35. The Tryptophanase-tryptophan Reaction

5. Possible Mechanisms for the Inhibition of Indole Production by Glucose in Cultures of *B. coli*

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The facts described by Happold & Hoyle [1936] and Evans *et al.* [1941] can be concisely stated as follows:

(1) In a complex medium (e.g. bouillon, casein-digest, etc.) in which the presence of glucose in sufficient amount inhibits completely the production of indole by *B. coli*, the complete tryptophanase system is absent from the cells.

(2) With a simple salt medium as used by Fildes, glucose only partly inhibits the production of indole (up to about 80%), but such inhibition can be completed by the addition of either phenylalanine or tyrosine in certain stoichiometrical relationships to the tryptophan present. This completion of the inhibition by these amino-acids is specific. The problem therefore resolves itself into two questions, (*a*) the actual mechanism of the glucose inhibition, and (*b*) the part played by these specific amino-acids.

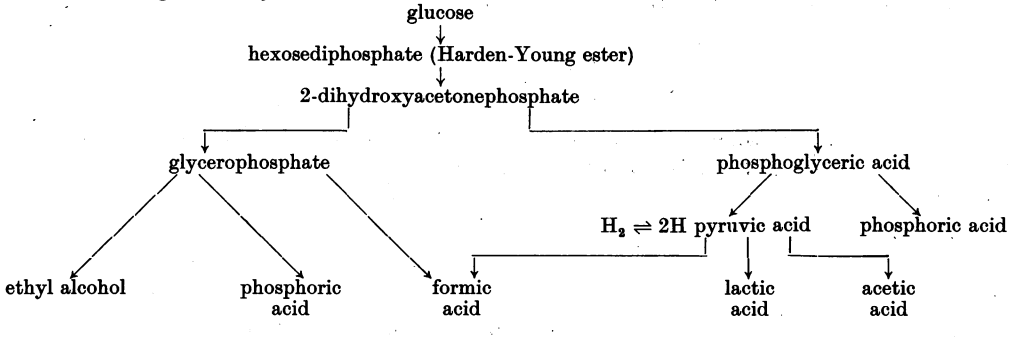
We have attempted to throw some light upon the glucose inhibition by using either:

(*a*) Complex media like bouillon or a casein-digest medium where the necessary amino-acids are present in ample quantity.

(*b*) Fildes synthetic medium together with the addition of the requisite amount of phenylalanine or tyrosine.

Effects of possible dissimilation products of glucose on indole production

Incomplete evidence supports the following scheme of the probable pathways of dissimilation of glucose by *B. coli*:



Thus, Tikka [1935] showed that when glucose was fermented with dried *B. coli* in phosphate buffer 15% of the glucose was phosphorylated after 42 hr. Hexosediphosphate and glucose were fermented by *B. coli* to give qualitatively the same fermentation products. Further, if hexosediphosphate was fermented by *B. coli* in the presence of fluoride, 95% of the reducing power disappeared, but only 54% of phosphate was liberated, thus supplying evidence for the formation of a non-reducing ester.

Stone & Werkman [1936] isolated phosphoglyceric acid from toluene-treated fermentations of *B. coli*. It is possible, however, that there may be two or more glycolytic systems present in the bacterial cell, one phosphorylating and one not.

In mammalian and yeast metabolism, the dissimilation of carbohydrate, i.e. the stages glucose → hexosediphosphate (Harden-Young ester), is a complex one involving two step-wise phosphorylations:

glucose → glucose-1-phosphate (Cori ester) → glucose-6-phosphate (Embden ester) ↓ hexosediphosphate (Harden-Young ester), fructosediphosphate

Willstätter [1940] claims to have shown that if glucose is added to a suspension of yeast cells, it disappears from solution and is built up into a polysaccharide (glycogen) inside the yeast cell. Only after this process, taking about 10 min., does fermentation (CO_2 production) begin. Whether or not this happens with *coli* cells is not known, but it appears likely from our findings [Evans *et al.* 1941], since a tryptophanase system reappears in *E. coli* cells grown on complex glucose-containing medium always after a definite lag period. This lag we assume is related to the metabolism of stored carbohydrate.

Hanes [1940] showed that glucopyranose-1-phosphate (Cori ester) added to enzymically active preparations of yeast, is partly converted into a true polysaccharide, apparently glycogen:

glycogen ⇄ (phosphorolysis) glucopyranose-1-phosphate (Cori ester) ↓ phosphoglucomutase glucopyranose-6-phosphate (Embden ester) ⇅ fructofuranose-6-phosphate ⇅ fructofuranose-1:6-diphosphate (Harden-Young ester) ⇅ 2-triosephosphate

The enzymic phosphorylation of glucose in position 1 has still to be demonstrated, if it is to be accepted as an intermediary in glycogen synthesis, although there is no doubt that yeast, muscle and liver can all utilize glucose for formation of glycogen.

It seems reasonable to suppose that a similar mechanism obtains for the bacterial dissimilation of carbohydrates. In order to gain some insight into the mechanism of the inhibition due to glucose, we have prepared all the known phosphorylated-hexose intermediates to see which, if any, of these can exert a similar inhibition.

Experimental

Hexosephosphates

Cori ester (glucopyranose-1-phosphate). The white amorphous Ba salt (prepared by the method of Cori *et al.* [1937] analysed as follows:

	Found	Calc.
P [Fiske & Subbarrow, 1925]	6·7 %	6·9 %
Glucose content after acid hydrolysis	37·5 %	40·1 %
($\alpha_D^{25°}$) of the free acid	+119	Cori *et al.* [1937] +120°

Embden ester. This ester was prepared enzymically from starch by the methods of Ostern *et al.* [1936] and Kendal & Stickland [1938]. The Embden ester so produced had a P content of 7·8 %; calc. 7·84 %. The Embden ester is not a pure substance but a mixture of glucose-fructose- and mannose-6-phosphates.

Harden-Young ester. Calcium hexosediphosphate, supplied by Bayer, was purified through the Ba salt, making use of the lower solubility of this salt in water-alcohol mixtures at 80° [cf. Harden, 1930].

Estimation of indole

Indole determinations have been made by the method of Happold & Hoyle [1934] which depends on the rapid extraction of the indole from the culture by light petroleum (B.P. 40–60°) and the addition of the Ehrlich reagent to the petroleum extract. The colours are matched in a colorimeter. The method is rapid, inexpensive and accurate to a degree not yet claimed by other methods. Nessler tubes can be used where no colorimeter is available.

Growth experiments in bouillon with the phosphorylated hexoses

The phosphorylated sugars were all added as the Na salts in concentration equivalent to the glucose used (1 %). The basal medium was ordinary bouillon. The indole, if any, was noted 8, 12 and 18 hr. after inoculation with *B. coli*.

There was no inhibition of indole formation with Harden-Young ester, glycogen, starch or maltose and no acid production during the period of growth. (A glycogenase, etc., develops by adaption on standing.) Glucose alone showed real inhibition though the Embden ester stopped indole formation up to 8 hr. and some slight inhibition may have occurred with the Cori ester, though this is less certain since growth too was retarded with this compound. Acidity developed with glucose and both monophosphoric esters but it was decidedly weaker with the Cori ester. It is clear therefore that the Harden-Young ester, maltose and the two polysaccharides do not influence the reaction because they are not absorbed by the cell with sufficient rapidity. (The late formation of a glycogenase system seems to indicate a possible slow absorption.) Knowledge of the rate of dissimilation of the monophosphoric esters, which again may be controlled by their rate of absorption, is vital to a correct assessment.

Results with synthetic media

Several experiments have been performed with synthetic media, that of Fildes and one of our own derivation. The results are qualitatively the same with both types so that a typical experiment with the former will be recorded (Table 1) and the rest of the results summarized.

Fildes's medium contained lactate as sole source of carbon except for the tryptophan essential to the reaction. We have added phenylalanine to 1 % and our tryptophan level has never exceeded 0·5 %. We have compared rates of growth and of indole formation in such media—with and without lactate ($M/2$–$M/25$) and with the substitutions of ($M/12{\cdot}5$–$M/25$) formate, acetate, pyruvate and succinate for the lactate.

The results with these substrates do not advance our knowledge but there is delay in the onset of indole production when pyruvate $M/12{\cdot}5$ is used and this effect is less marked at lesser concentrations. A similar effect with formate is less remarkable in that it can be correlated with a marked lag in the development of the logarithmic phase of growth of the organism.

Numerous experiments performed with the three hexosephosphate esters and glucose confirm that hexosediphosphate retards growth but not indole formation. The initial rate of growth with the two monophosphoric esters is generally less rapid than with the control medium and this is markedly true of the Cori ester. The rate of acid production and of disappearance from the media of these two esters is also less rapid than is that of glucose; this is especially true of the Cori ester, which in 7 days was reduced by 80 % (68·85 mg., as glucose, to 13·8 mg.) whereas a similar quantity of glucose disappeared entirely in 36 hr.

In this particularly we would draw attention to the fact that whilst Cori ester $\rightleftharpoons$ glycogen, is a demonstrable biological reaction, there is no evidence for a direct Embden ester $\rightarrow$ glycogen, change. The results can be amply accounted for by the differences in the rate of utilization of the carbohydrate derivatives involved.

Table 1. *Growth experiments in Fildes's synthetic medium with hexosephosphates*

Basal medium: Fildes's synthetic medium, 20 ml.; tryptophan, 0·1 ml. (0·1 g./20 ml.); phenylalanine, 0·5 ml. (0·2 g./20 ml.); glucose and substrates added in 1 % equivalent solution; indole determinations on 2 ml. of culture.

Time ...	3 hr.		9 hr.		12 hr.		18 hr.		31 hr.	
Medium	Indole μg.	Growth	Indole μg.	Growth	Indole μg.	Growth	Indole μg.	Growth	Indole μg.	Growth
+Lactate *M*/2	½	–	2	+	9	+ + +	14		22	
Cori ester	Nil	–	½	±	10	±	14	+ +	25	
Embden ester	Nil	–	1	+ +	1	+ + +	10		15	
Glucose	1	–	1	+ +	1	+ + +	Trace		Nil	
Control	1	–	4	+	12	+ +	18		30	

– = no visible growth.

The effect of other carbohydrates on indole production

Peptone water was used, the sugar to be tested was added to 1 % concentration and the solution sterilized by filtration. Observations were recorded after 24 hr. growth; different results are obtained with some carbohydrates which are not fermented here when they are subjected to prolonged incubation, but this involves adaptation. The results are summarized under three headings.

I. *No acid production or inhibition of indole formation* was obtained with: glycogen, starch, dextrin, saccharose, dulcitol, salicin, inulin, glycerol and potassium saccharate.

II. *Acid production and complete inhibition of indole formation* were obtained with: arabinose, lactose, glucose, fructose, mannitol and potassium *d*-gluconate.

III. *Acid production without marked inhibition of indole production* was obtained with: *d*-ribose, rhamnose, glucosamine HCl (20 μg. indole after 7 hr. growth = value for control medium), xylose, sorbitol and galactose (15 μg. indole at 7 hr.) and mannose (8 μg. indole).

Glyceraldehyde in concentrations down to *M*/1000 inhibited all growth in this medium but it should be noted that the effectiveness of the glyceraldehyde is not permanent and that complete inhibition can only be guaranteed if inoculation is made from a suspension of washed cells.

The third group of sugars is particularly interesting since we have an intense fermentation without inhibition of the indole production (mannose is at least as rapidly fermented as glucose). A common structural clue to this difference in action between groups II and III is not apparent. This difference in action between sugars in groups II and III is further support to the evidence previously presented [Happold & Hoyle, 1936] that the failure of indole production in glucose peptone water cannot be ascribed to competitive removal from the medium, by the glycolytic system, of a coenzyme factor essential to the tryptophanase system.

The effect of inhibitors of carbohydrate metabolism upon the production of indole

Fildes's [1938] medium and bouillon, both with and without glucose (1 %), were used. Na_2SO_3 and NaF (*M*/100) did not inhibit indole production in the control media nor prevent the inhibition of indole production in the glucose cultures. Phloridzin was equally ineffective, whilst sodium iodoacetate prevented all growth. The results with glyceraldehyde are presented in Table 2. The glyceraldehyde does not affect the *p*H of the bouillon cultures. It will be observed that glyceraldehyde prevents growth of the organisms at *M*/100 concentration, at a lesser concentration it inhibits indole formation without affecting growth and at still weaker concentrations is without any action. At 72, but not at 48, hr. the bouillon + glyceraldehyde (*M*/500) shows indole formation. These

Table 2. *Effect of glyceraldehyde*

Medium	After 24 hr. Indole production	Growth
Bouillon	+ + + (50 μg./2 ml.)	Good
Bouillon + glyceraldehyde, *M*/1000	+ + + „	„
„ , *M*/500	Nil	„
„ , *M*/100	Nil	Nil
Glucose bouillon + glyceraldehyde, *M*/1000	Nil	Good
„ , *M*/1000	Nil	Good

concentrations may be variable since Mendel *et al.* [1931] have shown that the glyceraldehyde effect is inhibited by small traces of pyruvic acid, and Needham & Lehmann [1937] report a similar effect with hexosediphosphate; the presence of some such reactant in the bouillon is the most likely explanation of the decreased activity in this medium as compared to peptone water. It should be noted that glyceraldehyde has been postulated as an intermediate in non-phosphorylating glucolysis.

Table 3. *Action of inhibitors of glucolysis on the appearance of indole when tryptophan is acted upon by suspensions of* B. coli *grown on casein digest-glucose agar*

Washed cells from 24 hr. culture (3 Roux bottles) were suspended in 70 ml. (½ vol. 0·85 % NaCl, ½ vol, *M*/15 phosphate buffer). There was no indole in the washings.

10 ml. of suspension + 10 ml. NaCl-phosphate buffer + 1 ml. inhibitor + 1 mg. tryptophan in 0·2 ml. were mixed in each flask and incubated at 37°. Indole determinations were made on 2 ml. samples, but are represented as the total amounts which would be present in the intact flask at the time of sampling.

Addition	μg. indole 0 min.	30 min.	60 min.	120 min.
None	Nil	21	105	525
Phloridzin, *M*/200	„	Nil	21	472
NaF, *M*/50	„	Trace 10	52	525
Iodoacetate, *M*/33	„	Nil	Nil	Nil
dl-Glyceraldehyde, *M*/100	„	10	21	262
Glucose, 0·1 %	„	Nil	Nil	Trace
Toluene	„	„	„	Nil

It may be that there is some slight delay in the recovery of tryptophanase action by the cells in the presence of phloridzin and NaF. The results with *dl*-glyceraldehyde and sodium iodoacetate are more definite.

A more extensive series with glyceraldehyde + 0·5 mg. tryptophan gave the following:

	μg. indole formed after 0 min.	30 min.	90 min.	150 min.	210 min.
Cells + glyceraldehyde, *M*/100	Nil	Nil	Nil	<1	8
„ , *M*/1000	„	„	20	150	220
Cells alone	„	„	22	182	240

The effect with glyceraldehyde is thus definite but not permanent as with toluene. When active tryptophanase suspensions were incubated for 10 min. with *M*/100 glyceraldehyde and then tested against tryptophan there was some loss of activity (11–15 %); when subjected to the action of *M*/20 glyceraldehyde the preparation was inactivated. There is thus no guarantee that the increased delay in indole production by cells grown in the presence of glucose and treated with glyceraldehyde is not due to direct action upon the tryptophanase system. That the iodoacetate effect is also non-permanent is shown

by the following results. The results with cells grown in the absence of glucose show that iodoacetate has little direct action upon the tryptophanase system.

	μg. indole formed after			
	0 min.	30 min.	50 min.	3 hr.
Normal cells + 1 mg. tryptophan	105	315	472	—
,, + $M/33$ iodoacetate	84	265	420	—
Glucose cells + 1 mg. tryptophan	Nil	Nil	105	420
,, + $M/33$ iodoacetate	,,	,,	Nil	21

When the concentration of iodoacetate is further reduced to $M/333$ indole formation is quickened and enhanced.

The effect of K^+ on tryptophanase regeneration in glucose agar cells

Pulver & Verzar [1940] suggest that the yeast cell respiring in the presence of glucose and salts first builds up the glucose into a polysaccharide (probably glycogen) before metabolizing the carbohydrate. The first phase it is suggested is associated with an absorption of K^+ from the medium, whilst during active fermentation K^+ is released from the cells into the surrounding medium.

The lag period in indole production which occurs when the washed cells of *B. coli*, grown in glucose agar, are placed in contact with tryptophan, had all the appearance of an intimate relationship with the breakdown of stored carbohydrate. Consequently tests were made on the effect of K and Na ions on the rate of recovery of indole production.

Cells grown on glucose bouillon agar for 18 hr. were washed off with sterile water, resuspended in $M/20$ sodium phosphate buffer and divided. One half was diluted 1/9 with 0·5 % NaCl (NaCl concentration 0·45 %, Na phosphate $M/200$) and the other half with 0·5 % KCl (KCl 0·45 %, Na phosphate $M/200$). They were again washed in the centrifuge and the deposited cells suspended again in these mixtures to which was added tryptophan so that there would be 100 μg. to every 5 ml. They were incubated at 37°. No indole was produced in either series up to 90 min. The results were as follows:

	Indole, μg./5 ml.	
min.	0·45 % KCl system	0·45 % NaCl system
90	Nil	Nil
120	25	,,
135	50	½
180	53	6

This experiment, frequently repeated, always showed indole formation in the K^+ series before the Na^+ series. These results give indirect evidence that the breakdown of reserve carbohydrate precedes the reactivations of the tryptophanase system.

With the assistance of three of our senior students (J. Dawson, F. M. Parsons and W. Whitaker) who have been working under the supervision of one of us (F. C. H.) it has been possible to carry out a more extensive series of determinations in which complete curves were obtained. The molar and osmotic relationships were varied and concentrations reduced. Suspensions have been washed with the salt solutions finally used.

(i) Sodium phosphate buffer, Na concentration = 47·7 mg./200 ml.

(ii) Sodium phosphate buffer, Na = 23 mg./ml., + equal vol. of potassium phosphate buffer, K = 21 mg./ml.

No NaCl or KCl was included. The tests were carried out in a series of 125 ml. Erlenmeyer pyrex flasks and each contained 12 ml. buffer, 2 ml. tryptophan (1/10,000) and

1 ml. of the bacterial suspension. The following values, typical of others were obtained:

min.	μg. indole in Na system	μg. indole in Na + K system
20 & 40	Nil	Nil
60	½	5
80	1·5	13
100	3	13·5
120	7	38·5
140	8	53·0
160	45	63·5
180	73	61·5
200	73	63

The increased lag in the absence of K^+ is apparent in the slower rate of acceleration in indole production after its commencement. These facts present further indirect evidence in support of the hypothesis that the metabolism of stored carbohydrate prevents the functioning of the adaptive tryptophanase system in cells grown in the presence of complex media containing glucose.

Discussion

The results presented are unavoidably incomplete in some directions. Studies on carbohydrate storage have made some progress and will show that the lag in tryptophanase recovery by the *coli* cells grown on a glucose nutrient medium is associated with the breakdown of stored carbohydrate [J. Dawson & F. C. Happold, unpublished]. This point is already indicated in the results presented in this paper concerning the effect of K ions on the rate of recovery of tryptophanase.

Needham & Lehmann [1937] have shown that freshly prepared glyceraldehyde inhibits glycolysis of glycogen in muscle extract in the same way as glucose does, the inhibition occurring at the stage glycogen $\rightleftharpoons$ Cori ester.

Stickland [1941] has shown that inhibition of the conversion of glucose into Cori ester occurs with the monomeric form. The preparations of glyceraldehyde used in our experiments were in the dimeric form, or predominantly so, and the results here reported might be explained by the inability of the tryptophanase system to function while glycolysis is occurring in the cell.

Iodoacetate it is claimed inhibits the reaction triosephosphate $\rightleftharpoons$ 3-phosphoglycerate, i.e. the first oxidation process in the glycolytic system: from the present experiments it would appear that interference in the glycolysis at this stage also blocks the recovery of tryptophanase action, neither is this recovery hastened in the presence of excess of cozymase. According to Adler *et al.* [1938] the action of the iodoacetate is upon the triose dehydrogenase itself.

The ineffectiveness of fluoride and sulphate as well as the results of experiments using lactate, pyruvate, formate etc., as energy sources in growth experiments, indicates quite definitely that the secondary oxidation system in glucolysis is without action upon the inhibition of tryptophanase action by the organism.

The inhibitory action upon the growth of *B. coli* of the presence of the hexosephosphates was surprising as was the fact that whilst the two monophosphates were fermented the rate was slower than with glucose, this was especially true of the Cori ester. The inhibition of indole production in cultures was less marked with these esters than with glucose, but until something is known of their rate of absorption as compared with that of the unphosphorylated carbohydrate, the results shed no new light upon the problem.

We must again stress that the tryptophanase system is adaptive and that the previous nutritional history of the cell during growth determines its activity. The combined action of the glucose and phenylalanine is only effective whilst the cells are still growing, they are without action upon the preformed tryptophanase system of suspensions which are still viable.

We must reject the suggestion that the system is rendered inoperative because of an increase in H-ion concentration during the fermentation of the carbohydrate, in view of the results obtained with mannose and the sugars grouped with it, and because of repeated failure over a period of years to obtain any tryptophanase action from preparations from cells grown in complex media containing glucose.

Summary

Glycogen, starch, dextrin, saccharose, dulcitol, salicin, inulin, potassium saccharate and hexosediphosphate are not fermented in 24 hr. cultures of *B. coli*, nor do they affect the production of indole when tryptophan is present in the medium.

Complete inhibition of indole production is obtained with arabinose, lactose, glucose, fructose, mannitol and potassium *d*-gluconate and some with Embden ester. The position with the Cori ester is less easy to assess since it is utilized less rapidly.

Acid production without marked inhibition of indole production was obtained with rhamnose, glucosamine hydrochloride, xylose, sorbitol, galactose, *d*-ribose and mannose.

Glyceraldehyde in freshly prepared solutions inhibits growth in some media in concentrations of $M/1000$. It continues to inhibit indole production at concentrations at which it does not affect growth. $M/100$ glyceraldehyde reduces the activity of the system tryptophanase-tryptophan → indole and at $M/20$ completely stops the reaction.

Iodoacetate ($M/33$) retards the recovery of tryptophanase action by cells grown on glucose-casein-digest agar, its effect upon preformed tryptophanase is slight. Other inhibitors of glycolysis are without action upon the rate of recovery of tryptophanase action by such suspensions. This recovery is delayed in the absence of K ions.

It is concluded that the inhibition of indole production by cells grown in the presence of glucose and phenylalanine is maintained by the metabolism of stored carbohydrate and that such inhibition may be operative down to the stage triosephosphate → phosphoglycerate.

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