The Assimilation of Amino-acids by Bacteria

4. The Action of Triphenylmethane Dyes on Glutamic Acid Assimilation

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SUMMARY: The level of glutamic acid concentration measured inside the streptococcal cell during assimilation represents a balance between the rate at which the amino-acid is withdrawn from the external medium and the rate at which it is metabolized within the cell.

Treatment of the cells, prior to or during assimilation, with dyes of the triphenylmethane series results in raising the level of free glutamic acid attained within the cell. Evidence is presented that the triphenylmethane dyes prevent the metabolism of glutamic acid within the cell. The activity of the triphenylmethane molecule as an antibacterial agent and in raising the level of glutamic acid assimilation is increased by alkyl substitution and can be correlated with the lipid solubility of the dye.

Dyes of the triphenylmethane series have long been known as effective antibacterial agents. Kligler (1918) showed that the quinonoid structure of the dye molecule is important for antimicrobial activity and that the effectiveness of the dyes is increased by substitution of alkyl radicles in the amino-groups of triamino- and diamino-triphenylmethanes. He showed that bacteria differ widely in their sensitivity to these dyestuffs and that, in general, Gram-positive organisms are more sensitive than Gram-negative species. The dyes fell into the following order of increasing effectiveness: fuchsin (1CH_3), methyl violet (5CH_3), crystal violet (6CH_3), malachite green (4CH_3), brilliant green ($4\text{C}_2\text{H}_5$). A series of studies by Stearn (1927) and Stearn & Stearn (1924, 1925, 1926, 1930) led these workers to put forward a theory of dye bacteriostasis which can be summarized as follows: Basic dyes combine reversibly with acidic groups of bacterial protein, and the action of a dye can be described in terms of a mass-action equilibrium between the dye molecule and the organism considered as an ampholyte. They showed that Gram-positive organisms have an isoelectric range lying between pH 2 and 3, while the isoelectric range of Gram-negative organisms averages pH 5; also that the degree of Gram-positiveness of bacterial cells parallels their sensitivity to triphenylmethane dyes and that the loss of Gram-positiveness (by mutation) is accompanied by a shift in the isoelectric range of the organism towards the range of Gram-negative organisms. McCalla (1941, 1942) and McCalla & Clark (1941) showed that dyes are taken up by bacteria by ionic interchange, basic dyes displacing cations and acidic dyes, anions; thus crystal violet displaces magnesium or hydrogen ions. Although, as suggested by Stearn & Stearn (1924), the basicity of the dye is one of the main factors determining the bacteriostatic effectiveness, these workers and Kligler (1918) realized that this is not the only factor, as methyl green (hepta-

methyltriaminotriphenylmethane) with a strongly basic quaternary-N group, is without antibacterial activity. Studies on other types of cell have suggested that the plasma membrane is a protein-lipid complex (Harvey & Danielli, 1938; Schulman, 1937), and that a further factor involved in the increasing activity of the dyes with alkyl substitution is the increasing lipid-solubility with such substitution in the molecule. Marshall, Lockwood & Dubos (1941) pointed out that Gram-negative bacteria have an outer surface of lipopolysaccharide antigens.

In the previous papers of this series it has been shown that Gram-positive bacteria differ from Gram-negative in that the former are able to assimilate certain amino-acids from the medium and concentrate them in the free state in the internal environment (Gale, 1947*a*; Taylor, 1947). Of the two amino-acids studied in detail, lysine passes across the cell wall by simple diffusion under an electrostatic gradient, while glutamic acid cannot pass across the membrane unless energy is supplied by some exergonic reaction. In either case an equilibrium is set up between the external and internal concentrations of the amino-acid such that the internal concentration is markedly greater than that holding in the external environment (Gale, 1947*a*). It has been suggested that this assimilation of free amino-acids is an essential stage in the anabolism of the Gram-positive organisms which are nutritionally exacting towards certain amino-acids (Gale, 1947*a*). In the present communication it is shown that the free glutamic acid measured in the internal environment of Gram-positive cells represents a balance between the rate of assimilation of glutamic acid from the external environment and the rate at which it is metabolized within the cell. Triphenylmethane dyes penetrate the cell wall and inhibit the internal metabolism of glutamic acid.

Methods

The organisms, growth media, preparation of suspensions and methods of assay of free amino-acids in the internal and external environments were the same as those described by Gale (1947*a*). In general, *Streptococcus faecalis* was grown in a medium containing little free glutamic acid; the cells were washed and their internal glutamic acid content assayed; the cells were then suspended in a salt solution containing a known amount of glutamic acid and glucose and incubated at 37° for 1 hr. (final suspension strength = 2–5 mg. dry weight of organism/ml.); the cells were then centrifuged, washed once and their internal glutamic acid content assayed.

Since glutamic acid was estimated manometrically in terms of CO₂ produced by the action of the specific decarboxylase (Gale, 1947*a*), it has been convenient throughout to express quantities of glutamic acid in terms of μ l.; 22.4 μ l. glutamic acid = 1 μ mol.

Effect of crystal violet on glutamic acid assimilation

Preliminary work. The action of various antibacterial substances has been tested on the assimilation of lysine and glutamic acid by washed suspensions of *Strep. faecalis* ST. In the course of these tests it was found that gentian violet

or crystal violet in a dilution of 1 : 1000 inhibited the uptake of glutamic acid by deficient cells, but the same dyes in 1 : 10,000 solution gave rise to an increased assimilation. Table 1 shows μ l. glutamic acid assimilated by 100 mg. deficient cells when incubated for 1 hr. at 37° in the presence of salts, 0.5 % (w/v) glucose and 200 μ l. glutamic acid/ml. The amount of glutamic acid assimilated was determined by measuring the increase in the internal glutamic acid content of the cells under these conditions. When glucose is omitted from the external environment, no significant assimilation occurs whether crystal violet is present or not; the addition of crystal violet (1:10,000) in the presence of glucose resulted in an increase of 148 μ l. of glutamic acid assimilated by 100 mg. of cells. Crystal violet forms micelles in solution, and it was thought possible that the extra glutamic acid might be carried into the cells in solution

Table 1. *Effect of crystal violet on glutamic acid assimilation*

Strep. faecalis grown in deficient medium B, cells washed and their internal glutamic acid content assayed, cells then incubated for 1 hr. at 37° as below and the increase in internal glutamic acid determined.

External environment during incubation	μ l. glutamic acid increase in internal environment/100 mg. cells
(a) Salt solution pH 7.4; 0.5 % (w/v) glucose; glutamic acid 200 μ l./ml.	113
(b) As (a) without glucose	9
(c) As (a) + crystal violet 1:10,000	261
(d) As (c) without glucose	3
Cells treated with crystal violet (1:10,000) for 10 min., washed and then tested:	
(e) As (a)	239
(f) As (b)	5

in the dye micelles. To test this the cells were exposed to 1 : 10,000 crystal violet for 10 min. at 37° and then washed before being incubated in the presence of glutamic acid and glucose. Table 1 shows that such pretreated cells showed the same increase as those in which the dye was present during the assimilation process. The action of the dye is therefore to affect the cells in such a way that within the stained cells a greater accumulation of free glutamic acid takes place than in the untreated cells.

Effect of dye concentration. Table 2 shows the effect of various concentrations of crystal violet on the assimilation of glutamic acid and also on the fermentation of glucose by the washed streptococcal cells. Crystal violet at 1:1,000,000 had no effect on either assimilation or fermentation; higher concentrations resulted in an increased accumulation of glutamic acid in the internal environment of the cells. The optimal effect on assimilation was given with 1:10,000 crystal violet whereas 1:1000 dye inhibited assimilation. These concentrations of dye had an inhibitory action on fermentation when this was determined manometrically by measuring the formation of lactic acid in bicarbonate/CO₂ buffer. Dye (1:10,000) inhibited acid production by approx. 45 %; 1:1000 dye

produced 96 % inhibition and this concentration of crystal violet was bactericidal.

The concentration of dye which is bacteriostatic for this organism was determined by taking tubes of casein digest glucose media, adding serial dilutions of crystal violet, inoculating with a standard inoculum of *c.* 10^6 cells/ml. and incubating at 37° for 24 hr. A dye concentration of 1:500,000 prevented growth while 1:700,000 allowed normal growth. No significant

Table 2. *Effect of crystal violet on glutamic acid assimilation and glucose fermentation*

Strep. faecalis grown in deficient medium B; cells washed and their internal glutamic acid content assayed; cells then incubated for 1 hr. at 37° in glutamic acid (200 μ l./ml.), glucose (0.5 % w/v) and crystal violet as below; the increase in internal glutamic acid determined and hence the effect of crystal violet on its assimilation. Fermentation rates determined in Warburg manometers containing 1.0 ml. suspension of cells (dry wt. 3 mg./ml.), 1.5 ml. M/80 NaHCO_3 and 0.5 ml. 1 % (w/v) glucose, and filled with 5 % CO_2 + 95 % N_2 gas mixture.

Concentration of crystal violet	Effect on glutamic acid assimilation (as % control without dye)	Inhibition of glucose fermentation (%)
0	100	0
1:1,000,000	100	0
1:100,000	168	4
1:50,000	509	20
1:10,000	581	46
1:2,000	234	—
1:1,000	30	96

alteration of this bacteriostatic concentration could be obtained by varying the amino-acid composition or concentration of the test medium. Stearn (1933) showed that the bacteriostatic concentration of dye was proportional to the bacterial mass present. The dry weight of cells/ml. in the assimilation and fermentation experiments quoted in Table 2 was 1–2 mg., which is approximately 100 times the bacterial mass present in the growth tests. Consequently the dye concentration (1:10,000) giving optimal glutamic acid assimilation bears the same relation to the bacterial mass in the assimilation experiments as the concentration (1:10⁶) found to be bacteriostatic in the growth inhibition tests. The amount of dye removed from solution by the cells can be estimated photoelectrically, and in a series of tests it was found that 33 μ g. crystal violet/mg. dry weight of cells were removed from 1:10⁵ dye solution when the cell-suspension strength was 2.5 mg./ml., while 28 μ g./mg. were taken up when both dye and bacterial suspension were diluted ten times; this confirms the finding of Stearn (1933) that the amount of dye taken up was proportional to the bacterial mass and was approximately constant when the ratio of dye/bacterial mass was constant.

Course of glutamic acid assimilation in presence of crystal violet. Fig. 1 shows the rate of appearance of glutamic acid in the internal environment of 20 mg. dry weight of deficient cells when these were suspended in salt solution containing glucose (0.5 % w/v) and glutamic acid (200 μ l./ml.) at pH 7.2 and 37° . Glutamic acid was taken up by the cells until the new equilibrium level was

reached after 15 min. When crystal violet ($1:10^4$) was present during the assimilation (or when the cells had been stained in such a solution before assimilation began) the rate of assimilation was slower, but the final level reached was higher than that attained in the absence of the dye. Since the assimilation of glutamic acid, in this case, is dependent upon energy obtained from glucose fermentation, the slower rate of assimilation was probably due to the inhibition of fermentation rate produced by the dye (Table 2). The difference between the final levels reached in the presence and absence of dye was variable and depended upon the age of culture of the deficient cells.

Fig. 1. Effect of crystal violet ($1:10,000$) on assimilation of glutamic acid into internal environment of *S. faecalis* cells. Cells incubated at 37° in (1) salt solution + 0.5% glucose + glutamic acid (200 μ l./ml.) ●—●, (2) as (1) + crystal violet $1:10,000$ ○—○.

Effect of the age of the culture. During the early stages of this work it was found that the 'dye effect' varied greatly from time to time, the same concentration of crystal violet increasing the glutamic acid assimilation from 25 to 600 % when tested under what appeared to be standard conditions. It seemed that the effect varied with the age of the cells tested, so this point was investigated in detail. Table 3 shows the μ l. glutamic acid assimilated/100 mg. cells in the presence and absence of crystal violet ($1:10^4$) when the cells had been harvested from deficient medium at 4–17 hr. after inoculation at 37° . From the final column of Table 3 it can be seen that the assimilation in the presence of the dye was approximately constant at 450–550 μ l./100 mg. from the earliest culture tested up to the tenth hour of incubation when growth has ceased; after this time the assimilation dropped rapidly. The assimilation in the absence of dye was much less from 4–10 hr. of incubation, and then that value also fell, but the two values approximated until at 17 hr. the dye had little or no enhancing effect. Cells taken at the third hour of growth also showed no dye effect, but in this case the assimilation value in the absence of dye was abnormally high and the same as that obtained in the presence of dye for all cultures up to the end of the growth period. The probable significance of

these values will be discussed below. From a practical point of view it follows from Table 3 that large 'dye effects' on glutamic acid assimilation can be obtained only with cells harvested after 6–10 hr. incubation, and that older cultures will display smaller or even negligible effects of this nature.

The estimations involved in this experiment are set out in detail in Table 3 in order that another property of the 'dye effect' can be shown. The internal environment of the cells was investigated by adding glutamic acid decarboxy-

Table 3. *Variation of effect of crystal violet on glutamic acid assimilation with age of culture*

Cells grown in deficient medium, washed and incubated for 1 hr. at 37° in the presence of (a) 0.5% (w/v) glucose + 200 μ l. glutamic acid/ml., (b) as (a) + crystal violet 1:10,000. In each case glutamic acid assayed on intact cells (external) and on cells disrupted by boiling (total); internal glutamic acid = total – external.

Age of culture (hr.)	Growth (mg./ml.)	Sample dry weight (mg.)	Glutamic acid determinations						Internal glutamic acid (μ l./100 mg. cells)	
			(a) No dye			(b) Crystal violet			(b)	
			Ext. (μ l.)	Total (μ l.)	Int. (μ l.)	Ext. (μ l.)	Total (μ l.)	Int. (μ l.)	(a) Alone	Dye present
3	0.050	6.8	31	68	37	—	—	—	545	—
4	0.137	21.6	2	58	56	67	166	99	260	460
6	0.294	34.0	24	116	92	34	212	178	286	534
8	0.310	30.0	0	84	84	50	188	138	280	460
10	0.330	33.0	20	66	46	105	289	184	138	552
12	0.330	33.3	15	52	37	65	172	107	110	320
17	—	31.6	38	72	34	34	75	41	102	123

lase to suspensions of the cells before and after disruption (Gale, 1947*a*). The glutamic acid obtained with intact cells represents that amount of the amino-acid carried down with and adsorbed on the surface of the intact cells; the value obtained with disrupted cells represents the total free glutamic acid content of the cell preparation, and the amount concentrated in the internal environment can be obtained by difference. It was noticed in the early studies of the 'dye effect' that the presence of the dye not only increased the amount of glutamic acid assimilated inside the cell but also increased the amount of glutamic acid carried down on the outside of the washed intact cells. This can be seen in Table 3: for example, cells harvested after 10 hr. growth, washed and treated by the assimilation procedure as usual gave a value of 30 μ l. glutamic acid/33 mg. cell preparation for the external environment, but similar cells put through the same procedure in the presence of crystal violet gave a value of 105 μ l./33 mg. It can be seen from the values for this culture in Table 3 that, while the dye caused an increased assimilation of 138 μ l. in the internal environment, it also caused an increase of 85 μ l. in the amount carried down outside the cells. The external effect is not always as marked as this, and it can be seen from Table 3 that the external effect is smaller in cultures both older and younger than 10 hr. This glutamic acid estimated in intact cell suspensions must exist on the outside of those cells, as the glutamic acid

decarboxylase preparation used for its determination is a washed suspension of *Clostridium welchii* (Gale, 1945) and obviously cannot penetrate the streptococcal cell wall. Because of this and since the amount assayed with the intact cells remained after washing the cells, it follows that this glutamic acid must be adsorbed on the surface of the cells and that the action of crystal violet was to increase the amount of amino-acid so adsorbed. It was shown in Table 1 that no increased assimilation of glutamic acid took place in the presence of crystal violet when glucose was absent; this increased adsorption of glutamic acid on the outside of the cells was also dependent upon the presence of glucose in these experiments.

Table 4. *A comparison of certain triphenylmethane dyes with respect to their inhibition of the growth of Streptococcus faecalis and its assimilation of glutamic acid*

(+ = Growth from inoculum of 10⁶ cells/ml.)

Name	Substituent groups	Conc. of dye								Effect on glutamic acid assimilation (crystal violet effect = 100; dye concentration = 1/10,000)	Partition coefficient isobutanol/water
		1/10,000	1/50,000	1/100,000	1/200,000	1/300,000	1/500,000	1/700,000	1/1,000,000		
<i>p</i> -Rosaniline	—NH ₂ , —NH ₂ , —NH ₂	—	—	+	+	+	+	+	+	4	12.5
Fuchsin	—NH ₂ , —NH ₂ , —NH ₂ , —CH ₃	—	—	+	+	+	+	+	+	25	14.7
Methyl violet	—N(CH ₃) ₂ —N(CH ₃) ₂ —NHCH ₃	—	—	—	—	—	+	+	+	85	62
Crystal violet	—N(CH ₃) ₂ —N(CH ₃) ₂ —N(CH ₃) ₂	—	—	—	—	—	—	+	+	100	76
Malachite green	—N(CH ₃) ₂ —N(CH ₃) ₂	—	—	—	—	—	—	—	+	100	125
Brilliant green	—N(C ₂ H ₅) ₂ —N(C ₂ H ₅) ₂	—	—	—	—	—	—	—	+	143	480
Methyl green	—N(CH ₃) ₂ —N(CH ₃) ₂ —N(CH ₃) ₃ Cl	+	+	+	+	+	+	+	+	0	0.2
Aurine	—OH, —OH	—	—	+	+	+	+	+	+	140	625
Soluble blue 3	3[—NH— —SO ₃ H]	—	—	+	+	+	+	+	+	7	0.1

Activities of triphenylmethane series of dyes

Table 4 summarizes the activities of various triphenylmethane dyes when tested against *Strep. faecalis*.

Inhibiting concentration. The inhibiting concentration of each dye was determined by the addition of serial dilutions of dye to tubes containing casein digest glucose medium, inoculation with 10⁶ cells/ml., and incubation for 24 hr. at 37°. Table 4 shows that the antibacterial activity of the dyes increased with the

degree of alkyl substitution in the molecule. The acid dyestuffs, aurine and soluble blue, had little antibacterial activity under the conditions tested. The strongly basic methyl green with a quaternary-N group in its structure was devoid of activity.

Effect on assimilation of glutamic acid. Each of the dyestuffs was tested for an effect on glutamic acid assimilation of the nature described above for crystal violet. In each series of experiments, deficient cells were incubated for 1 hr. at 37° in the presence of 0.5% (w/v) glucose and 200 μ l. glutamic acid/ml. The assimilation of glutamic acid was determined in the absence of dye, in the presence of crystal violet (1:10,000), and in the presence of the other dyestuffs in the same concentration. All the triphenylmethane dyes, with the exception of methyl green, produced an increase in the amount of glutamic acid assimilated (as measured by the increase in the internal concentration), and Table 4 shows their relative activities when compared with crystal violet. The effects on assimilation can be directly correlated with their antibacterial activities. Alkyl substitution increased the activity of the dyes ranging from *p*-rosaniline to brilliant green, but the strongly basic methyl green was inactive. Soluble blue was almost inactive but aurine had an activity equal to that of brilliant green and so forms an exception to the general correlation occurring between assimilation effects and antibacterial activities.

Many other dyestuffs were similarly tested. The following were without effect on the assimilation process: methylene blue, toluidine blue, congo red, auramine, dispersol diazo-black, chloracol brilliant yellow, chrysaphenine, rhodamine, safranine, primulin, acridine orange, solway green, solway violet and antrypol.

Partition coefficient. If the bacterial cell membrane is of lipid nature it is possible that the difference in activity of the triphenylmethane dyes might lie in their differing lipid-solubilities. As an approximate indication of this difference the partition coefficient between *isobutanol* and water was determined.

In each case 10 mg. of dye was shaken with 10 ml. water and 10 ml. *isobutanol* saturated with water. After equilibrium was established, the alcohol and water phases were separated and the partition of the dye determined colorimetrically. The values in Table 4 show that the assimilation effects and the antibacterial activities (with the exception of aurine) can be correlated with the partition coefficient in that the greater the partition is in favour of the *isobutanol* phase, the more active is the dye in the biological systems.

The balance between assimilatory and metabolic processes

In the work so far described, the assimilation of glutamic acid has been determined by the increase in the concentration of the amino-acid in the internal environment of the cells when these are removed from an external environment deficient in glutamic acid and transferred to an environment rich in this amino-acid. It does not necessarily follow that the total amount of glutamic acid assimilated or withdrawn from the external environment is

equal to this increase in the internal concentration. If, for example, some metabolism of glutamic acid takes place inside the cell, then the amount of glutamic acid withdrawn from the external environment would be equal to that appearing in the internal environment plus the amount metabolized. In this case the amount of amino-acid measured in the internal environment would in fact be a measure of the balance between the total amount withdrawn from outside and the amount metabolized within the time studied. Since the decarboxylase method estimates the free amino-acid only, such metabolism might consist of synthesis into peptide chains of glutamyl residues or of some breakdown or conversion of the glutamic acid molecule.

Effect of crystal violet on glutamic acid metabolism

The possibility that some metabolism of glutamic acid might take place during assimilation was investigated in the following manner. Deficient cells were prepared in washed suspension as usual and suspended in salt solution containing 30 μ l. glutamic acid/ml. and 0.5 % (w/v) glucose. Before incubation the glutamic content of the cells and medium was assayed accurately by taking samples equivalent to the experimental samples. The cells were incubated for 1 hr. at 37° in the presence of the glutamic acid and then centrifuged, washed and their glutamic acid content reassayed. The supernatant medium and the washings were combined, evaporated *in vacuo* and assayed for glutamic acid. In this way it was possible to determine the increase in the internal concentration of glutamic acid of the cells and compare this with the disappearance of glutamic acid from the external environment, allowance being made for the alteration in the amount adsorbed on the outside of the cells before and after incubation. Table 5 outlines results obtained in a series of experiments of this nature.

Some idea of the experimental error involved in these assays and manipulations was first obtained as follows: Since glutamic acid is not assimilated by the cells in the absence of a source of energy (Gale, 1947*a*), an experiment was first carried out in which glucose was omitted from the reaction mixture. There was no change in the internal glutamic acid concentration under these conditions but an overall loss of 64 μ l. out of 1800 μ l. in the external environment; this may be due to slight metabolic activity by the cells, but is probably due to loss during experimental manipulation. In the presence of glucose, a series of three experiments showed a variable increase in the amount of glutamic acid appearing inside the cells, while the amount disappearing from the external environment was greater than this increase by an average of 632 μ l. In these experiments c. 30 % of the total glutamic acid added to the external environment disappeared during the assimilation period of 1 hr.

The same type of experiment was then carried out in the presence of crystal violet 1:10,000. In the absence of glucose a greater loss of glutamic acid was found than in the absence of the dye. Since the dye adsorbs strongly on to all glassware and so renders quantitative recovery of solutions difficult, it is probable that this increased loss was again due to experimental error and not

to metabolism by the cells in the presence of the dye. In the presence of glucose the amount of glutamic acid appearing in the internal environment was markedly greater than that appearing in the absence of the dye, but the difference between the amount disappearing from the external environment and that appearing in the internal environment was now 163 μ l. as compared with 632 μ l. in the absence of the dye. When allowance is made for the experimental loss as measured in the absence of glucose, the glutamic acid disappearing in the absence of the dye was 568 μ l. as compared with 23 μ l. in the presence of the dye.

Table 5. *Effect of crystal violet on glutamic acid metabolism during assimilation*

In all cases initial external glutamic acid = 1800 μ l. total; concentration of glutamic acid in external medium initially = 30 μ l./ml.; total dry weight of deficient cells = 175 mg.

Present during incubation for 1 hr.				Glutamic acid determinations		Glutamic acid metabolized or lost	
Deficient cells	Glutamic (200 μ l./ml.)	Glucose (0.5 % (w/v))	Crystal violet (1:10 ⁴)	Change in internal environment (μ l.)	Change in external environment (μ l.)	Total (μ l.)	Corrected for blank (μ l.)
+	+	—	—	0	— 64	64	0
(1) +	+	+	—	+100	—712	612	} 632 . 568
(2) +	+	+	—	+290	—882	592	
(3) +	+	+	—	+303	—995	692	
+	+	—	+	— 5	—135	140	0
(1) +	+	+	+	+510	—785	275	} 163 . 23
(2) +	+	+	+	+416	—555	139	
(3) +	+	+	+	+366	—441	75	
Aurine 1:10 ⁴							
+	+	+	—	+320	—810	490	
+	+	+	+	+1010	—1160	150	

These experiments suggest that some metabolism of glutamic acid takes place during or after assimilation by the cells, and that this metabolism is inhibited by crystal violet. The level of glutamic acid concentration attained inside the cell thus represents a balance between the rate of entry into the cell and the rate of metabolism within the cell. Crystal violet inhibits the metabolic process and, as a consequence, the level of glutamic acid concentration within the cell rises.

A further test of this hypothesis can be obtained from a study of the metabolism of glutamic acid occurring during its migration out of the cell. It has been shown (Gale, 1947 *a*) that although the cell wall will not allow free diffusion of glutamic acid, this amino-acid can pass across the cell wall in either direction if glucose is metabolized by the cell at the same time, and that an equilibrium is then established between the internal and external concentrations of glutamic acid. Consequently, if the cells are grown in a medium rich in glutamic acid, such as casein digest medium, and then incubated in a salt solution containing

glucose, glutamic acid will pass out of the cell and can be detected in the external environment, although the amount found in the external medium is less than that lost from the internal environment (Gale, 1947*a*). Table 6 summarizes the results of a series of experiments of design similar to those already described, except that the cells initially contained a high concentration of glutamic acid and were incubated in an amino-acid-free salt solution. In a series of four such experiments, the net loss of glutamic acid during migration was 102 μ l., but in a parallel series carried out in the presence of crystal violet, the glutamic acid accumulating in the external environment was equal to that disappearing from the internal environment within experimental error.

Table 6. *Effect of crystal violet on glutamic acid metabolism*

'Saturated cells' = cells grown in casein digest glucose and containing a high internal concentration of free glutamic acid. Total dry weight of cells = 200 mg. in all cases.

Present during incubation for 1 hr.			Glutamic acid determinations		Glutamic acid metabolized or lost	
Saturated cells	Glucose (0.5 % (w/v))	Crystal violet (1:10,000)	Change in internal environment (μ l.)	Change in external environment (μ l.)	Total (μ l.)	Corrected (μ l.)
+	—	—	— 40	0	40	0
(1) +	+	—	— 335	+ 189	146	147
(2) +	+	—	— 240	+ 99	141	
(3) +	+	—	— 180	+ 30	150	
(4) +	+	—	— 202	+ 72	130	
(1) +	+	+	— 370	+ 335	35	26
(2) +	+	+	— 330	+ 272	58	
(3) +	+	+	— 102	+ 108	0	
(4) +	+	+	— 110	+ 100	10	

Aurine. In Table 4 it was shown that the effect of the dyes on glutamic acid assimilation was paralleled by the partition coefficient between *isobutanol* and water, and by the antibacterial activity. *Aurine*, an acidic dye, differs from the others examined in that although it has a marked effect on glutamic acid assimilation, it has no marked antibacterial properties. Its action in suppressing glutamic acid metabolism has therefore been tested in parallel with the experiments described above for crystal violet. Table 5 shows the results obtained. In the presence of *aurine* there is suppression of glutamic acid metabolism inside the cell, but the effect is not the same as that obtained with crystal violet; examination of the figures in Table 5 shows that crystal violet inhibited glutamic acid disappearance, and that the amount of glutamic acid withdrawn from the external environment in the presence of the dye was approximately the same as, or less than, the amount withdrawn in the absence of the dye. It is reasonable to suppose that less glutamic acid will be withdrawn from the external environment when no metabolism is occurring within the cell than otherwise. In the case of *aurine*, however, although there was approximate balance between the loss from the external environment and the gain in the internal environment, the amount of glutamic acid withdrawn from

the external environment was markedly increased by the presence of the dye. Somewhat similar results have been obtained with the acidic dye, Evans blue, which does not belong to the triphenylmethane series; in this case there was a greatly increased uptake of glutamic acid from the external medium in the presence of the dye, but there was also an increase in the amount of the glutamic acid metabolized within the cell. The dye has a feeble antibiotic action which seems to be fundamentally different from that of the triphenylmethane series.

Nature of glutamic acid metabolism

Peptide synthesis. The discovery of the structure of folic acid (Angier *et al.* 1946) and of the glutamic acid peptides associated with *p*-aminobenzoic acid (Ratner, Blanchard & Green, 1946) indicated that glutamic acid can form homoglutamic peptides in the living cell. This property is shown in the case of the D-isomer by the homo-D-glutamic acid nature of the capsular polypeptide

Table 7. *Absence of glutamyl-peptide formation during assimilation of glutamic acid by Streptococcus faecalis*

Preparation	μ l. glutamic acid/100 mg. cells		
	Free	Total after hydrolysis	Combined (peptide)
1. Washed deficient cells	40	965	925
2. Cells after incubation for 1 hr. in 0.5 % (w/v) glucose and glutamic acid (200 μ l./ml.)	225	1042	817
3. Cells treated as (2) in presence of crystal violet (1:10,000)	455	1302	847

of *B. anthracis* (Hanby & Rydon, 1946). Streptococcal protein possesses the normal complement of glutamic acid (Freeland & Gale, 1947), and it follows that one line of glutamic acid metabolism within the bacterial cell is that which leads to peptide and protein formation. If the metabolism which is blocked by crystal violet is this peptide formation, there should be an increase in combined glutamic acid during assimilation in the absence of dye, and this increase should be prevented by the presence of the dye. Combined glutamic acid of this nature would be released by acid hydrolysis and could then be estimated by the decarboxylase preparation (Gale, 1945). Table 7 shows the results of an experiment in which the free glutamic acid obtained before and after acid hydrolysis was determined with (a) deficient cells, (b) deficient cells after 1 hr. incubation with glutamic acid and glucose, and (c) deficient cells treated as (b) in the presence of crystal violet 1:10,000. The results show that there was, if anything, a decrease in the peptide-glutamic acid during assimilation. It seems, therefore, that the metabolism of glutamic acid which was blocked by crystal violet in these experiments was not peptide synthesis.

Deamination. No ammonia formation could be detected when glutamic acid was incubated with washed suspensions of *Strep. faecalis*.

Transamination. Lichstein & Cohen (1945) and Lichstein, Gunsalus & Umbreit (1945) have shown that bacteria, including the streptococci, possess an active transaminase catalysing the formation of aspartic acid + α -keto-glutaric acid from glutamic acid + oxalacetic acid. The action of crystal violet was tested on the disappearance of glutamic acid in the presence of oxalacetic acid and a dried cell preparation of transaminase from *Strep. faecalis* kindly given to us by Prof. I. C. Gunsalus. The transamination was inhibited 75 % by crystal violet in concentration 1:100, but no significant inhibition was obtained by concentrations of the order (1:10,000) used in the experiments on assimilation.

DISCUSSION

The material presented in this paper shows that glutamic acid is assimilated by streptococcal cells from the external environment when a source of energy such as glucose is available; inside the cell some of the assimilated glutamic acid then undergoes metabolic changes so that it is no longer estimated by the specific decarboxylase preparation. The level of glutamic acid concentration estimated inside the cell then represents the balance between the rate of entry of glutamic acid into the cell and the rate at which it undergoes metabolic change within the cell. It has been shown that certain dyes of the triphenyl-methane series bring about an increase in the internal concentration of glutamic acid and evidence has been put forward to show that this is accomplished by inhibition of the metabolism of glutamic acid inside the cell. The statement that the metabolism of glutamic acid takes place *inside* the cell rests upon the facts that no assimilation of glutamic takes place in the absence of glucose (Gale, 1947*a*), and no disappearance of glutamic acid from combined internal and external environments occurs in the absence of glucose. Further evidence will be presented in the next paper of this series (Gale & Taylor, 1947) that this metabolism is intracellular only. It follows that for a dyestuff to inhibit such metabolism it must first penetrate the cell wall, and this fact can probably be correlated with the variation of antibacterial efficiency of the triphenyl-methane dyes and its relation, in turn, to the *isobutanol*/water partition coefficient.

The nature of the internal metabolism of glutamic acid which is inhibited by crystal violet is at present obscure. It is highly probable that some glutamic acid becomes combined into peptide chains, but this type of synthesis apparently does not occur in the washed suspension experiments described in this paper. It is shown in a later paper (Gale, 1947*b*) that this form of synthesis occurs in growing as opposed to non-growing cells. It is also probable that transamination reactions occur within the cell and involve glutamic acid; it was not possible to show inhibition of transaminase preparations by crystal violet, but this is not necessarily significant as we do not know what is the concentration or location of the dye within the intact cell. It is becoming increasingly obvious to cytologists that the organization and orientation of enzyme systems within intact cells is of primary importance for their correlated

metabolic activities, and it is possible that a dye such as crystal violet may disorganize or disorientate the interior of the cell in such a way that enzyme activities may no longer be possible in ordered sequence although the dye may not be active against the cell-free enzyme systems.

It has been the custom to calculate the amount of amino-acid assimilated from the increase in the internal concentration of that amino-acid. From the results obtained in this paper it follows that such values are less than the amount of amino-acid withdrawn from the environment by that quantity metabolized during the assimilation process. A more satisfactory value can probably be calculated from the rise in internal concentration in the presence of crystal violet which prevents the internal metabolism. In Table 3 the internal glutamic acid level is given in absence (*a*) and presence (*b*) of crystal violet, and the latter series of values (*b*) presumably gives a measure of the assimilatory power of the organism at various stages during its growth. It can be seen that the μ l. glutamic acid assimilated/100 mg. organism remained approximately constant until growth ceased at 10 hr. and then fell rapidly. The values in the absence of the dye were approximately constant from 4–8 hr., but began to fall off before the parallel series in the presence of dye. The value obtained with the 3 hr. culture was high in the absence of dye and of the same order as the steady figure obtained in the presence of the dye; in this case the addition of dye sometimes inhibited the glutamic acid uptake, and the culture generally behaved as though it were very sensitive to outside influences of any description. It seems possible that the high value obtained with the 3 hr. culture was actually an artefact due to inactivation of the metabolic system during preparation, this inactivation having the same effect on assimilation as the dye had in older cultures. If the ideas put forward in this paper are correct the difference between the values in columns (*a*) and (*b*) of Table 3 form a measure of the glutamic acid metabolized inside the cells of the cultures. This metabolism was greatest at 10 hr. growth—when growth was stopping—and subsequently decreased rapidly.

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