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Apparent enhanced stability in acid media of the ethyleniminium ion derived from dibenamine

Studies on the inhibition reaction between α-chymotrypsin and dibenamine (Al Shabibi & Smith, 1974) showed that the alkylating species were derived from dibenamine and that this species, which was probably the ethyleniminium ion, was present and stable in acid media but was rapidly depleted in near neutral buffered media. We have assessed the relative levels of this ion in solutions of dibenamine in a qualitative manner using its known blocking action on the muscarinic receptor of guinea-pig isolated ileum (Furchgott, 1954).

Materials. Strips of terminal guinea-pig ileum were suspended in 5 ml of aerated Tyrode solution at 37°. Muscle contracture was recorded isotonically under a load of 1 g by means of a simple lever and a four-fold magnification. The time cycle for emptying and refilling the tissue bath with Tyrode solution was controlled by the relays of an automatic assay command module (Cassella Electronics).

Inhibition of the muscarinic receptor by dibenamine

In all experiments the maximal response of the tissue to acetylcholine was determined before a dose giving 50% maximal response (submaximal dose) was assessed from a log dose-response curve. The contact time was 30 s. This was followed by flushing the bath with its own volume of Tyrode solution. There was a rest period of 3 min between each addition of acetylcholine. When the response to the submaximal dose of acetylcholine was reproducible, the incubation with dibenamine solution was commenced.

The tissue was incubated for 2 min with a solution of dibenamine hydrochloride ($1{\cdot}6 \times 10^{-5}$ M) freshly prepared in Tyrode solution (pH 7·4). The bath was then washed out once with Tyrode solution and the submaximal dose of acetylcholine was applied on three occasions each of 30 s with intermediate washing with Tyrode as before. The average value for the height of contracture was used in subsequent calculations. The whole procedure of incubating the tissue with dibenamine and

Table 1. *The effect of pH on the reaction between dibenamine and the muscarinic receptors of guinea-pig isolated ileum.*

Cumulative incubation period (min) with dibenamine	% decrease in response						
	Experiment: 1	2	3	4	5	Mean	Standard deviation
At pH 7·4							
2	Nil	3·5	4·6	Nil	Nil	1·6	2·2
4	7·15	6·9	9·1	4·2	2·3	6·1	2·4
6	14·3	13·8	9·1	8·3	4·7	10·0	4·0
8	21·4	17·3	13·6	12·5	6·9	14·3	5·4
At pH 4·0							
2	20·0	27·5	23·2	24·3	23·1	23·6	2·6
4	40·0	48·3	38·5	45·5	55·8	45·6	6·9
6	56·0	69·0	61·5	66·7	67·4	64·1	5·3
8	76·6	93·1	80·8	84·9	80·2	83·1	6·3

subsequent determination of the response to the submaximal dose of acetylcholine was repeated four times. The results are summarized in Table 1.

The same tissue that had been incubated with dibenamine at pH 7·4 was washed out four times with Tyrode solution and then incubated for 2 min with a modified Tyrode solution previously adjusted to pH 4·0 with dilute hydrochloric acid. The incubation with the modified Tyrode was repeated three times and the response to the submaximal dose of acetylcholine was determined after each incubation. The response was only slightly greater (ca 10%) than that observed before incubation with modified Tyrode indicating that the lower pH had not had a deleterious effect on the tissue.

The tissue was then incubated with a solution of dibenamine hydrochloride ($1{\cdot}6 \times 10^{-5}$ M) in modified Tyrode solution (pH 4·0) for periods of 2 min and the response of the tissue determined at pH 7·4 as previously described.

After four incubations of the isolated preparation with dibenamine ($1{\cdot}6 \times 10^{-5}$ M) at pH 7·4 there was an average 14% decrease in the response of the tissue to the submaximal dose of acetylcholine (Table 1). Further incubation of the same preparation with dibenamine at pH 4·0 in a similar manner resulted in an average decrease of 83% in the response of the tissue to the submaximal dose of acetylcholine.

Fresh tissue incubated at pH 4·0 with dibenamine as previously described gave results similar to those shown in Table 1.

Influence of sodium thiosulphate. A solution of dibenamine hydrochloride ($7{\cdot}5 \times 10^{-4}$ M) was prepared in Tyrode solution containing sodium thiosulphate ($6{\cdot}67 \times 10^{-2}$ M) and the mixture was adjusted to pH 4·0. The tissue was incubated for 2 min with dibenamine-thiosulphate solution in Tyrode (pH 4·0) using a dibenamine concentration of $1{\cdot}6 \times 10^{-5}$ M in the gut bath. The bath was then washed out once with Tyrode solution and the dose-time cycle for acetylcholine was recommenced as before and the whole cycle repeated four times. There was an average decrease of 11% in the response of the tissue to acetylcholine after this procedure.

Dibenamine gives very low levels of the corresponding ethyleniminium ion as estimated by the reaction with thiosulphate ion (Harvey & Nickerson, 1953) the latter reacting but slowly with the parent β-halogenoethylamine (Bunte, 1874). The formation of the ethyleniminium ion from the dibenamine base by an intramolecular nucelophilic reaction would be facilitated to a greater extent in neutral media than in acidic media since dibenamine has pKa 5·5 (Beddoe & Smith, 1971) and exists as the

base to the extent of approximately 3 and 99% in solutions of dibenamine at pH 4·0 and 7·4 respectively. However, studies on the inhibition reaction between α-chymotrypsin and dibenamine indicated that the alkylating species which was probably the ethyleniminium ion derived from dibenamine (Al Shabibi & Smith, 1974) existed at a higher concentration in acid solution than at near neutral pH.

Dibenamine reacts with the muscarinic receptor of guinea-pig isolated small intestine through its ethyleniminium ion and blocks the response of the tissue to acetylcholine (Furchgott, 1954; Beddoe, Nicholls & Smith, 1971). The much increased block at the lower pH and its suppression by thiosulphate ion, showing that the ethyleniminium ion was the alkylating species present in acidic media, would appear to indicate that higher levels of ethyleniminium ion are present in acid solutions of dibenamine than in near neutral solutions. This supports the previous conclusion made by Al Shabibi & Smith (1974). It is suggested that although there is less tendency for the ion to be formed at pH 4·0 than at pH 7·4, its apparently greater stability in the acidic media can be accounted for by its rapid depletion by the added nucleophiles which are present only in the buffered near neutral media.

The degree of ionization of the receptors, which could affect the rate of reaction of the dibenamine with the receptors at the two pH values involved, was ignored for the following reasons:

The ethyleniminium ion is positively charged and is known to alkylate neutral nucleophilic groups (^-SH, R_3N) and negatively charged nucleophiles ($RPO_3^=$, $R.CO_2^-$). The change in the pH of the media from 7·4 to 4·0 would decrease the ionization of the phosphate and carboxylate ions, increase the ionization of α-amino and imidazole functions and leave unchanged the fully ionized lysine as well as the thiol groups. Consequently the reactive functions known to react with ethyleniminium ion which could be part of the receptor would either be converted to an unreactive form or remain unchanged by the decrease in pH. Thus, the inhibition reaction rate at pH 4·0 might well have been slowed due to an alteration in the state of ionization of the receptor but in such an event the *true* differences in the ethyleniminium ion levels in the two media would be even greater than those observed.

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