

A very large number of organisms belonging to many different groups have been isolated from discoloured Stilton cheeses, milk from which such cheeses were made and the water supplies of the farms from which the milk was obtained.

The bacteriological reactions of two of these groups have been studied.

Certain members of these two groups have been selected arbitrarily and their reactions upon caseinogen and various amino-acids resulting from its degradation have been investigated.

The results show that members of both groups are capable, under the conditions of experiment, of producing discolouration upon certain of the substances selected and that the two groups show very distinct differences in their capacity for producing colour upon the various media upon which they were tested.

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