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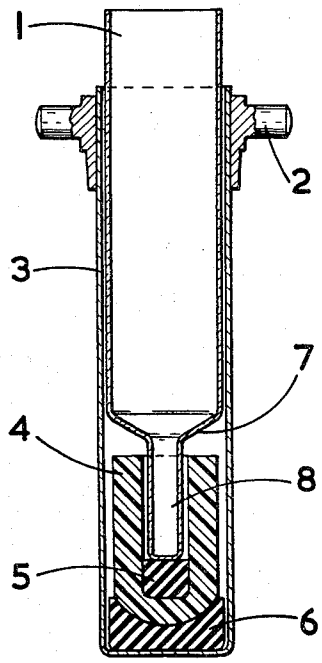

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CENTRIFUGATION OF WHOLE BLOOD TO SEPARATE EOSINOPHILS

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This invention relates to substances derived from blood.

It is known that mammalian blood contains a relatively small proportion of cells which are stained by eosin and are called eosinophil leucocytes or more shortly eosinophils. Attempts to obtain concentrations of these cells by centrifuging blood have hitherto been unsuccessful owing to the assumption, which has now been found to be erroneous, that the cells concentrate in the so-called buffy coat layer. The cells have been extracted from horse blood by using organic solvents such as acetone, but such procedure kills the cells.

It has now been discovered that eosinophils concentrate in that fraction of the blood which behaves as the heaviest fraction when the blood is subjected to centrifugation techniques. Thus, eosinophils concentrate in the erythrocyte or red cell layer of centrifuged mammalian blood or, in a sufficiently powerful centrifuge, in a yet lower layer.

According to the present invention, therefore, a process for the extraction of eosinophils from blood comprises subjecting eosinophil rich mammalian blood to centrifugation, collecting the fraction which behaves centrifugally as the heaviest fraction, i.e., heavier than the buffy coat fraction and recovering an eosinophil-containing preparation from said heaviest fraction.

For the purposes of this invention horse blood is the source of eosinophils which is most convenient and preferred at the present ime. For best results horses are selected which have an eosinophil blood count of at least 700 per cubic millimetre. Such horses are preferably isolated and stalled under proper conditions in order to preserve a high eosinophil count in their blood. They are bled from time to time and the blood taken from the selected horses is used for processing in accordance with the present invention.

For convenience further description of this invention will be given in terms of batch process extraction but it will be readily appreciated that an extension to continuous production for large scale working is possible.

Preferably the majority of the erythrocytes are separated from the blood before centrifuging, for example by allowing whole blood to stand in a separating funnel in the presence of an anticoagulant until the bulk of the erythrocytes have settled. The latter can then be run off. Frequently, however, some erythrocytes will still remain in the supernatant fluid and will be found in the heaviest fraction along with the eosinophils, unless the centrifuge is exceptionally powerful.

The procedure of this invention is preferably carried out at a temperature as cold as possible without freezing of the material being processed. It is not feasible to work below about 1° C. and the preferred operating temperature is about 2° C. The stability of the ultimate product is closely dependent on temperature and it is undesirable to exceed about 5° C. in any case. The initial centrifugation is preferably conducted under conditions of not less than 60,000 g.-min.

In order to obtain an improved resolution of the buffy coat layer and the erythrocyte layer, it is advantageous to carry out the centrifugation in a tube the lower part of which is of a smaller cross-section than the upper part.

The buffy coat and the erythrocytes then separate into two clearly distinct layers in the lower part of the tube. For example, if the upper part of the tube has a diameter of 30 mm. and a length of 120 mm., the lower part preferably has a diameter of about 7.5 mm. and a length of about 30 mm. the two layers in question then substantially fill the lower part of the tube between them.

The eosinophils may be separated from the erythrocytes in the erythrocyte layer obtained by centrifugation by lysing the erythrocytes and then centrifuging again, sufficient salt solution being added to the medium after lysis of the erythrocytes is complete to provide a stable medium for the eosinophils for example a medium equivalent to 0.6% saline. This centrifugation is comparatively light, e.g., the order of about 10,000 g.-min.

By this method according to the invention it is possible to obtain a suspension in an aqueous saline solution of eosinophils mixed with contaminating leucocytes, mainly neutrophils, the proportion of eosinophils being greater than 50%, usually at least 70% and in some cases as much as 98%.

Another aspect of the invention consists in an aqueous extract of eosinophils, which may be obtained by disruption of the cells, for example by means of an homogenising mill, preferably after freezing and thawing. This extract has an anti-histamine activity substantially greater than known anti-histamine agents. It is thought that the activity may be a general anti-inflammatory rather than a specific anti-histamine one.

The invention is illustrated by the following example:

Isolation of eosinophil leucocytes from whole blood

The apparatus employed is shown diagrammatically in the accompanying drawing. Into a metal centrifuge bucket 3 supported by a trunnion 2 is inserted a tube 1 which will contain the sample to be centrifuged. Towards the lower end the tube 1 converges at 7 into a bottom extension 8 of much narrower section than the upper portion of the tube. The extension 8 fits into a hard plastic insert 4 which prevents distortion of the bucket 3 by the localised pressure which would otherwise be applied to it. The extension 8 sits on a hard rubber cushion 5 fitting into the insert 4 and the whole is supported in the bucket 3 on another hard rubber cushion 6 disposed at the bottom of the bucket.

All glassware is carefully coated with silicone and the entire procedure is carried out at 2° C. except where otherwise stated.

750 ml. of blood are taken from the jugular vein of a horse having an eosinophil blood count of from 700 to 2,000 per cubic millimetre, into a 1,000 ml. separating funnel containing 45 ml. of 2.5% di-sodium ethylenediaminetetraacetic acid (Na_2 E.D.T.A.) adjusted to pH 7 with 10 N sodiumhydroxide. After mixing, the funnel is fixed in retort stand and the blood is left to sediment for one hour at room temperature (20° C.). The sedimented erythrocytes are run off and discarded. Eight of the centrifuge tubes 1 described above are filled with the leucocyte-rich plasma, each tube containing about 60 ml. These tubes are centrifuged at 2,000 r.p.m. (1,000 g.) for one hour in a swing-out head.

After centrifuging, the supernatant plasma and the whole of the buffy coat layer which extends part of the way down into the extension 8 is sucked off with a Pasteur pipette leaving intact the red cell layer. Platelets tend to adhere to the constricting part 8 of these special tubes and particular care is needed to ensure their removal. For this purpose, about 5 ml. of an ice-cold solution of 1% Na_2 E.D.T.A. in saline buffered with glyoxaline buffer to pH 7.0 is added gently to each tube. This is again sucked off with the pipette and discarded

without disturbing the red cell layer. Any remaining platelets and other leucocytes are removed by repeating this process if the tubes appear cloudy above the red cell layer.

Each tube will now contain in the extension 8 about 0.15 ml. of packed erythrocytes and eosinophils. About 5 ml. of ice-cold glass-distilled water is added to each tube. A Pasteur pipette is filled with this water, the tip is advanced to the bottom of the tube and the packed cells are blown out into the remainder of the water. It is very important to mix thoroughly the suspension at this stage by repeatedly sucking in and out of the pipette until no clumps of cells are visible to the naked eye.

The contents of all eight special tubes are then transferred to a single 100 ml. centrifuge tube. The volume at this stage should be about 40–45 ml. About an equal volume of ice-cold 1% E.D.T.A. in buffered saline is added to a total volume of about 80 ml. This is centrifuged in the same head at 2,000 r.p.m. for 10 minutes.

The supernatant, which contains haemoglobin, erythrocyte envelopes and a few platelets is discarded. The deposit contains about 70% eosinophils and 30% leucocytes mainly neutrophils.

Small volumes of human blood are processed by a modification of this method. 20 to 40 ml. of human blood preferably taken into Na_2 E.T.D.A. is placed immediately (without sedimentation on the bench) into a 50 ml. centrifuge tube. The tube is centrifuged at 2,000 r.p.m. for ½ hour and the whole of the supernatant plasma, together with the buffy coat layer and the uppermost 2 or 3 mm. of packed erythrocytes is transferred to one of the special leucocyte tubes already described. This mixture is diluted with ½ to 1 volume of ice-cold E.D.T.A.-saline, and centrifuged for 1 hour at 2,000 r.p.m. The plasma and buffy coat layer are pipetted off and discarded and thereafter the method is as already described from the addition of ce-cold water, except that it is preferable to use about 10 ml. of water for each 20 ml. of original whole human blood.

Preparation of extracts from isolated eosinophils

Isolated eosinophils are suspended in sterile, pyrogen-free water to a concentration of about 10,000 per c. mm. and carefully counted. They are then frozen at —20° C., thawed and homogenised for about 10 mins., in a Potter-Elvehjem type mill (see Journal of Biological Chemistry, vol. 114, at page 495). The homogenate is again frozen at —20° C., thawed and shaken for 5 mins. The homogenate is filtered by suction through a glass fibre filter paper and then through a membrane of 1μ porosity. The final filtrate is stored either frozen at —20° C. or is freeze-dried. It contains about 2 mg. dry matter per ml.

In a modification of this procedure ultrasonic disintegration can profitably replace grinding cells in a Potter mill thus expediting the production of extracts since the time-consuming sequence of repeated freezing and thawing is unnecessary.

The instrument we used was an M.S.E.-Mullard 60 w. disintegrator working at a frequency of about 20 kcs. In an experiment the eosinophil concentration was fixed at about 50×10^6/ml. This suspension was blanketed under argon and the actual disruption carried out in an ice-cold bath. Initial runs in saline showed that after exposure to ultrasonic energy of a calculated input to the probe head totalling 2,000 joules no intact cells could be observed in the mixture and the concentration of free granules reached a maximum yield of about 7%. After 25,000 J. the cell debris resembled that from the Potter Mill in microscopical appearance and this energy was employed in subsequent experiments when extracts were prepared.

To prepare an extract, the cells were suspended in water, as opposed to saline, for ultrasonic treatment. After

subjection to a total flux of 25,000 J. the cell debris was filtered, as in the orignal method, first through a glass fibre paper followed by a Courtauld's membrane (porosity ca. 1μ) and finally freeze dried. The prepared material possessed a biological activity indistinguishable from that of an identical control batch of cells processed by the original method. The sole disadvantage of ultrasonic disintegration is that the debris tends to clog the glass fibre filter. If cystein (concn.: 1 mg./ml.) as recommended by M.S.E., is added before irradiation, the potency of the extract is not improved and the membrane filter clogs.

The process described herein may be applied to any mammalian blood containing eosinophils, and the blood of animals that are slaughtered for food purposes (cows, pigs, sheep) may provide a source of raw material. Large scale extension of the work described above will probably involve continuous centrifugation apparatus, e.g., the falling film centrifuge, and with any given apparatus it will be a relatively straightforward matter in the light of the foregoing disclosure to arrange for collection of the fraction containing the bulk of the eosinophils.

I claim:

1. Process for producing an aqueous preparation of eosinophils which comprises removing from eosinophil-rich mammalian blood the bulk of the erythrocytes therein, centrifuging the residual blood, discarding the lighter blood fractions up to and including the buffy coat layer, subjecting the heavier remaining fraction to treatment in an aqueous medium to cause selective lysis of erythrocytes present in said heavier fraction, centrifuging the resultant material to remove cell debris arising from said selective lysis, and recovering the aqueous preparation of eosinophils so obtained.

2. Process according to claim 1, in which the operations are conducted at 2° C.

3. A freeze-dried preparation containing a substance having anti-inflammatory activity and derived by disruption of eosinophils isolated according to claim 1.

4. Process according to claim 1, in which the mammalian blood is horse blood.

5. Process according to claim 4, in which the blood contains at least 700 eosinophils per cubic millimetre.

6. Process which comprises subjecting an aqueous eosinophil-containing preparation produced according to claim 1, to treatment adapted to disrupt the eosinophils, filtering the material from cell debris, and concentrating the filtrate.

7. Process according to claim 6, in which the filtrate is freeze-dried.

8. The process which comprises subjecting to centrifugation horse blood containing at least 700 eosinophils per cubic millimetre and from which the majority of the erythrocytes have been removed, said centrifugation being conducted at a temperature in the range of about 1 to about 5° C. and under conditions equivalent to not less than about 60,000 g.-min., collecting the heaviest fraction in which the eosinophils are concentrated, disrupting any erythrocytes present in said heaviest fraction by lysis at a temperature not higher than about 5° C. in an aqueous liquid medium in which eosinophils are stable followed by centrifugation of about 10,000 g.-min. to separate the eosinophils, disrupting the eosinophils in an aqueous medium selected from the group consisting of water and aqueous saline medium by disintegrative treatment at a temperature not above about 5° C., filtering the product from cell debris and freeze-drying the filtrate.

9. Process according to claim 8, in which the centrifugation operations are conducted at 2° C.

10. Freeze-dried material produced according to the process defined in claim 8.

11. Process according to claim 8 in which said disintegrative treatment of the eosinophils is a combined freezing, thawing and grinding treatment.

12. Process according to claim 8 in which said dis-

integrative treatment of the eosinophils is an ultrasonic disintegration treatment.

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