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The Histamine Content of Rabbit Leucocytes and its Release During *In Vitro* Anaphylaxis

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Summary. A method has been devised for obtaining from rabbit blood suspensions of leucocytes rich in basophils but depleted of platelets. The preparation contains histamine equivalent to 0·2 μg/ml of blood or 1·3 pg/basophil. *In vitro* anaphylactic histamine release by suspensions obtained from actively sensitized rabbits has been demonstrated and some properties of this new system have been described.

INTRODUCTION

In human blood almost all the histamine is contained in the leucocyte fraction and about half of this is located in the basophil polymorphonuclear cells (Graham, Lowry, Wheelwright, Lenz and Parish, 1955). Anaphylactic histamine release *in vitro* from leucocytes of allergic subjects has frequently been demonstrated (Katz, 1940; Noah and Brand, 1955; Van Arsdel, Middleton, Sherman and Buchwald, 1958; Lichtenstein and Osler, 1964). Although the part played by basophils in these reactions is uncertain, the system has yielded useful information on the mechanism of anaphylactic histamine release from human blood (Middleton and Sherman, 1960; Middleton, Sherman, Fleming and Van Arsdel, 1960; Lichtenstein and Osler, 1964, 1966a, b).

Rabbit blood contrasts with that of man and other domestic and laboratory animals in that in this species the major part of the blood histamine is contained in the platelets (Humphrey and Jaques, 1954). Although the rabbit basophil makes a numerically high contribution to the total white cell count in blood (Casey, Rosahn, Chu'an-K'uei and Pearce, 1936) it does not provide the main reservoir of circulating histamine. In the present study we have presented evidence that, despite this unusual situation, rabbit basophils contain histamine and that *in vitro* anaphylactic release of histamine from these cells can be demonstrated.

METHODS

Separation of platelets from rabbit leucocytes

Young adult New Zealand albino female rabbits weighing 2–5 kg were used. Blood cells were kept chilled in melting ice throughout the procedure. Twenty-six millilitres blood was collected from the marginal vein of the ear into polythene tubes containing 1 mg ethylene diamine tetra-acetic acid (EDTA) and 30 μg heparin per millilitre of blood. After centrifugation at 220 *g* for 20 minutes the leucocyte-rich zones (total about 1·2 ml) interposed between the platelet-containing supernatant plasma and the packed erythrocytes were isolated using a tube slicer and transferred to a polythene tube. The volume was

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made up to 8 ml with calcium-free Tyrode solution and the tube was centrifuged for 5 minutes at 100 *g*. The platelet-containing supernatant was then removed by aspiration, the interior of the tube wiped to remove adhering platelets and the cell button resuspended in 4 ml calcium-free Tyrode solution. Final platelet separation was achieved on a density gradient prepared as follows. Fifty grams of powdered bovine serum albumin was dissolved in 50 ml isotonic sodium phosphate buffer, pH 7·6. Phosphate buffer was then added to adjust the specific gravity (at 20°) to 1·092. Aliquots of this solution were then diluted appropriately to the required densities for the three remaining fractions of the gradient (1·078, 1·064 and 1·052). One-millilitre aliquots of each fraction were then pipetted into a polythene tube in decreasing order of density. The tube was then allowed to stand for 1 hour to achieve a smooth gradient. Two-millilitre portions of the leucocyte suspension were introduced over the albumin in each of the two tubes and centrifugation carried out at 220 *g* for 30 minutes. After centrifugation the cells were found in three zones. At the bottom of the tube were aggregated platelets and erythrocytes. A clear cell-free zone separated these from the middle greyish-red layer consisting of leucocytes and erythrocytes. Above this was a milky zone containing platelets. The tube slicer was used to isolate the middle layer (about 2·5 ml) from each tube. This was then transferred to a polythene tube and diluted with calcium-free Tyrode solution to 10 ml. After centrifugation at 220 *g* for 20 minutes the supernatant (containing nearly all the remaining albumin) was discarded and the cells resuspended in the required volume of Tyrode solution, to give a basophil count of about 400 cells/μl.

Cell counts

Basophils were counted using toluidine blue stain (Moore and James, 1953) and a Fuchs–Rosenthal haemocytometer. Eosinophil counts were performed by the same method as for basophils, eosin stain being used instead of toluidine blue. Platelets were counted using cresyl violet stain and a Burker haemocytometer. In some experiments leucocytes were stained supravitally with neutral red in a Helber bacteria counting chamber. Granules of basophil leucocytes stain brick red, and eosinophil and neutrophil leucocyte granules stain light brown and yellow, respectively.

Histamine release

Aliquots of the leucocyte suspension (usually 0·5 ml) were pre-warmed to 37° in polythene tubes and then incubated with the required concentration of antigen in Tyrode solution, the final volume being 4 ml. All samples were duplicated. After 15 minutes incubation the reaction was terminated by plunging the tubes into an ice bath. The cells were spun down (220 *g* for 20 minutes at 4°) and the histamine released into the supernatant was assayed. Spontaneous release was determined in each experiment and subtracted from antigen release. The histamine remaining in the cells was released by resuspending the residue of each tube in Tyrode solution and boiling for 3 minutes. Release was expressed as a percentage of the total histamine present.

Assays were done on the isolated atropinized guinea-pig ileum with the automatic bioassay apparatus of Boura, Mongar and Schild (1954), and histamine was identified using the competitive antagonist mepyramine.

Viability of leucocytes

The leucocytes were considered to be viable and in good condition following differential and gradient differential centrifugation on the following grounds:

(1) Phase contrast microscope studies of leucocytes at 37° revealed normal amoeboid and cell movement (Greaves, 1968).

(2) Leucocyte suspensions in Tyrode solution incubated at 37° for 15 minutes or stored at 2° for 18 hours leaked less than 10 per cent of their total histamine content.

Histamine content of whole blood

The histamine content of whole rabbit blood was determined by the method of Barsoum and Gaddum (1935), with modifications by Code (1952) and Greaves (1968). The average histamine recovery for this method was 75 per cent.

Values obtained in blood from six rabbits ranged from 0·9 to 3·8 μg/ml (mean 2·3 μg). Although the variation between animals was great, a second estimation 14 days later in two animals gave results which differed by 20 per cent or less from the original estimate.

Sensitization

Ovalbumin (Albumin egg powder, B.D.H.) for injection was prepared in two forms. For intraperitoneal injection a solution of 10 per cent ovalbumin was made using 0·5 per cent phenol in isotonic aqueous NaCl as a solvent. For subcutaneous injection, an aliquot of aqueous ovalbumin for intraperitoneal injection was emulsified with an equal volume of Freund's incomplete adjuvant (Difco Laboratories Ltd) giving a final ovalbumin concentration of 5 per cent. Rabbits were sensitized by a single injection of 1 ml 10 per cent ovalbumin intraperitoneally accompanied by 2 ml of 5 per cent ovalbumin with incomplete adjuvant subcutaneously.

RESULTS

HISTAMINE CONTENT OF PLATELETS AND BASOPHILS

The leucocyte suspensions obtained after differential and gradient differential centrifugation contained about 0·2 μg histamine/ml blood. The contribution by the residual platelet impurities was small (about 5 per cent) as can be seen from the results of the following experiments.

TABLE 1

HISTAMINE CONTENT OF PLATELETS IN FINAL LEUCOCYTE SUSPENSION

Total histamine (μg)	Platelets		
	Count $\times 10^6$	Estimated histamine content (ng)	Platelet histamine (% of total)
2·7	44	105	4·0
2·3	65	156	6·7
2·4	47	112	4·6

The histamine content of platelets was determined on an aliquot of the upper layer of the density gradient, which after centrifugation contained platelets but no cells. It amounted to 2·4 pg ± 0·3 (SE)/1000 platelets. This value was used to determine the contribution of the platelets to the histamine content of the final leucocyte suspension (Table 1).

The final leucocyte suspension is rich in basophils. The relative amounts of basophil

leucocytes, non-basophil leucocytes and erythrocytes in suspensions from two rabbits were studied in four experiments carried out on different occasions using neutral red supravital staining. Lymphocytes and erythrocytes which do not stain characteristically with neutral red were identified by their size and morphology. The results indicate that the final leucocyte suspension contains about twenty erythrocytes and seven non-basophil leucocytes for each basophil. The corresponding values for whole blood of the New Zealand albino rabbit are approximately 780 and 12, respectively (Casey *et al.*, 1936) Thus differential and gradient differential centrifugation as well as causing platelet depletion, also causes substantial concentration of basophils.

TABLE 2
BASOPHIL COUNT AND HISTAMINE CONTENT OF FINAL LEUCOCYTE SUSPENSIONS

	Experiment No.								
	1	2	3	4	5	6	7	8	9
Basophil count $\times 10^4$	47	34	13	39	7	64	4	8	23
Histamine content (ng/ml)	510	50	170	360	90	1160	50	380	110

TABLE 3
COMPARISON OF EOSINOPHIL COUNT AND HISTAMINE CONTENT OF FINAL LEUCOCYTE SUSPENSIONS

	Experiment No.					
	1	2	3	4	5	6
Eosinophil count $\times 10^4$	4·4	9·3	3·2	3·8	9·6	7·0
Histamine content (ng/ml)	190	150	500	390	120	290

There is a statistically significant correlation between the histamine content and basophil count in the leucocyte suspensions. The relationship was determined between the histamine content and basophil count in nine leucocyte suspensions in which there was a sixteen-fold variation in basophil counts. Allowance was made as described above for the histamine content of the platelets present. The mean value obtained was $1{\cdot}3 \pm 0{\cdot}3$ (SE) pg histamine/basophil. The individual values are given in Table 2 which shows the highly significant correlation between basophil count and histamine content ($r = 0{\cdot}86$, $0{\cdot}01 > P > 0{\cdot}001$).

Blood eosinophils contain significant amounts of histamine in some species (Graham *et al.*, 1955; Code and Mitchell, 1957) although studies on rabbit eosinophils have not been reported. The relationship between histamine content and eosinophil count in the final rabbit leucocyte suspension was therefore investigated in preparations from six different rabbits (Table 3). The eosinophil counts varied over a three-fold range but were not positively correlated with the histamine content ($r = -0{\cdot}81$; $P = 0{\cdot}1$).

Thus the results of this study support the view that rabbit basophils in the final leucocyte suspensions contain the majority of the non-platelet histamine.

SPONTANEOUS HISTAMINE RELEASE FROM LEUCOCYTE SUSPENSIONS

Table 4 shows that only 5 per cent of histamine content is lost in 15 minutes at 37°. At 2° release is about 100 times slower.

TABLE 4

SPONTANEOUS HISTAMINE RELEASE FROM RABBIT LEUCOCYTES

Duration of incubation	Temperature (°C)	No. of observations	Mean histamine release (%)
15 minutes	37	8	$4{\cdot}9 \pm 2{\cdot}0$ (S.E.)
18 hours	2	2	6·9 —

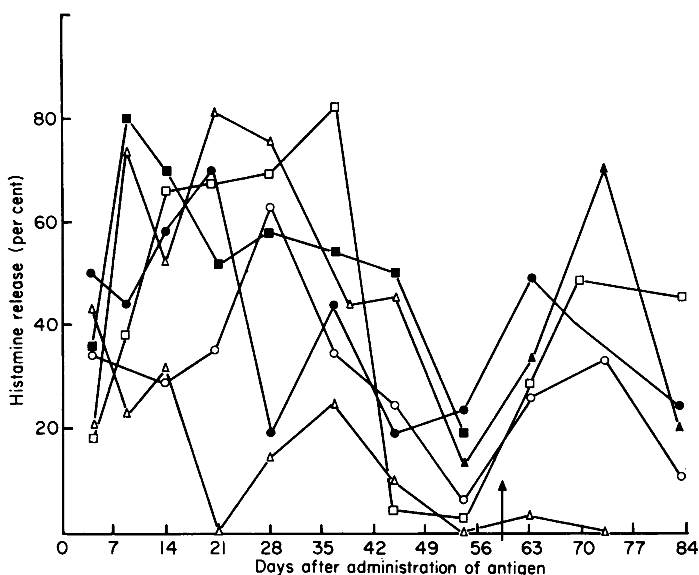

FIG. 1. Time course of sensitization of basophils (as measured by *in vitro* anaphylactic histamine release) following a single dose of antigen administered intraperitoneally together with a single dose of an emulsion of egg albumin with incomplete adjuvant given subcutaneously. Arrow represents 'booster' dose of antigen given intraperitoneally. Duration of incubation at 37° = 15 minutes. Antigen concentration = 100 μg/ml.

ANAPHYLACTIC HISTAMINE RELEASE FROM LEUCOCYTE SUSPENSIONS

When leucocytes from a rabbit previously sensitized by injection with ovalbumin were challenged by antigen *in vitro*, release of up to 80 per cent of their histamine content took place. The possibility that other smooth muscle contracting agents were being released in significant quantities was excluded by demonstrating abolition of all smooth muscle-contracting activity using mepyramine in a concentration which just abolished the effects of an equivalent histamine standard. Results from six rabbits are shown in Fig. 1. Each of the

rabbits was bled at 6–9-day intervals in order to follow the time course of sensitivity as determined by *in vitro* anaphylactic histamine release. Four days after injection of the rabbits appreciable sensitization was evident. This was maintained fairly constantly, but began to decline at 30–45 days, and at 54 days was absent or negligible.

Fig. 1 also shows the effect of a second 'booster' dose of antigen (1 ml 10 per cent aqueous ovalbumin) given intraperitoneally to five of the rabbits on the 58th day. Sensitization of leucocytes from each animal was studied at intervals of 6–14 days. Restoration of sensitization was seen in four of the rabbits and in one of these substantial anaphylactic histamine release from leucocytes could still be demonstrated 83 days after the first dose of antigen. In the remaining three sensitization had declined at this time.

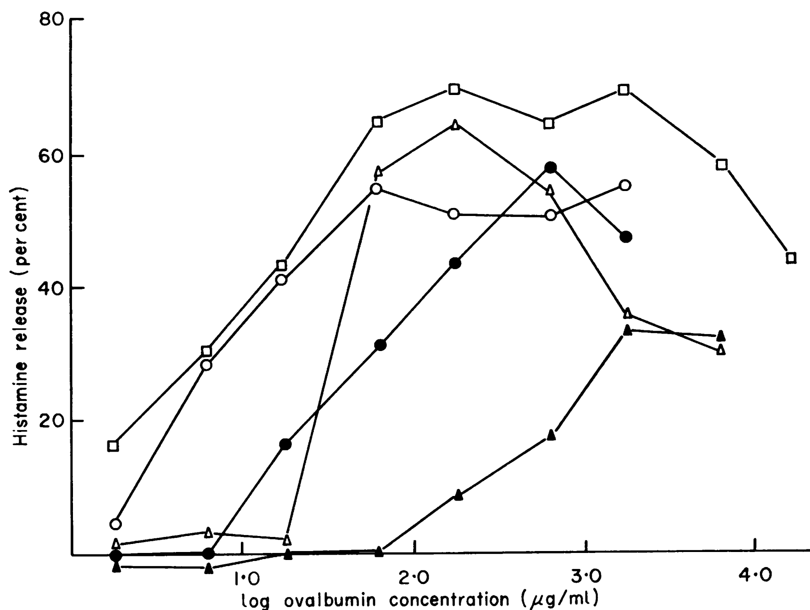

FIG. 2. Relationship between antigen concentration and histamine release in five rabbits. Duration of incubation at 37° = 15 minutes.

RELATIONSHIP BETWEEN CONCENTRATION OF ANTIGEN AND ANAPHYLACTIC HISTAMINE RELEASE FROM RABBIT LEUCOCYTES

Control experiments on the effect of ovalbumin on normal rabbit leucocytes were first carried out. Aliquots of a leucocyte suspension were incubated with a series of concentrations of five-times crystallized ovalbumin (Koch-Light) ranging from 0·125 μg to 12·5 mg/ml. Following incubation for 15 minutes at 37° even the highest concentration released only 1·8 per cent histamine after correction for spontaneous release. The lower concentrations released less than 1 per cent.

The effect of ovalbumin on leucocyte suspensions from five sensitized rabbits was then studied. Measurement of histamine release in response to concentrations of antigen ranging from 1·25 μg to 12·5 mg/ml enabled the dose–response curves to be drawn (Fig. 2).

Only in one animal did appreciable histamine release occur at 1·25 μg/ml. At 12·5 μg/ml

leucocytes from three rabbits released significant amounts of histamine. Maximum release was achieved at about 100 μg/ml in four rabbits. In the fifth maximum release was only

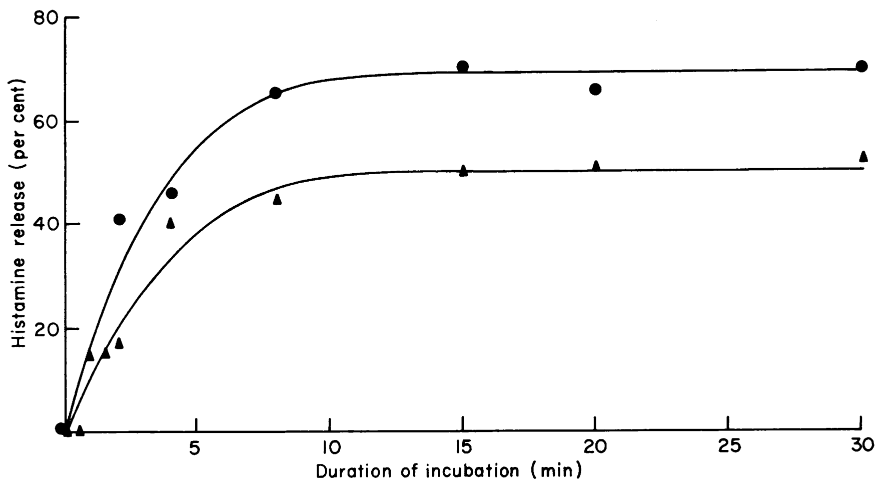

FIG. 3. Relationship between duration of incubation with antigen and histamine release. Two experiments are shown using leucocyte preparations from different rabbits. Reaction terminated by cooling. Antigen concentration = 625 μg/ml.

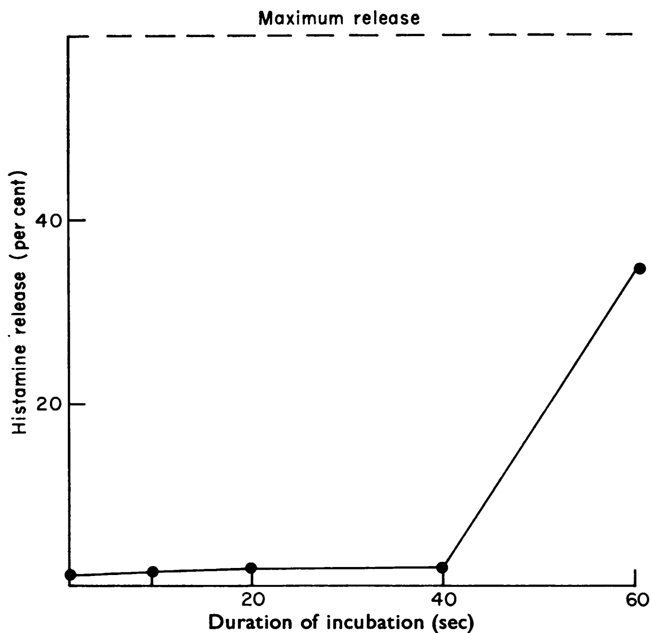

FIG. 4. Relationship between duration of incubation with antigen and histamine release in a single experiment. Reaction terminated by decalcification with EDTA. Antigen concentration = 625 μg/ml.

obtained if the concentration was raised to 1·25 mg/ml. In two of the rabbits concentrations of antigen above those required for maximum release led to inhibition of release. It of is interest that no single concentration of antigen gave maximum release in more than

one rabbit but a concentration of 625 μg/ml gave approximately maximum release in four out of the five rabbits and was, therefore, used in all subsequent studies.

RATE OF HISTAMINE RELEASE

Sensitized rabbit leucocytes were incubated with antigen, the duration of incubation being varied from 1 to 30 minutes. The relationship between duration of incubation and histamine release is shown in the two experiments of Fig. 3. Maximum release was achieved in about 10 minutes, significant release being evident at 1 minute.

In the above experiments the reaction was stopped after the required period of incubation by plunging the reaction tubes into an ice bath. For shorter periods of incubation an alternative method of stopping the reaction was adopted. The anaphylactic histamine release from rabbit leucocytes needs free calcium ions (Greaves and Mongar, 1968), so EDTA (1 mg/ml) was added after antigen had been in contact with the cells for 10–60 seconds to terminate the reaction abruptly. In Fig. 4 it can be seen that there is a delay of at least 40 seconds following challenge with antigen before significant release occurs.

DISCUSSION

The finding that rabbit leucocyte suspensions which are almost free of platelets but rich in basophils contain substantial quantities of histamine is of great interest since it provides a means of studying anaphylactic histamine release from isolated highly sensitized cells. It also refutes the widely held view that in the rabbit the blood histamine is contained almost entirely in the platelets (Zon, Ceder and Crigler, 1939; Code, 1952; Waalkes, Weissbach, Bozicevich and Udenfriend, 1957; Barvaro, 1961).

The average histamine content of blood of New Zealand Albino rabbits is 2·3 μg/ml, and the average histamine content of 1000 platelets is 2·4 pg in these animals. Assuming an average blood platelet count of 790,000/mm^3 (Casey *et al.*, 1936) and assuming that plasma and erythrocyte fractions contain negligible histamine (Code, 1952) then leucocytes contain approximately one-fifth of the total blood histamine.

The demonstration of a highly significant correlation between number of basophils and histamine content of the suspensions suggests that the majority of the histamine is present in the basophils but it does not exclude the participation of other cell types. However, the absence of a demonstrable positive relationship between eosinophil count and histamine content in the leucocyte suspensions indicates that eosinophils do not contribute an appreciable portion of the histamine.

If all the non-platelet histamine in the suspensions is contained in the basophils the results of the present study suggest that each rabbit basophil contains approximately 1·3 pg histamine. The corresponding value in man is 1·1 pg (Graham *et al.*, 1955) and in the guinea-pig 0·8 pg (Aures, Winquist and Hansson, 1965).

Addition of ovalbumin *in vitro* to leucocytes from a rabbit previously injected with ovalbumin resulted in liberation of histamine from these cells. In most experiments releases of 50–80 per cent could be achieved under suitable conditions. Onset of sensitization was rapid, 20–50 per cent histamine release being recorded within 1 week of sensitization. Seven out of eight rabbits developed satisfactory sensitization as judged by *in vitro* histamine release from their leucocytes. This was sustained for 5–6 weeks, thus enabling a single animal to be used repeatedly as a source of sensitized leucocytes. If necessary, availability

of sensitized leucocytes could be further prolonged for about 3 weeks by an additional dose of intraperitoneal ovalbumin.

Anaphylactic histamine release from rabbit leucocytes was independent of gross serum factors, since the leucocytes are thrice washed during differential and gradient differential centrifugation. The possibility that traces of an essential plasma factor, remaining closely adherent to the cell surface, may participate in anaphylactic histamine release from the leucocytes, cannot be excluded.

The nature of the anaphylactic antibody involved in rabbit leucocyte hypersensitivity awaits elucidation. Recently Zvaifler and Becker (1966) identified an antibody in rabbits appearing 6–7 days after antigenic stimulation which reached a peak at 9 days and disappeared at about 21 days. This antibody is thermolabile and sensitizes rabbit skin for passive cutaneous anaphylaxis, being demonstrable at the site of injection for 17 days.

Sensitized rabbit leucocytes show a low sensitivity to the antigen used, high concentrations of antigen being required to produce maximum histamine release. In five rabbits studied 10–1000 μg ovalbumin/ml were required. By contrast, using sensitized human leucocytes, Lichenstein and Osler (1964) were able to obtain maximum release with as little as 0·001 μg/ml antigen, although this was in a highly purified state. In the rabbit, a slight inhibition with higher concentration of antigen was seen in leucocytes from two out of the five animals. Lichenstein and Osler (1964) observed a similar phenomenon using human leucocytes and the effect has been observed in other types of *in vitro* anaphylactic systems (Brocklehurst, Humphrey and Perry, 1961; Liacopoulos, Halpern and Frick, 1963).

Anaphylactic histamine release from rabbit leucocytes in the presence of optimum concentrations of antigen is slow. In the present study 50 per cent of maximum histamine release took place 2–2½ minutes after challenge with antigen. Release from human leucocytes is also slow, 50 per cent being released in about 10 minutes (Lichenstein and Osler, 1964). Release from guinea-pig lung is much faster, 50 per cent of maximum release being achieved in ¾ minutes despite the added factor of diffusion within the tissue fragments (Austen and Brocklehurst, 1961). The corresponding value for isolated rat peritoneal mast cells is less than ½ minute (Perera and Mongar, 1963; Humphrey, Austen and Rapp, 1963).

A latent period of about ½ minute before any anaphylactic histamine release from rabbit leucocytes takes place is detectable. Its interpretation is uncertain and it can hardly be due to the time taken for antigen to reach the site of cell-bound antibody since the antigen–antibody reaction probably takes place on the cell surface (Humphrey and Mota, 1959). On the other hand if the release process is a multi-stage reaction similar to that described by Mongar and Schild (1962) the site of the delay could be a step following antigen–antibody union.

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