LXXII. THE ACTION OF DYESTUFFS ON ENZYMES.

I. DYESTUFFS AND OXIDATIONS.

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DURING the past year, a series of investigations has been carried out in this laboratory on the toxic action of dyestuffs on enzymes and on the catalytic activities of tissues. About thirty dyestuffs of various types have so far been examined and it is already clear that not only do the dyestuffs vary very widely amongst themselves in their attack upon enzymes but that the enzymes exhibit a considerable specificity of behaviour towards the dyestuffs. Marston [1923] showed that the proteolytic enzymes are specifically precipitated by dyes of the safranine type, the compounds produced still being proteolytically active. In the investigations to be described, however, the main object has been to determine the amount of poisoning action a dyestuff has upon an enzyme, and how far the union of dyestuff and enzyme is reversible. Whilst this work was in progress, Bamann and Schmeller [1931] published a paper showing that substances of the indicator type, *e.g.* phenolphthalein, bromothymol blue, *etc.*, have a toxic action on lipase and there is little question but that a study of the action of these substances and of dyestuffs, such as those which are discussed in this paper, will lead to valuable information bearing on the constitution of enzymes.

The fact that dyestuffs have specific toxic actions which can be accurately estimated is a very great aid in distinguishing between enzymes which are closely related and between which there appears at present to be no satisfactory method of differentiation. Moreover, as will be shown subsequently, it is possible, by making use of the knowledge that certain dyestuffs have toxic actions upon an enzyme which is normally intracellular, to gain information on the permeability of cells (*e.g.* bacteria) to these dyestuffs.

The enzymes at present under investigation are those concerned with oxidations and reductions and also with hydrolytic changes, *e.g.* fumarase, urease, diastase.

The present communication is concerned with the effects of dyestuffs upon the oxidations of a number of substrates by bacteria (*B. coli*), and by muscle and brain tissues, succinate in particular being investigated.

EXPERIMENTAL.

The method, in brief, was to determine the amount of oxygen consumed in a certain time by a suspension of bacteria, or a quantity of muscle or brain tissue, in presence of a substrate, and to compare this with the oxygen consumed after the bacteria or tissue had been exposed to the action of a dyestuff (usually at a concentration of 1/5000) for 30 minutes at 37°. The consumption of oxygen was measured in the Barcroft differential manometer, all experiments being carried out at 37°.

There were placed in each vessel of the apparatus 1 cc. of a suspension of bacteria, 1 cc. of *M*/5 phosphate buffer, p_H 7.4, and 0.5 cc. of a 1/1000 solution of the dyestuff. The apparatus was shaken at 37° at the usual rate for half an hour, the taps of the vessels being open. After this interval, 0.5 cc. of a solution of the substrate under investigation was placed in the right hand vessel and 0.5 cc. of saline in the left hand vessel. The taps were closed after a few minutes' further shaking to secure temperature equilibrium, and the rate of oxygen uptake was measured. A control experiment, in which water was substituted for the dyestuff, was carried out in every case. The rates of oxidation were approximately linear, and the oxidation was allowed to proceed for 2 hours (or a less period if this proved more convenient). The oxygen uptakes in a series of experiments at the end of this period were measured and the percentage inhibition effected by the dyestuff calculated. To give an example, the oxygen taken up in 1½ hours at 37° by 1 cc. of a suspension of *B. coli*, unexposed to dyestuff, in presence of 0.067 *M* sodium lactate was 1367 mm.³ The oxygen uptake by the same amount of the suspension of *B. coli* under the same conditions but after exposure to Congo red (1/5000) was 1161 mm.³, and after exposure to ethyl violet (1/5000) 201 mm.³ The percentage inhibition in the first case was 15 and in the second case 85.

The acid substrates were used in the form of their sodium salts, the p_H of solutions being 7.4.

The *B. coli* suspension was prepared by 48 hours' growth in tryptic broth, the organism being centrifuged from the broth and washed twice with normal saline. It was made into a thick suspension in normal saline and stored at 0°.

In the cases of muscle and brain, 0.5 g. of the tissue was used. Muscle, after dissection from the animal, was cooled and minced. Brain was similarly treated, the whole brain being used.

Choice of dyestuffs.

A fairly representative series of dyestuffs has been chosen. This includes 10 members of the triphenylmethane series (malachite green, brilliant green, methyl violet, crystal violet, ethyl violet, gentian violet, acid green, acid fuchsine, water blue, soluble blue), 3 monoazo-dyestuffs (orange G, chrysoidine, and crystal scarlet) and 3 tetrazo-dyestuffs (Congo red, benzopurpurine, and

Bismarck brown). There is 1 diphenylmethane dyestuff, auramine. The pyronine series is represented by pyronine, eosin and erythrosin; the thiazines by methylene blue and toluidine blue; the safranines by safranine and methylene violet; and the eurhodines by neutral red and Janus green. There are also included acriflavine, haematoxylin, and an anthocyanin, cyanin chloride. It is proposed later to add to this series, but it has been felt that there are sufficient at present under investigation to indicate the main features of the action of dyestuffs on enzymes.

Dyestuffs and oxidations by B. coli.

The results of the action of dyestuffs on the oxidations by *B. coli* of glucose, lactate, succinate and formate are shown in Table I, the final concentrations of these substrates in the Barcroft vessels being 0.017 *M*, 0.067 *M*, 0.067 *M*, and 0.05 *M*, respectively.

It will be seen that, under the experimental conditions employed, whereas 15 of the dyestuffs have a relatively large inhibitory action on the oxidation by *B. coli*, the remaining 14 have little or none.

With regard to the toxic dyestuffs it may be imagined, in the first instance, that the toxicity of the dyes to the oxidations of *B. coli* is due primarily to the lethal action of these substances. Previous evidence, however, has shown with certainty that lethal action is not necessarily connected with a poisoning action on the oxidative mechanisms of the cell. For instance, toluene, which has a lethal action on *B. coli*, does not inhibit the oxidative action of this organism on succinate, lactate or formate [Quastel and Wooldridge, 1927]. Again ultra-violet light which induces great mortality among the cells of *B. coli* has little action on the oxidation by this organism of glucose or lactate [Cook and Stephenson, 1928]. It would be unreasonable, therefore, to ascribe the action of the dyestuffs which inhibit the oxidations of *B. coli* to a lethal action of these substances. An analysis of the results shown in Table I bears out this conclusion. Firstly it will be seen that in many cases the oxidation of lactate or glucose is affected to a greater extent than that of succinate. If a lethal action were responsible, such difference in effects would not be anticipated. Secondly, a specificity of behaviour is observable, for whereas one dyestuff will inhibit lactate oxidation much more powerfully than that of succinate, another will inhibit both to the same extent. Such specificity of behaviour would not be expected if the dyestuffs acted solely by a lethal action.

It is more reasonable to suppose that the toxic action of the dyestuffs is due to their combination with certain enzymes involved in the oxidations. It would be expected, if this were true, that the dyestuffs which are toxic would also be capable of staining the cell and this actually is the case. The reverse conclusion, however, *i.e.* that a dyestuff capable of staining the cell is necessarily toxic to oxidations, does not follow; for (a) the combination of enzyme

Table I. *Percentage inhibitions by dyestuffs of oxygen uptakes by B. coli in presence of various substrates. $p_H = 7.4$. Temp. 37° .*

Dyestuff (1/5000)	Nature of dyestuff	Glucose	Lactate	Succinate	Formate
Malachite green	Basic	100	92	72	71
Brilliant green	"	68	66	67	46
Methyl violet 6 B	"	100	88	46	86
Crystal violet	"	97	91	53	92
Ethyl violet	"	82	85	69	84
Gentian violet	"	98	80	36	75
Methylene blue	"	57	33	75	28
Toluidine blue	"	46	38	72	24
Auramine	"	93	87	51	54
Acriflavine	"	100	95	45	58
Neutral red	"	92	72	37	50
Janus green	"	100	69	29	45
Chrysoidine	"	95	34	19	44
Pyronine	"	39	20	6	17
Safranine	"	98	86	22	52
Methylene violet	"	19	5	10	25
Bismarck brown	"	0	4	8	27
Acid fuchsin	Acid	0	0	2	0
Acid green	"	0	17	3	0
Soluble blue	"	0	0	5	9
Water blue	"	0	8	7	0
Orange G	"	0	4	2	14
Crystal scarlet	"	1	0	7	16
Congo red	"	0	15	10	0
Benzopurpurine	"	0	31	22	22
Eosin	"	4	21	2	3
Erythrosin	"	6	18	16	9
Haematoxylin	"	17	8	39	35
Cyanin chloride	"	15	12	14	13

and dyestuff may not be such as to prevent activation of the substrate occurring and (b) the dyestuff may combine with structures in the cell which are not connected with the enzymes under consideration. It may be pointed out, in this connection, that the compounds of safranine and proteolytic enzymes are still proteolytically active and that methylene violet which has but little toxic action on the oxidations of *B. coli* (under the conditions employed, see Table I) will stain a smear of *B. coli* as well as some of the dyestuffs which are powerfully toxic.

The most important fact which emerges from a study of the results embodied in Table I is that all the highly toxic dyestuffs are basic in character, whilst the acid dyestuffs have comparatively little or no action at the concentrations and p_H used. This leads to the conclusion that the dehydrogenases responsible for the activation of glucose, *etc.*, are essentially acidic in character and are able to adsorb or combine with the basic and not with the acidic dyestuffs under the experimental conditions employed.

This conclusion explains the fact that dehydrogenases, in presence of their substrates, do not reduce dyestuffs in the order expected from their oxidation-reduction potentials. It is clear that the rate of reduction is dependent not only on the reduction potential of the dye but on its access to, *i.e.* its adsorption or combination with, the centre responsible for the activation of the substrate. The basic dyestuffs are more accessible, the acidic less

so, and it is thus found that the sulphonated (acidic) dyes are reduced at a much smaller speed than would be anticipated from the magnitude of their reduction potentials [Quastel and Wooldridge, 1927].

It is of considerable interest that Bismarck brown, which is a basic dye, has but little toxic action on the oxidations of *B. coli*. It is therefore apparent that although basicity of a dyestuff plays an important part in determining the combination of dyestuff and dehydrogenase, the basicity is not by any means the only factor involved; the structure of the dyestuff molecule is also concerned. It is presumably this fact which explains the relatively different actions of the dyestuffs on the oxidations of succinate and lactate. The importance of even slight differences in the structures of the dyestuffs is seen in the effects of safranin and methylene violet. The former is highly toxic to the oxidations of *B. coli*, the latter negligibly so.

It is evident from these results that it is of the highest importance, in using dyestuffs, *etc.*, for the measurement of r_H of cells, to have some knowledge of their effects on the oxidative mechanisms of the cells. Entirely fictitious results will be obtained by the use of dyestuffs having selective and toxic actions.

It is of interest that cyanin chloride¹, a natural anthocyanin, has but little action on the oxidative mechanisms of *B. coli*. It would be expected *a priori* that the natural colouring matters would not have a poisoning action on the oxidative mechanism of the cell.

Phosphates and the toxic action of dyestuffs.

The results given above all relate to suspensions of *B. coli* exposed to a 1/5000 aqueous solution of dyestuff in presence of phosphate buffer at p_H 7.4. It is found when using the organism at various concentrations and of varying activity, that (quantitatively) variable results are obtained. This is particularly the case with methylene blue, with which, in examining the effect of the dyestuff on the oxidation of succinate by *B. coli*, an inhibition of 70 % has been sometimes observed and at other times of only 30 %. The reason is doubtless partly connected with the state of the organism, but it is connected to a far greater degree with the nature of the constituents present in the solution in which the organism is suspended.

In the course of an investigation on the action of methylene blue on the rate of succinate oxidation by *B. coli*, it was found that the presence of phosphate had a most marked influence on the toxicity of the dyestuff. To study the rate of oxidation in the absence of phosphate, use was made of veronal buffer, p_H 7.4, made up as suggested by Michaelis [1930]. It was shown in the first place that the replacement of phosphate buffer by veronal buffer of the same p_H (7.4) had little or no effect on the rate of oxidation of succinate by normal *B. coli*. If, however, the *B. coli* were exposed to methylene blue (1/5000) in presence of veronal buffer, p_H 7.4, for 30 minutes at 37°

¹ We are indebted to Miss Rose Scott-Moncrieff for a specimen of this substance.

previous to the addition of the succinate, the oxidation of the latter was inhibited by over 90 %; whereas exposure to the dyestuff under the same conditions but in the presence of phosphate buffer, p_H 7.4, resulted in an inhibition of about 30 %. The following figures illustrate this:

O₂ uptake in 1½ hours in mm.³ at 37° (in presence of 0.067 *M* succinate) by:

1 cc. <i>B. coli</i> in presence of phosphate buffer, p_H 7.4	...	...	...	...	665
1 cc. <i>B. coli</i> in presence of veronal buffer, p_H 7.4	...	...	...	...	625
1 cc. <i>B. coli</i> , previously treated with 1/5000 methylene blue in presence of phosphate buffer, p_H 7.4	...	...	...	...	475
1 cc. <i>B. coli</i> , previously treated with 1/5000 methylene blue in presence of veronal buffer p_H 7.4	...	...	...	...	29

It was then shown that the toxic effect of methylene blue on *B. coli*-succinate oxidation in presence of veronal buffer could be partly reversed by the subsequent addition of phosphate buffer at the same p_H . This is indicated by the following figures:

Oxygen uptake in 2 hours in mm.³ by:

(a) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of phosphate buffer <i>M</i> /25, p_H 7.4, for 30 minutes, succinate alone then being added	...	...	...	505
(b) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of veronal buffer, p_H 7.4, for 30 minutes, succinate alone then being added	...	...	...	25
(c) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of phosphate buffer <i>M</i> /25, p_H 7.4, for 30 minutes, a mixture of succinate and veronal buffer, p_H 7.4, then being added	...	...	...	538
(d) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of veronal buffer, p_H 7.4, for 30 minutes, a mixture of succinate and phosphate buffer, p_H 7.4, then being added	...	...	...	255

The fact that there is a recovery in oxygen uptake when phosphates are added subsequently to the exposure of the organism to the dyestuff in presence of veronal buffer, indicates that the phosphates must be able to remove methylene blue to some extent from combination with the enzyme. This seems the only feasible explanation, for the fact that replacement of phosphate buffer by veronal buffer does not result in a diminished rate of oxidation of succinate by the untreated organism shows that phosphates are not essential to the oxidation of succinate. Moreover veronal cannot have the effect of making methylene blue more toxic, for the addition of veronal to the organism exposed to the dye in presence of phosphate buffer does not result in a diminished rate of oxidation of succinate. There is the possibility that phosphates and methylene blue compete for the same groups in the enzyme but there is no evidence as yet for this.

Other basic dyestuffs such as toluidine blue and malachite green behave in a similar manner to methylene blue. The acid dyestuffs, however, *e.g.* water blue and Congo red, which in phosphate buffer at p_H 7.4 have little or no toxic action on succinate oxidation by *B. coli* have just as little action in veronal buffer at the same p_H .

It seems likely that the action of the phosphate is to combine with the basic dyestuff ions and prevent their combination with the acid groups of the enzyme, or if such combination has been effected, to accomplish a reversal,

by what is tantamount to an eluting action on the adsorbed or combined dyestuff.

If for veronal buffer, p_H 7.4, there be substituted $M/20$ glycine solution brought to p_H 7.4 by the addition of a little alkali, the toxic action of methylene blue on *B. coli* (towards succinate oxidation) is greatly reduced, the oxygen uptake being nine times that which is obtained after exposing the organism to the dyestuff in veronal buffer and about half that which occurs after exposing the organism to the dyestuff in phosphate buffer. Glycine has a protective action similar to, but much less than, that of phosphate at p_H 7.4. Clearly the magnitude of the protective action of the buffer on the organism will be dependent not only on the concentration of buffer ions but on the relative affinities of the buffer ions and the acid groups of the enzyme for the dyestuff. In this connection the work of Hirschfelder and Wright [1930] and of Stearn and Stearn [1930] on bacteriostasis and the equilibria between dyestuff and proteins is of importance.

Oxidations by muscle and brain.

Meyerhof [1917, 1918] showed that the respiration of micro-organisms (staphylococci) was inhibited 10 to 40 % by methylene blue but that the respiration of the same organisms, after exposure to acetone, increased in the presence of methylene blue. Fleisch [1924] demonstrated that the addition of methylene blue to muscle tissue in presence of HCN brought about an increased rate of oxidation of succinate. Barron [1929] found that the addition of methylene blue to living cells (sea urchin, star fish eggs) and red blood cells produced an increased oxygen consumption. It is clear that the dyestuff is able to play a catalytic role in oxidations on account of its reversible nature and the fact that its leuco-derivative can be oxidised by molecular oxygen without the intervention of a dehydrogenase system. The catalytic action of dyestuffs, generally, on the oxygen consumption of living cells is dependent, as shown by Barron and Hoffman [1930], on their reduction potentials and their ability to penetrate into the cell. The results given in Table I show that the acid or basic nature of the dyestuff and its access to (or adsorption or combination with) the centre at which the hydrogen donator is activated influence considerably its effect on oxidations. In these experiments the organism was exposed to the dyestuff for a certain period before the addition of the substrate under investigation.

It has been a usual practice, in investigations on dyestuffs, to add the substrate (*e.g.* glucose) at the same time as the dyestuff to the suspension of cells. If the oxidation of the substrate, under these circumstances, is rapid, there may be insufficient time for the dyestuff to exert a marked toxic action on the enzymes involved and the observed rate of oxidation will not be complicated by this factor. This, however, is only an ideal condition, for circumstances are usually such that the rate of reduction of the dyestuff is not

extremely rapid. The nature of the buffering substances, too, will have a decided influence on the rates of oxidation in presence of the toxic dyestuffs.

When 0.5 g. fresh muscle is exposed to dyestuff prior to the addition of succinate, the experiment being carried out in precisely the same way as has been described for the bacteria, the toxic action of such basic dyestuffs as malachite green and methyl violet becomes apparent. Illustrative results are shown in Table II.

In a typical experiment 0.5 g. freshly minced rabbit muscle was placed in each vessel of the Barcroft apparatus, and phosphate buffer, p_H 7.4 (to give a final concentration of $M/15$), and dyestuff (to give a final concentration of $1/5000$) added. The apparatus was allowed to shake at 37° for 30 minutes, and then succinate (to give a final concentration of $0.067 M$) was added to the right-hand vessel and saline to the left-hand vessel. The rate of oxygen uptake was measured and compared with the control where the muscle was not exposed to dyestuff. It is important in these experiments to have in the control vessels a volume of distilled water equal to that of the dyestuff solution which is placed in the experimental vessels. If normal saline be used instead of distilled water, confusing results are obtained owing to differences in osmotic tension.

In one experiment, for instance, the oxygen uptake by 0.5 g. muscle, unexposed to dyestuff, in presence of succinate was 386 mm.^3 in 2 hours; the oxygen uptake by the muscle, exposed to $1/5000$ malachite green, in presence of the succinate was 150 mm.^3 in 2 hours. When acid fuchsine and Congo red were substituted for malachite green, the oxygen uptakes in 2 hours were 355 and 375 mm.^3 respectively.

Muscle tissue deteriorates, on standing at 0° , at a far greater rate than a suspension of bacteria, so far as the oxidation of succinate is concerned, and this factor is of importance in estimating the effects of dyestuffs. The results with muscle cannot be compared quantitatively with those found with bacteria, for there is a great difference in the quantity of biological material used. Qualitatively, however, there appears to be a parallel between the action of dyestuffs on bacteria and on muscle.

Methylene blue and toluidine blue must be considered separately, for on certain occasions both these dyestuffs have given rise to an increased rate of oxidation of succinate and on others to a decided inhibition. Much depends on the state of the muscle at the time of experiment, and it seems clear that the variability in results is due to the interplay of two factors, (a) a toxic action on the enzymes involved in the oxidation of succinate, leading to inhibition of the rate of oxidation, (b) a catalytic action, due to reduction of the dyestuff and subsequent oxidation, leading to an increased rate of oxidation. If these two effects happen to balance each other, the net effect of the addition of methylene blue is zero—this has occurred on several occasions. On the other hand, on two occasions accelerations of 42 and 46 % have been noted and on another an inhibition of 31 % was found. Much the same has been found with toluidine blue.

If instead of fresh muscle, well-washed muscle be used, methylene blue has a slightly more vigorous toxic action. If, however, using washed muscle, phosphate buffer be replaced by veronal buffer at the same p_H , methylene blue has a very decided toxic action—an effect precisely similar to that found with the bacteria. Thus in one experiment, using well-washed rabbit muscle, methylene blue brought about a 6 % inhibition in presence of phosphate buffer ($M/15$, p_H 7.4) and a 60 % inhibition in presence of veronal buffer (p_H 7.4).

Brain tissue (sheep's whole brain has been chiefly studied) appears to be almost unaffected by the dyestuffs. In no case (see Table II) has an inhibition

Table II. *Percentage inhibition by dyestuffs of succinate oxidation by muscle and brain*

Dyestuff (1/5000)	Muscle	Brain
Methyl violet	30	0
Malachite green	50	0
Gentian violet	15	0
Methylene blue	0	0
Toluidine blue	0	0
Acid fuchsine	0	0
Water blue	4	0
Congo red	3	0

of the oxidation of succinate, due to the exposure of the tissue to dyes, been observed. Slight accelerations, *e.g.* with methyl violet, malachite green, acid fuchsine, toluidine blue, have been noted but these are irregular. For example:

The O_2 uptake in $1\frac{1}{2}$ hours in presence of phosphate buffer, p_H 7.4, and succinate in mm.³

(a) by 0.5 g. sheep's brain	...	...	...	...	...	...	...	...	514
(b) by 0.5 g. sheep's brain exposed to 1/5000 methyl violet	...	...	...	...	...	...	...	...	560
(c) by 0.5 g. sheep's brain exposed to 1/5000 toluidine blue	...	...	...	...	...	...	...	...	571
(d) by 0.5 g. sheep's brain exposed by 1/5000 malachite green	...	...	...	...	...	...	...	...	553

The difference between the effects of, say, methyl violet or malachite green, on the oxidations of brain and muscle tissue is very striking. It seems unlikely that the enzymes involved in succinate oxidation in brain are so differently constituted from those in muscle or bacteria that they are not attacked by the basic dyestuffs—it is more reasonable to suppose that the enzymes are protected either by an inability of the dyestuffs to reach the site of the enzymes in the cell or because there are constituents present in the tissue which combine preferentially with them.

Clearly, the locality of the enzymes in the tissues and the nature of their immediate environment are most important factors in determining the toxicity of dyestuffs.

SUMMARY.

1. The action of 29 dyestuffs on the oxidations of glucose, lactate, succinate and formate by *B. coli* has been investigated.

2. It is shown (a) that all the dyestuffs which have a vigorous toxic action are basic in character, whilst the acid dyestuffs have little or no action; (b) that the molecular structure of the dyestuff plays an important part in

determining toxicity; (c) that specificity of behaviour towards the dehydrogenases is exhibited.

3. The toxicity of the basic dyestuffs is greatly dependent on the nature of the buffering agent present.

4. The action of dyestuffs on the oxidation of succinate by muscle tissue is similar to that found with bacteria. In the case of methylene blue or toluidine blue two effects take place, (a) a toxic action leading to inhibition, (b) a catalytic action leading to an increased rate of oxidation, due to the reversible nature of the dye. As in the case of bacteria, the presence or absence of phosphate influences considerably the toxicity of the dyestuff.

5. The dyestuffs, so far examined, appear to have no inhibitory action on the oxidation of succinate by brain tissue.

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