612.125:547.781

THE QUANTITATIVE ESTIMATION OF HISTAMINE IN THE BLOOD

By C. F. CODE[1]

From the Department of Physiology and Biochemistry, University College, London

(*Received* 3 *November* 1936)

SINCE the discovery of the powerful biological properties of histamine, various methods have been devised for its quantitative estimation. These have been of two general types, chemical and physiological. Chemical methods have not been successfully applied to blood. Methods involving as their final test one or more of the physiological responses to histamine have been used to determine the blood histamine equivalent.

In order to carry out an accurate biological assay of histamine, preliminary treatment of the blood has been necessary. Harris [1927] took blood direct from the vein of human subjects into absolute alcohol, allowed it to stand for some hours, filtered, dried the filtrate *in vacuo*, took up the residue in 0·9 p.c. saline and estimated it directly by the cat's blood pressure. Best & McHenry [1930] determined the histamine content of various tissues by preliminary treatment with hot HCl, removal of acid by drying *in vacuo* and distillation with alcohol, extraction of the residue with water, neutralization, filtration and final estimation of the filtrate on the blood pressure of the etherized cat. Dog's blood treated in this manner was found to contain 0·4 mg. histamine per kg. blood.

Barsoum & Gaddum [1935*a*] have developed a method for estimating the histamine content of blood, involving the preparation of an extract followed by its pharmacological assay. Preparation of the extract requires, briefly, the five following steps:

(1) Precipitation with trichloroacetic acid followed by filtration.

(2) Removal of trichloroacetic acid from the filtrate with ether.

(3) Hydrolysis of the filtrate with HCl with subsequent removal of excess acid by drying *in vacuo* and distillation with alcohol.

[1] Fellow of the Mayo Foundation.

(4) Extraction of the residue with hot alcohol saturated with NaCl, filtration and removal of the alcohol from the filtrate.

(5) The dried residue is taken up in water, neutralized to litmus and is ready for pharmacological assay.

The method as outlined above has been used in various types of physiological experiment [Barsoum & Gaddum, 1935*b*; Anrep & Barsoum, 1935; Anrep *et al.* 1936].

Recently we commenced the routine use of this method in order to follow the changes in the histamine content of dog's blood under certain conditions. All estimations were done in duplicate or triplicate. The process of extraction was time consuming, and inconsistencies occurred in the histamine equivalents of extracts from the same sample of blood, which we felt could not be attributed to variations in technique. Duplicate extracts did not always show discrepancies. In the estimation of twenty different samples of dog's blood from each of which two or three separate extracts were prepared, a variation between the duplicate or triplicate extracts of over 30 p.c. occurred in seven instances. Duplicates of the remaining thirteen samples had an average percentage difference of 6. In comparing known standard solutions of histamine we observed the extreme sensitivity of the lower ileum of the guinea-pig to minute variations in histamine. It was decided therefore to subject the method of extraction to analysis with the hope of shortening the time necessary to prepare the extract and, if possible, increasing the accuracy of the method.

Experimental procedure

The Barsoum & Gaddum [1935*a*] method was first applied to histamine added to water, in quantities comparable to that found in blood. The facts acquired from this analysis were then applied to whole blood and a modified procedure evolved. The histamine equivalents were estimated as a routine on the lower ileum of the guinea-pig. The results were at times checked on the blood pressure of the etherized cat. Finally it was necessary to demonstrate that the substance estimated in the extracts of the modified method was similar pharmacologically to pure histamine. In the routine use of the Barsoum-Gaddum method we followed two suggestions made by Prof. J. H. Gaddum. 10 c.c. of concentrated HCl were added prior to boiling in place of 10 c.c. *N* HCl. Also the alcoholic extraction was carried out at a temperature of 50–52° C., with absolute alcohol saturated with NaCl at that temperature. Apart from these two differences we applied the method as originally published. All estimations are expressed in terms of histamine base.

Estimation of histamine added to water

The occurrence of differences in the histamine equivalents of duplicate samples of blood suggested that a variable amount of histamine was being lost in the extraction process. To determine the loss of histamine in each step of the process, we carried out an analysis of the method with histamine added to water.

During these experiments it was found unnecessary to extract with ether for removal of trichloroacetic acid. Boiling with concentrated HCl destroyed most of the trichloroacetic acid, the remainder being removed by evaporation with hot alcohol. Inconsistent losses of histamine occurred in the final extraction with hot alcohol saturated with NaCl, while extraction with water resulted in complete recovery (Table I).

TABLE I. Recovery of histamine from water

Steps performed	Estimated histamine γ per c.c.	Histamine originally present γ per c.c.	Percentage recovery
Group 1. Trichloroacetic acid, ether, HCl, alcoholic extraction	0·055	0·05	110
	0·035	0·05	70
	0·347	0·5	69
	0·307	0·5	61
Group 2. Trichloroacetic acid, HCl, alcoholic extraction	0·040	0·05	80
	0·034	0·05	68
	0·363	0·5	73
	0·344	0·5	69
Group 3. HCl, alcoholic extraction	0·034	0·05	68
	0·034	0·05	68
	0·032	0·05	64
	0·032	0·05	64
	0·347	0·5	69
	0·363	0·5	72
Group 4. HCl, water extraction	0·050	0·05	100
	0·052	0·05	104
	0·049	0·05	99
	0·500	0·5	100
	0·500	0·5	100

The modified method of extraction

Upon the basis of the above experiments a modified method of extraction has been evolved. It consists of the following steps:

(1) 10 c.c. of blood are added to 15 c.c. of 10 p.c. trichloroacetic acid and after standing for $\frac{1}{2}$–1 hour filtered by suction.

(2) 10 c.c. of concentrated HCl are added to the filtrate and the solution boiled for 90 min. Excess acid is removed by repeated evaporation in the presence of alcohol.

(3) The dried residue is extracted three times with 2 c.c. of water, and the combined extracts are filtered. The filtrate is neutralized and made up to 10 c.c. with water. The extract is then assayed pharmacologically.

Step 1. Filtration by suction must be complete. Cloudy filtrates tend to give high values in the final estimation. The precipitate is washed four times with 5 c.c. trichloroacetic acid.

Step 2. The boiling with concentrated HCl is carried out with a reflux air condenser, water being added to prevent desiccation. Towards the end of the boiling period the volume is reduced to less than 5 c.c., 10 c.c. absolute alcohol are added and the residue dried *in vacuo* on a hot bath.

Step 3. 2 c.c. of water are thoroughly mixed with the dried residue and allowed to stand for a few minutes. Filtration is commenced and the extraction with water repeated until 6 c.c. have been used. The filtrate is neutralized with *N*/5 NaOH. Neutralization to litmus has in our experience been unsatisfactory. Excess alkali may be added without change in the reaction to litmus. We have found the guinea-pig intestine extremely sensitive to slight variations in *p*H. By the use of brom-thymol blue and thymol blue a satisfactory adjustment of *p*H is possible. Brom-thymol blue is used merely to indicate when the correct *p*H is being approximated. The colour given by one drop of thymol blue plus one drop of Tyrode's solution is accurately matched with similar quantities of the indicator and the extract. 1·0–1·5 c.c. of *N*/5 NaOH is generally necessary. To reduce the acid content of extracts from dog's whole blood to within this range usually requires drying three times with alcohol, while with rabbit's whole blood the process must be repeated four to five times. In the neutralization of extracts from whole blood a fine precipitate forms. Its presence does not interfere with the final estimation.

The potassium content of the extracts. It was anticipated that in extracts from whole blood the potassium content might be sufficiently high to interfere with the reaction of the guinea-pig ileum to histamine. We are indebted to Mr R. A. Gregory for carrying out potassium estimations on a series of extracts by the Kramer & Tisdall [1921] method. Extracts from whole blood contained on the average 21 mg. p.c. potassium which is approximately twice that present in Tyrode. Small volumes of the extracts are added to a relatively large quantity of Tyrode's solution. Addition of the largest dose of the extract used in testing to the volume of Tyrode's solution bathing the intestine would elevate the potassium concentration from 10·4 to 12·5 mg. p.c. Raising the potassium content to this and slightly higher levels had no effect upon the intestine, and did not interfere with its contraction in response to histamine.

Total molar concentration of the extracts. Estimates of the total molar concentration of the extracts have been made by the Baldes [1934] modification of the Hill thermoelectric method. We are grateful to Mr G. H. Benham for carrying out these determinations. Early in this research it was found that, in order to obtain a molar concentration similar to that of Tyrode's solution, it was necessary to dilute the extracts to 10 c.c. Adoption of this procedure gave molar concentrations approximately that of Tyrode (Table II).

TABLE II. The total molar concentration of extracts

	Sample	Method of extraction	Total molar concentration expressed as p.c. NaCl
1.	Whole blood	Modified	0·899
2.	,,	,,	0·950
3.	,,	,,	0·977
4.	,,	,,	1·009
5.	,,	,,	0·875
6.	,,	,,	0·860
7.	,,	,,	0·946
8.	,,	,,	0·884
9.	,,	Barsoum-Gaddum	0·883
10.	,,	,,	1·040
11.	Tyrode's solution	Nil	0·957

The pharmacological assay of the extracts

The lower portion of the ileum of the guinea-pig has been used for the routine estimation of histamine. Approximately 2 in. of the intestine are suspended in a bath containing 2·5–3 c.c. Tyrode's solution. Titration is carried out according to a definite time schedule. One-half minute is allowed for contraction, one-half minute for washing and relaxation and one-half minute for rest. The unknown solution is alternated with a constant dose of standard histamine solution (Fig. 1). The standard consists of Tyrode's solution containing 0·2γ or 0·1γ histamine per c.c. (Fig. 1). We have observed that such solutions lose approximately 30 p.c. of their activity on standing for 24 hours in a refrigerator. The 1 : 10 million standard has been adopted in the assay of blood containing very little histamine. The intestine is sufficiently sensitive to respond proportionally to small variations in the dose of this dilute standard (Fig. 2). Blood with a histamine equivalent of 0·02γ per c.c. may be accurately estimated.

Following a suggestion by Prof. J. H. Gaddum, atropine has been added to the Tyrode. Concentrations of 1·0–0·5γ per c.c. in the stock solution facilitate the titration by ironing out spontaneous movements and shortening the relaxation time of the gut. The presence of atropine

reduces the sensitivity of the intestine to histamine and may cause difficulty in the estimation of weak extracts.

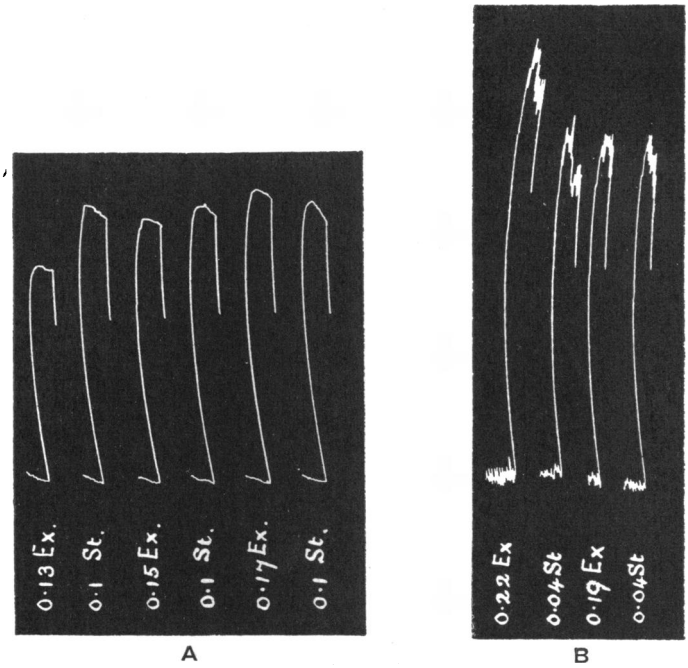

Fig. 1. Histamine equivalent estimation by the guinea-pig intestine. Doses of extract (*Ex*) and standard histamine solution (*St*) added to 3 c.c. perfusion fluid are indicated below the contractions.

A. Rabbit's blood. 0·16 c.c. extract equivalent to 0·1 c.c. 1 : 5 million standard.
B. Dog's blood. 0·19 c.c. extract equivalent to 0·04 c.c. 1 : 10 million standard.

Blank extracts prepared by passing water through the entire process had a negligible effect on the intestine. Histamine added to these extracts in known amounts gave correct titrations. Histamine added to extracts of blood was also quantitatively estimated. The process of extraction did not produce substances which interfere with the action of histamine.

The recovery of histamine added to blood

In estimating the recovery of histamine added to blood it was considered necessary to commence with blood containing little or no histamine. Advantage was taken of the observation that histamine is rapidly removed from blood by perfusion through the dog's kidney [Best & McHenry, 1930; Steggerda *et al.* 1935]. Forty-five minutes

perfusion of defibrinated dog's blood through the isolated kidneys by the heart-lung preparation reduced the histamine concentration to a level which we have called zero (Table III). Less than 0·01 γ per c.c. may have been present, but extracts prepared by both the Barsoum-Gaddum and the modified methods had little or no effect on the intestine.

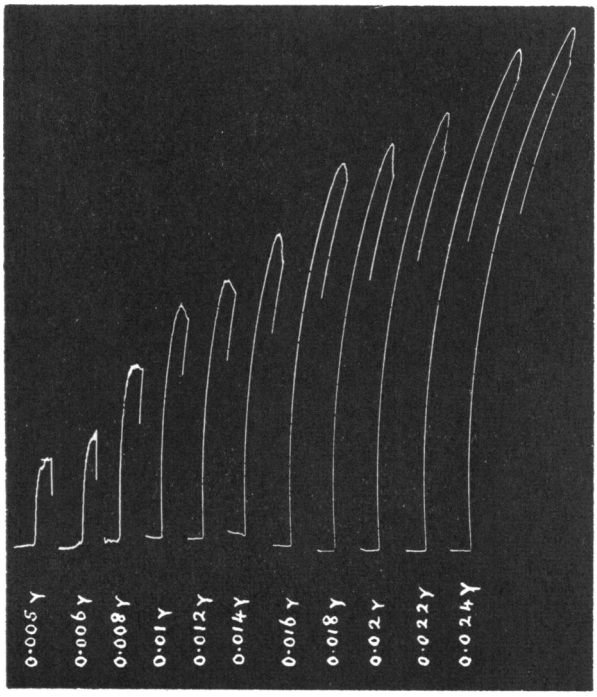

Fig. 2. Contraction of the guinea-pig intestine to increasing doses of 1 : 10 million standard histamine solution. The doses in γ histamine added to 2·5 c.c. perfusion fluid are indicated below the contractions.

Histamine was then added in varying concentrations, and extracts prepared by both methods. Careful estimation of these extracts showed that 90 p.c. or more of the added histamine had been recovered by the modified process, while recoveries with the Barsoum-Gaddum method were lower and less consistent (Table III). In addition to the experiments incorporated in Table III, twelve estimations have been carried out in which histamine was added to blood in quantities unknown to the investigator. The modified method yielded consistent recoveries of approximately 90 p.c.

TABLE III. The recovery of histamine added to blood

To reduce the initial concentration of histamine the blood was perfused through the isolated kidneys

Sample no.	Method of extraction	Estimated histamine γ per c.c.	Added histamine γ per c.c.	Percentage recovery
1, 2, 3, 4	Modified	0·000	0·000	—
5, 6, 7, 8	Barsoum-Gaddum	0·000	0·000	—
9	Modified	0·061	0·066	92
10	,,	0·062	0·066	94
11	,,	0·061	0·066	92
12	Barsoum-Gaddum	0·045	0·066	70
13	,,	0·043	0·066	65
14	,,	0·050	0·066	76
15	Modified	0·091	0·1	91
16	,,	0·094	0·1	94
17	,,	0·095	0·1	95
18	Barsoum-Gaddum	0·050	0·1	50
19	,,	0·050	0·1	50
20	,,	0·062	0·1	62
21	Modified	1·032	1·0	103
22	,,	1·000	1·0	100
23	,,	0·909	1·0	90
25	Barsoum-Gaddum	0·727	1·0	72
26	Modified	9·303	10·0	93
27	,,	9·526	10·0	95
28	Barsoum-Gaddum	6·545	10·0	65
29	,,	6·261	10·0	62

The histamine equivalent of blood to which no histamine has been added

The modified method has been applied to the routine estimation of dog's and rabbit's blood. Blood has been withdrawn under ether anæsthesia and immediately defibrinated. By using a proportional quantity of the various reagents, satisfactory extracts have frequently been prepared from 5 c.c. of blood. The average histamine equivalent of fourteen estimations on blood from five different dogs was 0·032γ histamine per c.c. blood, while that from seven different rabbits was 1·85γ per c.c. blood (Table IV). The agreement between duplicates was within 10 p.c. There was, however, considerable variation between the individual animals of each group.

Pharmacological evidence that the active principle of the extract is histamine

Modification of the method of extraction made it essential to test the extracts by methods other than the guinea-pig intestine. Rabbit's blood has been used throughout this series of experiments, because of its

comparatively high histamine equivalent. The small amount of histamine estimated to be present even in these extracts limits the number of tests which may be carried out.

The histamine equivalent of blood from five rabbits was estimated first by the blood pressure of the etherized cat, and then by the guinea-pig intestine (Table IV). In accord with the observation of Barsoum & Gaddum [1935*a*], atropinization of the cat considerably reduced the histamine equivalent of the extracts (Fig. 3). Following atropinization the values remained slightly higher than those derived from the guinea-pig intestine. With the exception of one experiment, the difference in the histamine equivalents of the two methods did not exceed 10 p.c.

TABLE IV. The histamine equivalent of rabbit's blood estimated on the guinea-pig intestine and the cat's blood pressure

		Histamine equivalent in γ per c.c. blood		
			Cat's blood pressure	
Exp.	Sample	Guinea-pig intestine	After atropine	Before atropine
18	1	1·60	1·72	2·66
	2	1·66	1·81	2·85
19	1	1·33	1·43	2·00
	2	1·33	1·43	2·00
20	1	0·59	0·62	—
	2	0·63	0·62	—
21	1	2·50	3·12	—
	2	2·66	3·33	—
22	1	2·50	2·66	—
	2	2·50	—	—
23	1	2·15	—	—
	2	2·00	—	—
24	1	2·42	—	—
	2	2·22	—	—
	Average	1·85		

Large doses of the extracts, producing a marked fall in the blood pressure of the cat, had no effect on the blood pressure of the etherized rabbit. The extracts caused a contraction of the uterus of the virgin guinea-pig, similar in character to that produced by histamine.

Large doses of histamine, allowed to act for a prolonged period, greatly reduce or abolish the sensitivity of the guinea-pig intestine to histamine [Feldberg & Schilf, 1930; Barsoum & Gaddum, 1935*a*]. 50–100 γ of histamine in contact with the intestine for 15–30 min. completely abolished its response to the standard histamine solution and the extracts, while that to barium chloride was only slightly diminished.

Doses of this magnitude could not be delivered by the extracts. Large quantities of the extracts allowed to act for ½–1 hour greatly reduced, although they did not completely abolish, the response to the extracts and to the standard histamine solution.

Following the technique of Koessler & Lewis [1927], we have tested the effect of the extracts on the bronchial musculature of the guinea-pig.

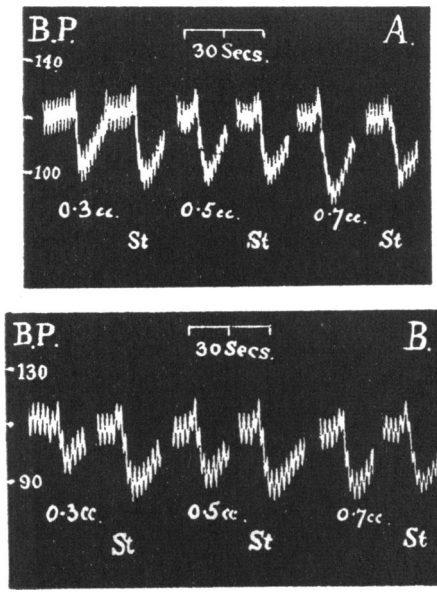

Fig. 3

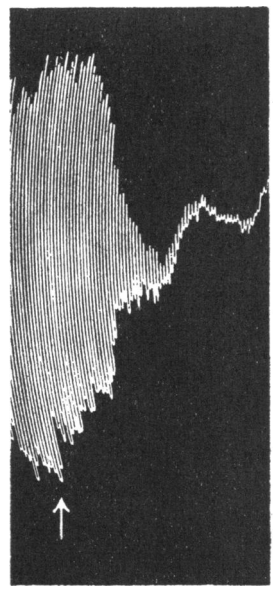

Fig. 4

Fig. 3. The histamine equivalent of an extract from rabbit's blood estimated by the cat's blood pressure, A before atropinization and B after atropinization. *St* is response to 1γ histamine standard. Doses of extract injected indicated in c.c. Before atropinization 0·5 c.c. extract equivalent to 1γ histamine, after atropinization 0·7 c.c. extract equivalent to 1γ histamine.

Fig. 4. Respiratory pulmonary excursions of the decerebrate guinea-pig, recorded by means of a perforated needle placed in the pleural cavity and connected to a bellows recorder. At arrow, 1 c.c. extract of rabbit's blood injected intravenously. Pulmonary excursions cease.

Intravenous administration of the extract produced responses exactly similar to pure histamine (Fig. 4). Large doses of the extracts caused death of the guinea-pig, and at autopsy the voluminous lungs typical of histamine overdose were found [Dale & Laidlaw, 1910].

Discussion

The chief modifications in the preparation of the extract have been the omission of the unnecessary removal of trichloroacetic acid with ether and the substitution of final extraction with water in place of alcohol. Our results indicated that repeated extraction with hot absolute alcohol saturated with NaCl did not remove all the histamine present. By extracting three times with moderate amounts of dilute alcohol, Best & McHenry [1931] report approximately 95 p.c. recovery of histamine. Hot 95 p.c. alcohol saturated with NaCl did not increase the recovery of histamine in our experiments. Hanke & Koessler [1920] used preliminary hot alcoholic extraction, thereby dividing their material into alcohol extract and alcohol insoluble residue. In applying their method to human blood serum, they added 5·0 mg. histamine to 75 c.c. serum, and recovered 3·5 mg. in the alcohol extract and 0·3 mg. in the alcohol insoluble residue. The remainder 1·2 mg. they believed had been absorbed by the charred material formed in acid hydrolysis and had not been removed by their final alkali-amyl-alcohol extraction. In the isolation of histamine from liver and lung [Best *et al.* 1927] and from spleen [Dale & Dudley, 1929], the most serious loss of histamine occurred in the final alkaline extraction with absolute alcohol. In our experiments, extraction with alcohol did not completely remove the histamine from the dried acid residue. Substitution of aqueous extraction gave higher and more consistent recoveries.

The histamine equivalents of blood from normal dogs and rabbits have in our experiments been consistently lower than the values reported by Barsoum & Gaddum [1935*a*]. The difference cannot be attributed to the adoption of the modified procedure. Using the Barsoum-Gaddum method we obtained an average value of 0·026 γ per c.c. blood from eleven estimations on the blood of five dogs. A histamine equivalent of 2·6 γ per c.c. is the highest value we have yet obtained from whole rabbits' blood.

The effect of active substances other than histamine, which may be present in the extracts, has as far as possible been eliminated. When estimated by the cat's blood pressure, atropinization definitely reduced the histamine equivalent values (Table IV). The effect of the extracts on the blood pressure of the unatropinized cat is, as Barsoum & Gaddum [1935*a*] have stated, probably partly due to choline. When determining the histamine equivalent by the guinea-pig intestine, we have added atropine to the stock Tyrode solution.

The pharmacological evidence indicates that the active principle estimated in the extract is histamine. The chemical proof that histamine is present in the blood is, however, still lacking.

Summary

1. The Barsoum-Gaddum method of extracting histamine from blood has been investigated.

2. A shorter method of extraction has been developed.

3. Application of this method to whole blood yields consistent histamine equivalent values.

4. Pharmacological evidence is submitted in support of the view that the active principle of the extracts is histamine.

I wish to express my sincere thanks to Prof. C. Lovatt Evans and Prof. J. H. Gaddum for their help and advice throughout this investigation. Part of the expense of this research was defrayed by a grant from the Donald C. Balfour Educational Fund.

REFERENCES

Anrep, G. V. & Barsoum, G. S. (1935). *J. Physiol.* **85**, 409.
Anrep, G. V., Barsoum, G. S. & Talaat, M. (1936). *Ibid.* **86**, 431.
Baldes, E. J. (1934). *J. sci. Instrum.* **11**, 223.
Barsoum, G. S. & Gaddum, J. H. (1935*a*). *J. Physiol.* **85**, 1.
Barsoum, G. S. & Gaddum, J. H. (1935*b*). *Ibid.* **85**, 13 *P*.
Best, C. H., Dale, H. H., Dudley, H. W. & Thorpe, W. V. (1927). *Ibid.* **62**, 397.
Best, C. H. & McHenry, E. W. (1930). *Ibid.* **70**, 349.
Best, C. H. & McHenry, E. W. (1931). *Physiol. Rev.* **11**, 371.
Dale, H. H. & Dudley, H. W. (1929). *J. Physiol.* **68**, 97.
Dale, H. H. & Laidlaw, P. P. (1910). *Ibid.* **41**, 318.
Feldberg, W. & Schilf, E. (1930). *Histamin.* Berlin: Julius Springer.
Hanke, M. T. & Koessler, K. K. (1920). *J. biol. Chem.* **43**, 543.
Harris, K. E. (1927). *Heart*, **14**, 161.
Koessler, K. K. & Lewis, J. H. (1927). *Arch. intern. Med.* **39**, 163.
Kramer, B. & Tisdall, F. F. (1921). *J. biol. Chem.* **46**, 339.
Steggerda, F. R., Essex, H. E. & Mann, F. C. (1935). *Amer. J. Physiol.* **112**, 70.