

Procedures for immunochemical study of histamine release from leukocytes with small volume of blood

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Procedures are described for in vitro immunochemical study of release of histamine from leukocytes of peripheral blood. Only 10 ml. blood is required to measure the effect of 10 concentrations of antigen or to carry out 10 tests with individual antigens or compounds. The procedures have been applied extensively for 3 years in a variety of studies, and the convenience, reliability, and usefulness have been demonstrated.

While we were engaged in development of procedures for in vitro measurement of antigenic release of histamine from leukocytes, a method was published which yields 25 determinations from 100 ml. blood.¹ As one of our goals was to conduct studies with children, we continued with the development of procedures which would utilize a smaller amount of blood and might be more convenient than existing micromethods.² By combining features of published methods^{1, 8, 9} with technical devices, we devised procedures which employ 10 ml. of blood to yield 10 measurements of histamine release from leukocytes. Extensive application of these procedures by us^{3, 4} and others has demonstrated the convenience, reliability, and usefulness. A full description is now presented as an aid to studies of histamine release in which a requirement for small amounts of blood is particularly advantageous, for example, in infants and children, in small animals, in frequent serial determinations in adults, in clinical management, etc.

MATERIALS AND METHODS

Reagents

Reagents from companies indicated have been satisfactory as to freedom from interfering fluorescence, but other sources may be equal or better: dextran, average molecular weight

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Received for publication Aug. 13, 1969.

*Career Scientist, Health Research Council of the City of New York. Supported by Public Health Service Grant HD-01892.

280,000 (Pharmachem); sodium heparin, United States Pharmacopeia, 1,000 units per milliliter; Trisma 7.6, a tris buffer prepared to give pH 7.4 at 37° C. (Sigma); albumin, human (Behringwerke, distributed by Certified Blood Donor Service, Inc.); normal human serum, group AB, separated from blood of nonallergic donor and stored at -20° C. in approximately 4 ml. aliquots; NaOH, pellets (Fisher S-318); n-butanol (Fisher A-399); n-heptane (Fisher 2215); 1N HCl (Fisher So-A-48); o-phthalaldehyde (Mann); methanol (Fisher A-412); phosphoric acid, concentrated (85 per cent H_3PO_4) (Fisher A-242); ligroin, practical (Matheson, Coleman & Bell LX-260); histamine dihydrochloride (Mann); and Kimwipe paper tissues (Kimberly-Clark).

Equipment

The following equipment was used: 4 ml. and 15 ml. round-bottom polycarbonate centrifuge tubes (Autoclear Nos. 2804, 1648, International Equipment Co.); Pasteur pipettes, 5¾ in. disposable glass, siliconized before use by means of Siliclad (Clay-Adams); 5 ml. graduated, polystyrene, serologic pipettes (Falcon); Propipette, rubber bulb with valves to facilitate control in pipetting (major laboratory supply companies); extraction tubes, screw-top, 13 × 100 mm., glass culture tubes with Teflon liner tops (Pyrex No. 9826); automatic repetitive pipettor such as Repipet (Labindustries); 4 place mechanical mixer, Vortex Model K-500-4 (Scientific Industries); syringe microburet, by screw propulsion of calibrated syringes with removable tips can deliver 1 or 5 μl per division of a dial (Micro-metric Instrument Co.); 10 × 75 mm. glass culture tubes (Pyrex No. 9820); centrifuge with horizontal head (Model HN, International Equipment Co.).

Washing of glassware

A laboratory detergent, Sparkleen (Fisher), is used in careful cleaning of glassware. Do not use highly fluorescent household detergents. Use of nitric acid or chromic acid solutions is unnecessary.

Solutions*

The following solutions were used: 3 per cent dextran—3 per cent glucose—3 Gm. dextran in 100 ml. 0.9 per cent NaCl solution, to 25 ml. 3 per cent dextran add 750 mg. D-glucose; tris-buffered saline concentrate (10×)—3.75 Gm. Trisma 7.6, 6.95 Gm. NaCl, 370 mg. KCl, to 100 ml. with distilled water and diluted 10 ml. to 100 ml. for use; Ca^{++} concentrate (100×)—900 mg. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to 100 ml. with distilled water; Mg^{++} concentrate (100×)—2.35 Gm. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to 100 ml. with distilled water; human albumin concentrate (0.3 per cent)—33 mg. dried human albumin to 1 ml. 0.9 per cent NaCl solution, prepare an amount estimated to last only a week, then discard any remaining; tris-A buffer—10 ml. tris-buffered saline concentrate, make up to about 98 ml. with distilled water in volumetric flask, add 1 ml. human albumin concentrate (0.3 per cent), adjust to mark with water and mix; Tris-ACM buffer—10 ml. tris-buffered saline concentrate, 1 ml. Ca^{++} concentrate, 1 ml. Mg^{++} concentrate, add to about 98 ml. with distilled water in volumetric flask, add 1 ml. human albumin concentrate (0.3 per cent), adjust to mark with water and mix; 7.5 N NaOH—15.1 Gm. NaOH pellets to 50 ml. distilled water; 3N NaOH—20 ml. 7.5N NaOH to 50 ml. with distilled water; 0.75N NaOH—5 ml. 7.5N NaOH to 50 ml. with distilled water. 0.12N HCl—24 ml. 1N HCl to 200 ml. with distilled water; 1.25M H_3PO_4 —8.5 ml. concentrated phosphoric acid to 100 ml. with distilled water; 0.05 per cent o-phthalaldehyde (OPT solution)—5 mg. recrystallized OPT weighed into a tube and 10 ml. methanol added just before use, unused portion discarded. OPT is recrystallized from ligroin, 200 mg. per 15 ml., by warming to 80° C. on hotplate and allowing to crystallize at 5° C. for 2 hours. Decant mother liquor. Dry crystals on filter paper in

*Gross contamination of solutions by bacteria and fungi is avoided by use of sterilized water or buffer in preparation. Concentrates should be dated and used within 2 months or discarded, and dated dilutions within 1 week or discarded. Store all solutions at 5° C. Water is double distilled, last distillation from all-glass still.

Table I. Scheme for leukocyte sensitivity

Agents	Milliliters in tubes for				
	Total histamine	Control	Antigen*	Internal standards	Reagent blank
Normal AB serum†	0	0.1	0.1	0	0
Tris-ACM buffer	0.2	0.1	0	0	1.0
Antigen solution	0	0	0.1	0	0
Leukocyte suspension	1.0	1.0	1.0	0	0
Histamine std.‡: 20, 40, 60 µg/L.	0	0	0	1.0	0

*A series of 8 concentrations of antigen can be used, making 10 tubes in all containing leukocyte suspension (prepare 10.5 ml.). Materials used as antigens are dissolved in Tris-CM buffer and must not contain a preservative such as phenol which may affect release of histamine. The stock antigen solutions are sterilized by passage through a Swinney filter, and preparation of dilutions with sterile buffer and usage is by strict aseptic technique. Antigen solutions are stored at 5° C., prepared fresh methodically as the stability dictates, and are discarded if turbidity develops or contamination is suspected.

†Efficiency of extraction of histamine is sharply reduced in presence of concentrations of protein above 8 per cent. The concentration of protein derived from reagents, serum, or leukocytes as employed in schema of Tables I and II was found by experiment to permit better than 90 per cent over-all recovery of histamine through ensuing steps of the procedure (Fig. 1). Any imbalance introduced by departures from the recommended schema in Tables I and II may introduce serious differences in per cent recovery during extraction and lead to misleading results in subsequent calculation of percentages of total histamine released.

‡For preparations of standards, see Solutions.

air (subdued light) about 30 minutes. Store in air-tight container at 5° C. in darkness. Keep dry by allowing to warm to room temperature before weighing samples.

Histamine standards

Reference standard, 10 µg per milliliter. Histamine, 16.6 mg. dihydrochloride is dissolved in 100 ml. 0.01N HCl to give 100 µg histamine base per milliliter; 10 ml. of this diluted to 100 ml. with 0.01N HCl provides a stock standard of 10 µg per milliliter. Solution is stored at -20° C.

Stock standards, 200 µg per liter. For external standards, 5 ml. of stock standard (10 µg/ml.) diluted to 250 ml. with 0.12N HCl; working standards are prepared by diluting with 0.12N HCl. For internal standards, 5 ml. of stock standard (10 µg per milliliter) diluted to 250 ml. with 0.01N HCl; working standards are prepared by diluting with Tris-ACM buffer. Divide stock standards into aliquots of about 8 ml. and store at -20° C.

Procedures

Preparation of leukocyte suspension. Before venipuncture, aspirate 0.1 ml. heparin solution and 2 ml. 3 per cent dextran-3 per cent glucose solution into a 12 ml. plastic syringe. Draw in 10 ml. of blood and mix thoroughly. Strap syringe vertically, needle end up, and allow erythrocytes to separate at room temperature (25° C.).

When separation of plasma-leukocyte layer is sharp, usually in ½ to 1 hour, push upper plasma-leukocyte layer out of syringe through plastic tubing into a plastic 15 ml. round-bottom centrifuge tube. Centrifuge at 1,000 r.p.m. (170 g) for 8 minutes, with the use of horizontal rather than angle head to keep cells off sides of tubes. Decant the supernatant plasma.

Wash the cells twice by first adding 1 ml. ice-cold Tris-A buffer and, forcing the entire volume in and out of a siliconized Pasteur pipette until a smooth, clump-free suspension is achieved, and then add another 2 ml. ice-cold Tris-A buffer. Centrifuge at 1,000 r.p.m. (170 g) for 8 minutes after each wash.

Finally resuspend leukocytes in Tris-ACM buffer at room temperature (25° C.), first in 1 ml. with Pasteur pipette, and then add a volume of Tris-ACM sufficient to provide 0.5 ml.

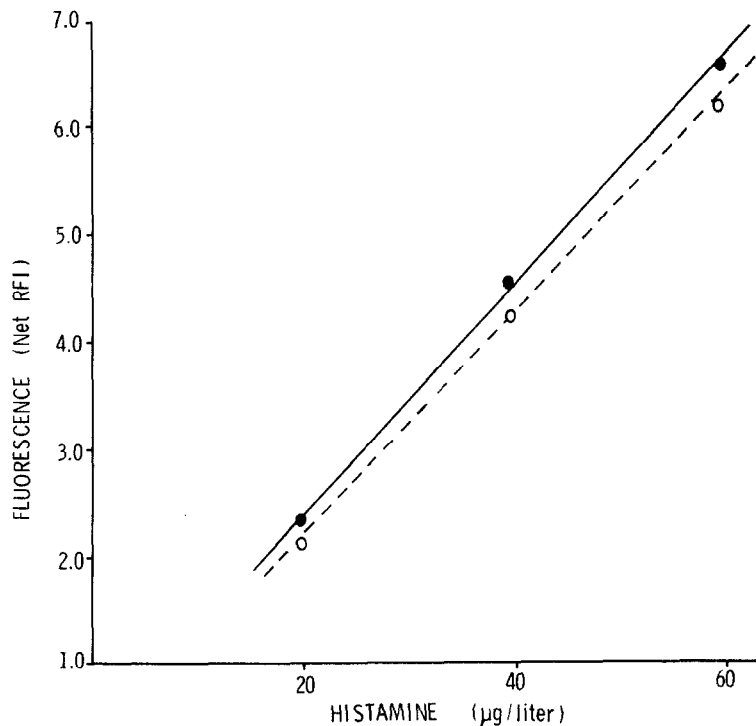


Fig. 1

Plot of internal and external histamine standards. Preparation and analysis of histamine standards is described in text. Recovery of histamine in internal standards is 95 per cent. Means of 10 comparisons during 6 week period are shown. (Actual values for means $\pm$ S.D. at 20, 40, and 60 μg per liter are: for external standards (●) 2.23 ± 0.28 , 4.47 ± 0.36 , and 6.43 ± 0.42 ; for internal standards (○), 2.15 ± 0.14 , 4.26 ± 0.10 , and 6.16 ± 0.28 .)

more leukocyte suspension than needed for the proposed experiment—but do not dilute the leukocytes derived from 10 ml. of blood to more than 11.5 ml. of suspension.*

Reaction of leukocytes with antigen. Mix leukocyte suspension gently and repeatedly to keep cells evenly distributed while quickly pipetting exactly 1 ml. aliquots from a 5 ml. graduated plastic serologic pipette into 4 ml. plastic tubes previously set up as shown in Table I for leukocyte sensitivity or in Table II for antigen neutralization capacity of patient's serum. Mix contents of all tubes gently by brief touch on slow speed of rotary mixer. Close tubes with tight-fitting stoppers and incubate in water bath at 37°C . for 1 hour, mixing gently at 20 minute intervals. As a further means of preventing significant evaporation, adjust the level in the bath even with level of fluid in the tubes.

Extraction of histamine. Immediately upon completion of the incubation, set tube containing leukocytes for total histamine aside and centrifuge cells from the remainder at 2,000 r.p.m. (600 g) for 10 minutes. Then proceed at once with extraction of histamine. In preparation, place in 13×100 mm. screw-top glass culture tubes approximately 300 mg. NaCl (spooned in through a funnel) and precisely 1.25 ml. of n-butanol (automatic pipettor). Into these tubes transfer 0.8 ml. of the suspension of leukocytes from the tube for total histamine

*Such leukocyte suspensions average 3×10^6 leukocytes per milliliter and contain sufficient histamine for reliable results. If a larger volume of leukocyte suspension is desired for a study, about 1 ml. of blood should be obtained for each milliliter of leukocyte suspension needed. The volumes of reagents used in collecting the blood and preparing the leukocyte suspension are adjusted proportionately.

Table II. Scheme for antigen neutralization capacity of patient's serum*

Agents	Milliliters in tubes for†				
	Total histamine	N.S. control	P.S. control	Antigen‡ + N.S.	Antigen‡ + P.S.
Normal AB serum (N.S.)	0	0.1	0	0.1	0
Patient's serum (P.S.)§	0	0	0.1	0	0.1
Tris-ACM buffer	0.2	0.1	0.1	0	0
Antigen solution	0	0	0	0.1	0.1
Leukocyte suspension	1.0	1.0	1.0	1.0	1.0

*Antigen neutralization capacity of serum refers to a property of serum regularly found even in untreated allergic patients, which is ascribed to the content of specific "blocking" or neutralizing antibody, considered to be of the IgG class.^{5,6} Sera of nonallergic persons apparently do not contain appreciable antibodies of this type, as the effect of normal serum is to enhance antigenic histamine release from leukocytes. Thus the incubation of antigen with normal and patient's sera reveals the increment by which the histamine-releasing activity of antigen was neutralized by prior incubation with antibodies in the patient's serum.⁵

†Internal standards and reagent blank are set up as in Table I.

‡From results of a previous determination of leukocyte sensitivity as in Table I or of antigen neutralization capacity of the patient's serum, 4 concentrations of antigen are chosen for each serum that are likely to release percentages of histamine in the most nearly linear portion of a log concentration-response curve (ideally 20 to 80 per cent) (Fig. 2). The two sets of 4 concentrations of antigen chosen are incubated with normal serum or patient's serum, in the amounts shown, at 37° C. for ½ hr. before addition of the leukocyte suspension. This is done while preparing the suspension of leukocytes.

§Patient's serum is obtained from 2½ ml. of clotted blood secured by exchanging syringes while needle remains in vein at time blood is being collected for separation of leukocytes; this yields more than the 0.5 ml. required.

and 0.8 ml. supernate from the remainder, taking care to avoid aspirating the sedimented cells (a device for controlled pipetting such as a Propipette is essential). In convenient batches of 4 to 8 tubes, quickly add 0.1 ml. 3N NaOH to one tube at a time, immediately touching each one briefly to a mixer for vigorous shaking. When the entire batch of tubes is ready, shake all together again on a mechanical mixer for exactly 3 minutes. These steps should be carried out expeditiously as the histamine will be vulnerable to the aqueous alkali until extracted by the butanol.*

After 3 minutes' shaking, the tubes are centrifuged at 2,500 r.p.m. (900 g) at room temperature, *not* in cold, for 3 minutes to break the emulsion and separate the layers. Each tube is then touched briefly to a mechanical mixer to loosen strands of precipitate and centrifuged an additional 8 minutes at 2,500 r.p.m. (900 g) to compress the precipitate into a thin pad. Then 1 ml. of the butanol layer can be readily recovered with the aid of a device for controlled pipetting (Propipette).

Transfer the 1 ml. of butanol layer to 13 × 100 mm. screw-top extraction tubes into which 0.6 ml. of 0.12N HCl and 1.9 ml. of n-heptane had been introduced with automatic pipettors and shake exactly 1 minute. Allow to stand in cold and dark until clear layers separate or centrifuge 5 min. at 2,000 r.p.m. (600 g) if histamine analysis is to be performed at once.†

*Uniform handling and shaking without delay is critical. In this one step proteins are precipitated and histamine extracted into butanol with the aid of the salt-alkali mixture. Almost indispensable is one or more mechanical mixers accommodating 4 or more tubes at once. A rapid repeating device for measuring out the NaOH is helpful (see Equipment section).

†It is preferable to carry out the procedures without interruption from collection of blood through extraction of the histamine into the 0.12N HCl. For convenience in scheduling work, the procedure may be interrupted after incubation when cells and supernate have been transferred into extraction tubes containing NaCl and butanol but before addition of NaOH. These can be kept in a freezer at -20° C. for at least 3 days without loss of histamine. Or the procedure can be stopped before going on to histamine analysis after extraction into 0.12N HCl by keeping tubes in dark at 5° C. up to at least 5 days.

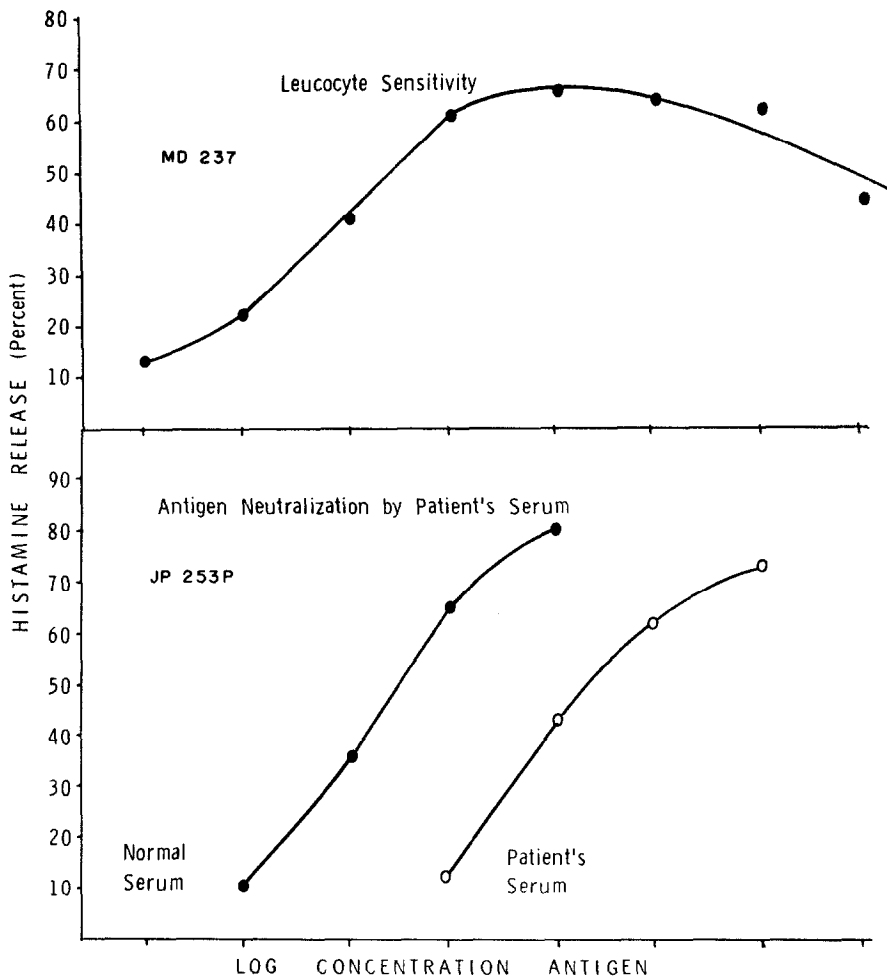


Fig. 2

Concentration-response curves. The upper curve illustrates how a range of 8 log-fold concentrations of antigen usually serves to determine point of maximal histamine release, as carried out in scheme of Table I. Note linear response occurs in range before maximal release, beyond which response is more erratic. The lower curves are typical of the findings when antigen is incubated with an allergic patient's serum and with a normal serum before reacting with sensitive leukocytes, in accord with the scheme in Table II. Four concentrations of antigen are selected to be incubated with each serum on the basis of preliminary measurement of leukocyte sensitivity of the patient (as above) or previous study in this manner.

Histamine analysis. Histamine extracted into the HCl layer is determined by the well-known fluorometric reaction with o-phthalaldehyde (OPT).⁷ All but the last 0.3 ml. of the heptane layer in the extraction tubes is removed by cautious suction to facilitate removal of an aliquot of the underlying HCl layer without contamination. A further precaution is to bubble air gently out of a 0.5 ml. serologic pipette with a Propipette while inserting it into the HCl layer to prevent entry of the heptane layer. Transfer 0.5 ml. HCl layer from each tube and 0.5 ml. of external histamine standards into 10 × 75 mm. glass culture tubes.

Table III. Results of typical experiment of antigenic release of histamine from leukocytes (No. 102B, Patient W. L.)

	T	C	Rgt. blk.	Ragweed extract ($\mu\text{g PN/ml.}$)						
				6×10^{-5}	2×10^{-4}	6×10^{-4}	2×10^{-3}	6×10^{-3}	2×10^{-2}	6×10^{-2}
MM	0.1	0.01	0.01	0.01	0.03	0.03	0.03	0.1	0.1	0.1
Rdg	39	75	60	84	42	53	75	34	37	38
RFI	3.90	0.75	0.60	0.84	1.26	1.59	2.25	3.40	3.70	3.80
- Rgt. blk.	0.60	0.60								
- Control				0.75	0.75	0.75	0.75	0.75	0.75	0.75
Net RFI	3.30	0.15	(C/T	0.09	0.51	0.84	1.50	2.65	2.95	3.05
Hist. release			= 5%)	3%	15%	26%	45%	80%	89%	92%

For protocol see Table I.

MM = setting of meter multiplier; Rdg = galvanometer reading; RFI = relative fluorescence index; Rgt. blk. = reagent blank.

Then cool tubes *thoroughly* in an ice water bath before adding in rapid succession 200 μl 0.75N NaOH and 60 μl of 0.05 per cent OPT solution with a syringe microburet, mixing *at once* by a touch on a mixer before returning tube to bath and proceeding to next tube. Allow tubes to remain in the ice-water bath another 40 minutes. Add 100 μl 1.25M H_3PO_4 as each tube is removed from the ice water, mix, and allow to stand at room temperature 20 minutes before measuring fluorescence.* Fluorescence may be measured in any suitable fluorometer with excitation at 360 $m\mu$ and emission at 450 $m\mu$. Herein an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Model 4-8202) was used with slit arrangement No. 4, a cell adapter for the 10 $\times$ 75 mm. tubes, and position of tubes adjusted by resting on a plug so that the light beam passes through the mid-portion of the 0.8 ml. contained in the tube. After wiping the tubes thoroughly with paper tissue (Kimwipes), readings of fluorescence are obtained quickly in a uniform fashion, as the fluorescence readings may drift considerably during even brief exposure to the light beam.

RESULTS

For calculation of results, the concentrations of each antigen or other agent tested for effects on histamine release in the schema of Tables I and II must be examined for contributory fluorescence in the procedure. This is done by adding 0.1 ml. of appropriate concentrations to 1.1 ml. Tris-ACM, carrying through the procedure, and subtracting fluorescence of the reagent blank to obtain a net value for any fluorescence from the antigen (antigen blank) or agent under consideration.†

*The manipulations in the OPT reaction should be carried out uniformly and consistently to keep timing regular in development of fluorescence. The OPT-methanol solution is prepared freshly just before use. A device for rapid accurate delivery of microliter amounts is mandatory, such as the syringe microburet described in the Equipment section. Other size tubes might be selected to carry out the OPT reaction according to specifications for cuvettes of a particular fluorometer.

†Many chemicals may either contribute fluorescence or quench the fluorescence derived from histamine in the procedure, because the extraction process may not exclude them. Quench may be detected by adding the compound to an internal standard. Compounds under study for effects on release of histamine from leukocytes must be examined for both contributory fluorescence and quench. If interference is proportionately too great, some preliminary measure to remove the agent without loss of histamine will have to be devised before applying the procedure described herein.

By reference to the internal histamine standards the actual histamine contents and percentage release can be calculated, but for percentage release the instrument readings may be more conveniently employed directly. In terms of instrument readings (RFI for Aminco-Bowman spectrophotofluorometer), calculations are performed as follows (see also sample experiment in Table III): Total tube minus reagent blank = net total histamine; antigen tube minus (corresponding control and antigen blank) = net release; net release/net total $\times 100$ = per cent release. Note that correction for any fluorescence derived from serum is covered by the control which includes serum. Plotting results on semilog, 4 cycle, graph paper allows proper comparisons to be made. Actual histamine content is calculated as follows:

$$\text{Micrograms per liter in internal standard} \times \frac{\text{net total}}{\text{net of internal standard}} = \text{micrograms per liter in leukocyte suspension.}$$

Fifteen successive duplicate determinations by an experienced technician agreed within ± 2 per cent (S.D. 1.5 per cent). When this degree of reproducibility is achieved, duplicates become unnecessary. Values for net fluorescence from controls normally range from 5 to 15 per cent of net fluorescence of the total histamine extracted from the leukocytes; for 15 experiments selected randomly the mean C/T was 6.2 per cent (S.D. 3.5 per cent).

For the internal and external standards, plots of concentrations of histamine versus values of fluorescence afford a straight-line relationship in the range of concentrations encountered in the extracts of most samples, namely 20 to 60 μg per liter (Fig. 1). Histidine added in concentrations 150-fold greater than the histamine content in leukocyte suspensions does not interfere.

COMMENTS

The procedure is based in part on McIntire's⁸ method for extraction of small amounts of histamine from biological materials. Other features are taken from Lichtenstein and Osler.¹ Economy of blood required was achieved principally through technical refinements and combining protein precipitation and histamine extraction into one operation. Efficiency of separation of leukocytes from blood and consequent increase in available histamine per unit of blood is facilitated by use of high-molecular weight dextran.⁹ This enhances accuracy by affording a wider separation between net values for fluorescence from total histamine and controls. Dextran was found by experiment to give no interference and did not cause release of histamine when used as described.

The procedures do not require exceptional skill to perform, but strict adherence to the detailed instructions, as well as meticulous care in preparation of reagents and in cleanliness of glassware and apparatus is essential. Two to three assays of leukocyte sensitivity (Table I) or antigen neutralization capacity of serum (Table II) can be completed by a technician in 6 to 8 hours.

The technical assistance of Emily Kostecki and Janet Strollo was most helpful in the course of development of the procedures described.

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