CXXVII. TRYPANOCIDAL ACTION AND TOXICITY TO ENZYMES.

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It has been shown in a previous communication [Quastel, 1931] that the Congo red series of dyestuffs is highly toxic to the enzyme fumarase, prepared either from bacteria or from mammalian tissues. The presence of proteins greatly affects the toxicity of the dyes; this is due to the adsorption or combination of the dyestuff with protein, there resulting a diminution in the concentration of free dyestuff. With fumarase extracted from brain tissue and prepared in such a way as to be relatively free from protein and phosphate, Congo red is toxic at a molar concentration of $1{\cdot}2 \times 10^{-8}$.

Trypan-blue is highly toxic and it has now been found that trypan-red is also toxic, though its activity is not as great as that of the former. It is not to be supposed that these substances are general enzymic poisons, for experiment has shown that neither Congo red nor trypan-blue has any toxic action upon the enzyme urease prepared from Soya bean. It is evident that there must be some close connection between the structure of fumarase and that of the Congo red series of dyestuffs, which allows this specificity of behaviour to obtain.

The fact that trypan-blue and trypan-red have highly toxic actions upon fumarase led the writer to consider the possibility that other trypanocidal agents, not belonging to the Congo red series, might affect the activity of fumarase. Accordingly, Bayer 205 was tested and it has been found that this substance is also very toxic, its behaviour, as in the case of Congo red and trypan-blue, being specific, for it has no toxic effect on urease.

It is necessary now, for a proper appreciation of what follows, to give a brief résumé of recent work on the mechanism of trypanocidal action.

Following the discovery by Ehrlich and Shiga that trypan-red (I),

(I) $C_{10}H_4(SO_3H)_2(NH_2).N:N.C_6H_4.C_6H_3(SO_3H).N:N.C_{10}H_4(NH_2)(SO_3H)_2$,

a member of the Congo red series of cotton dyes, can cure an experimental infection of trypanosomiasis in mice, Nicolle and Mesnil examined a large number of cotton dyes belonging to the Congo red series and found that the substance having the best curative action on infection with *Trypanosoma gambiense* was a *s*-carbamide (II) derived from H-acid.

(II) $C_{10}H_3(SO_3H)_2(NH_2)(OH).N:N.C_6H_4.NH.CO.NH.C_6H_4N:N.C_{10}H_3(SO_3H)_2(NH_2).OH.$

This was followed by the discovery, by the Bayer firm, that the replacement of the azo-linkage in II by the peptide (—NH—CO—) linkage gave rise to a colourless substance also possessing trypanocidal properties.

It was known from work on dyes that the substantive character to cotton was favoured by the presence of chains of aminobenzoyl groups, and, by the introduction of such groups into the *s*-carbamide of an aminobenzoylnaphthylaminepolysulphonic acid, the important trypanocidal agent Bayer 205 (III) was evolved.

(III) $C_{10}H_4(SO_3H)_3NH.CO.C_6H_3(CH_3).NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.C_6H_3(CH_3).CO.NH.C_{10}H_4(SO_3H)_3$.

As Balaban and King [1927] point out, it is clear that the discovery of Bayer 205 arose from the recognition of the fact that it is possible to prepare colourless substances of substantive properties which, being of similar molecular build to trypanocidal dyes, also exhibit trypanocidal properties.

The next important step was taken by Balaban and King [1927], who started with six readily obtainable naphthylaminedisulphonic acids and from these prepared the *m*-aminobenzoyl and the *m'*-aminobenzoyl-*m*-aminobenzoyl derivatives. They then formed the *s*-carbamides of these derivatives by phosgenation. The *s*-carbamide of the original naphthylaminedisulphonic acid will be referred to as the first *s*-carbamide derivative; that of the *m*-aminobenzoyl derivative as the second *s*-carbamide derivative and that of the *m'*-aminobenzoyl-*m*-aminobenzoyl derivative as the third *s*-carbamide derivative.

Thus:

$NH_2.R \rightarrow R.NH.CO.NH.R$
First *s*-carbamide derivative

$NH_2.C_6H_4.CO.NH.R \rightarrow R.NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.R$
m-aminobenzoyl derivative — Second *s*-carbamide derivative

$NH_2.C_6H_4.CO.NH.C_6H_4.CO.NH.R \rightarrow R.NH.CO.C_6H_4.NH.CO.C_6H_4.NH.CO.NH.C_6H_4.CO.NH.C_6H_4.CO.NH.R$
m'-aminobenzoyl-*m*-aminobenzoyl derivative — Third *s*-carbamide derivative.

Balaban and King found, on examining the trypanocidal properties of the aminobenzoyl derivatives and of the first, second and third *s*-carbamides of the six naphthylaminedisulphonic acids, that trypanocidal activity only occurred among the second and third *s*-carbamides, *i.e.* at the *s*-carbamide stage of combination of the aminobenzoyl and aminobenzoyl-aminobenzoyl derivatives. Bayer 205, it is to be noted, is a third *s*-carbamide derivative. Following this, these workers then showed, on examining the substantive properties of these substances to cotton, that a pronounced maximum of substantivity occurred at the *s*-carbamide stage of combination and was greater with the third *s*-carbamides than with the second. Thus there appeared to be a definite parallelism between the substantive properties of these carbamides to cotton and their trypanocidal activity. Bayer 205 was also shown to be substantive to cotton.

Turning now to fumarase and knowing that trypan-blue, trypan-red and Bayer 205 were toxic to the enzyme, it became of interest to see whether the *s*-carbamides investigated by Balaban and King had any effect upon the

enzyme. Experiment has shown that toxicity only appears at the second *s*-carbamide stage of combination and that the third *s*-carbamides are highly toxic. The effect, moreover, is specific to fumarase, for urease is unaffected by these derivatives.

Method.

The method adopted throughout this work is that described fully in a previous communication [Quastel, 1931]. It consists, briefly, in mixing a preparation of fumarase, obtained from brain, with phosphate buffer solution, p_H 7·4, and the substance under investigation and in incubating the mixture for 30 min. or 60 min. at 45°. Sodium fumarate solution is then added to the mixture to make a final concentration of 0·08 *M*. Incubation is allowed to proceed for 2 hours at 45°, and the *l*-malate produced is estimated polarimetrically. From a comparison of the *l*-malate produced in presence of the toxic substance with that formed in its absence, an estimate of the inhibiting effect of the toxic agent is obtained. Details of the estimation of the *l*-malate will be found in the previous communication.

The effects of trypan-blue and trypan-red.

To a mixture of 2 cc. fumarase preparation and 1 cc. *M*/5 phosphate buffer, p_H 7·4, was added 1 cc. trypan-blue solution or 1 cc. trypan-red solution, to give a concentration of 1/4000. The mixture was incubated for 30 min. at 45°, and then 1 cc. 0·4 *M* sodium fumarate solution was added. Incubation was carried out for 2 hours at 45°. A mixture of 1 cc. glacial acetic acid and 10 cc. 14·2 % ammonium molybdate solution was then added, and the solution shaken with a little decolorising charcoal and filtered. The filtrate was examined polarimetrically in a 1 dm. tube, compared with a control experiment in which no dyestuff was present, and the amount of inhibition calculated.

The experiment was repeated with various concentrations of the dyes and the results are recorded in Table II. It will be seen that trypan-blue is more toxic than trypan-red but that both are toxic at concentrations of 1/16,000.

The effects of naphthylaminedisulphonic acids.

The following six acids were used:

Freund's acid: 1-naphthylamine-3 : 6-disulphonic acid.
Amino-G-acid: 2-naphthylamine-6 : 8-disulphonic acid.
C-acid: 2-naphthylamine-4 : 8-disulphonic acid.
Amino-J-acid: 2-naphthylamine-5 : 7-disulphonic acid.
H-acid: 8-hydroxy-1-naphthylamine-3 : 6-disulphonic acid.
2R-acid: 8-hydroxy-2-naphthylamine-3 : 6-disulphonic acid.

Experiments were carried out, as described for trypan-blue and trypan-red, but the incubation period prior to the addition of fumarate was one hour and in the estimation of the malic acid it was unnecessary to shake with charcoal.

Results are noted in Table I from which it will be seen that these acids at a concentration of 1/4000 have little or no toxic action on fumarase.

Table I. *Percentage inhibitions of fumarase activity by naphthylaminedisulphonic acids and their* s-*carbamide derivatives. Concentration* = 1/4000.

	Freund's acid	Amino-G-acid	C-acid	Amino-J-acid	H-acid	2R-acid
Free acid	1	0	0	0	3	2
1st *s*-carbamide	—	—	3	4	—	5
2nd *s*-carbamide	5	32	17	19	8	95
3rd *s*-carbamide	83	90	94	95	32	95

The effects of s-*carbamides of naphthylaminedisulphonic acids.*

Specimens of these substances were kindly supplied by Dr H. King. The first *s*-carbamides of Freund's acid, amino-G-acid, and H-acid were not available and were not tested.

Experiments were carried out exactly as with the naphthylaminedisulphonic acids and the complete set of results is given in Table I.

The most striking result is that, whereas the first *s*-carbamide derivatives are practically inert, the third *s*-carbamide derivatives are highly toxic.

There is a great difference in toxicity between the carbamide derivatives of H-acid and 2R-acid, the only chemical difference between these acids being that the former is a 1-naphthylamine derivative and the latter a 2-naphthylamine derivative. The second *s*-carbamide of 2R-acid is as toxic as any of the third *s*-carbamide derivatives of the acids investigated, and far more so than the other second *s*-carbamide derivatives. It would seem that the extra toxicity of 2R-acid is determined by the presence of the hydroxyl group in the naphthalene nucleus and this is supported by the fact that trypan-blue (which contains such a group) is more toxic than trypan-red (from which the hydroxyl group is absent). H-acid, however, which also contains the hydroxyl group in the naphthalene nucleus, forms *s*-carbamides which are not only less toxic than those from 2R-acid but less toxic than those from the non-hydroxylated naphthylaminedisulphonic acids. It is impossible, therefore, to attribute any increased toxicity solely to the hydroxyl group.

It is, however, quite clear that toxicity to the enzyme becomes apparent only at the second *s*-carbamide stage of combination and is most marked at the third *s*-carbamide stage. (Bayer 205 which is a third *s*-carbamide derivative gives a 68 % inhibition of fumarase at a concentration of 1/4000.) Now this is precisely the result obtained by Balaban and King [1927] who found that trypanocidal activity became apparent only at the second *s*-carbamide stage of combination and was most marked at the third *s*-carbamide stage.

It must not be supposed that the toxicity of the higher *s*-carbamides to fumarase is an explanation of their trypanocidal activity, for Balaban and King found that the derivatives of Freund's acid and 2R-acid have no trypanocidal action. It is evident, however, that there must be some structure common to the trypanosome and to fumarase which accounts for the parallelism which

exists between fumarase toxicity and the trypanocidal action of the second or third *s*-carbamides.

Variation in toxicity with concentration of s-*carbamides.*

The experiments described above were repeated with the more toxic *s*-carbamides at various concentrations, the incubation period prior to the addition of fumarate being 30 min. The results are recorded in Table II. The most important result emerging from this Table is that the *s*-carbamide compounds which retain a high toxicity at considerable dilution are all derived from 2-naphthylamine structures. Derivatives of the 1-naphthylamine compounds (Freund's acid and H-acid) have relatively little toxicity at high dilution.

Table II. *Percentage inhibition of fumarase activity by by* s-*carbamides* etc. *at various concentrations.*

	Concentration		
	1/4000	1/8000	1/16,000
Trypan-blue	100	100	92
Trypan-red	74	43	21
3rd *s*-carbamide of Freund's acid	76	52	14
3rd *s*-carbamide of H-acid	29	11	6
2nd *s*-carbamide of amino-G-acid	16	11	3
3rd *s*-carbamide of amino-G-acid	87	78	60
3rd *s*-carbamide of C-acid	97	95	85
3rd *s*-carbamide of amino-J-acid	91	79	45
2nd *s*-carbamide of 2R-acid	93	87	75
3rd *s*-carbamide of 2R-acid	90	80	53

Substantive properties and toxic action.

Balaban and King suggested from their experiments that there is a rough parallelism between the substantivity to cotton and the trypanocidal action *in vivo*, in that the salient peaks of substantivity occurred at the *s*-carbamide stage of combination. They were only able to determine the substantivity of the derivatives of H- and 2R-acids, but since Bayer 205 was also found to be substantive to cotton, it seems likely that the phenomenon holds also for the nonhydroxylated derivatives.

It is obvious that there is also a parallelism between substantivity to cotton and toxicity to fumarase, in that the latter appears only among the second and third *s*-carbamides. The parallelism, however, must be very rough, for whereas the substantive power of the second carbamide of 2R-acid is $2\frac{1}{4}$ times that of the second carbamide of H-acid, the toxic power of the former on fumarase is about 12 times that of the latter.

It is significant in this connection that Congo red and benzopurpurin which are highly toxic to fumarase are substantive to cotton.

On the whole it would seem that the fumarase enzyme, which is very widespread biologically both in plant and animal tissues, must possess structures which are common to the cotton fibre and to the trypanosome and which are responsible for the specific combination or adsorption of the higher

s-carbamide derivatives of the naphthylaminedisulphonic acids. Other factors, specific for the type of biological material, doubtless account for the variations which are observed between the effects of these derivatives on the different biological types.

It is of particular interest that there should be an enzyme to which these trypanocidal agents exhibit a specific toxicity (urease, for instance, is unaffected by them) and the elucidation of the factors involved should be of material assistance in the development of this branch of chemotherapy.

Protection by fumarate.

It has been shown [Quastel, 1931] that fumarase is "protected" by its substrate, fumarate, from such toxic dyestuffs as Congo red and methyl violet. This holds also for the *s*-carbamide derivatives.

To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, p_H 7·4, 1 cc. water, and 1 cc. 0·4 *M* sodium fumarate solution. After incubation at 45° for 2 hours the rotation was 1·12°.

To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, p_H 7·4, 1 cc. 1/1000 solution of the third *s*-carbamide derivative of 2R-acid, and 1 cc. 0·4 *M* sodium fumarate solution. After incubation for 2 hours the rotation was 1·03°. (Inhibition = 8 %.)

To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, p_H 7·4, and 1 cc. of 1/1000 solution of the third *s*-carbamide derivative of 2R-acid. The mixture was incubated at 45° for 30 min. and then 1 cc. 0·4 *M* sodium fumarate solution was added. After incubation at 45° for 2 hours the rotation was 0·11°. (Inhibition = 90 %.)

The experiment shows that incubation with the *s*-carbamide for 30 min. prior to the addition of fumarate gave an inhibition of 90 %, whereas when the derivative was added together with the fumarate the inhibition was only 8 %.

This holds also for other *s*-carbamide derivatives.

Effects of proteins on the toxic action of s-*carbamides.*

As occurs in the case of the toxic dyestuffs, proteins exert a marked protective action against the *s*-carbamides.

This is indicated in Table III where the effects of addition of guinea-pig serum at different concentrations to fumarase are noted.

Table III. *Percentage inhibitions of fumarase by* s-*carbamides* (1/5000) *in presence of serum.*

Concentration of serum	3rd carbamide of amino-J-acid	3rd carbamide of 2R-acid	Bayer 205
1/25	36	40	20
1/50	81	70	33
1/100	89	84	37
1/200	92	91	43
0	92	92	43

A typical experiment was as follows. To 2 cc. fumarase preparation were added 1 cc. *M*/5 phosphate buffer, 1 cc. of diluted guinea-pig serum, and 1 cc.

of 1/1000 solution of the *s*-carbamide. This was allowed to incubate at 45° for 30 min. and then 1 cc. 0·4 *M* sodium fumarate solution was added. After further incubation at 45° for 2 hours the rotation was determined and the percentage inhibition calculated.

There is little doubt that, as in the case of the dyestuffs, the protective action of the protein is due to a combination of the protein with the *s*-carbamide derivative, resulting in a diminution of the concentration of the free derivative and hence in a reduced inhibition of fumarase activity.

SUMMARY.

1. Trypan-blue, trypan-red, and Bayer 205 are toxic to fumarase.

2. The toxicity of six naphthylaminedisulphonic acids and their *s*-carbamide derivatives on fumarase has been investigated. It is shown that the free acids and their first *s*-carbamide derivatives are inert. Toxicity begins to be apparent with the second carbamide derivatives (*s*-carbamide of *m*-aminobenzoylnaphthylaminedisulphonic acid) and is very marked with third carbamide derivatives (*s*-carbamide of *m'*-aminobenzoyl-*m*-aminobenzoylnaphthylaminedisulphonic acid). Attention is drawn to the parallelism which exists between this effect and trypanocidal action which begins only at the second *s*-carbamide stage of combination and is most marked at the third *s*-carbamide stage. The parallelism with the substantive properties of these derivatives to cotton is also discussed, and it is shown that there must be some structure in common between the fumarase enzyme, cotton fibre and the trypanosome which makes for specific combination or adsorption with the second and third *s*-carbamide derivatives of the naphthylaminedisulphonic acids. These derivatives are not toxic to urease.

3. Derivatives of the 2-naphthylaminedisulphonic acids are much more toxic to fumarase than those of the 1-naphthylaminedisulphonic acids.

4. Fumarate "protects" the enzyme, fumarase, from the toxic action of these derivatives.

5. The presence of proteins diminishes or eliminates the toxic action of these derivatives.

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REFERENCES

Balaban and King (1927). *J. Chem. Soc.* 3068.
Quastel (1931). *Biochem. J.* **25**, 898.