On the Concentration of Eosinophile Leucocytes

The eosinophile leucocytes are still very little known as far as their function is concerned. The great increase in their count in parasitic infestation and in allergic conditions has attracted great interest. The concentration and separation of these cells from other cellular elements is, however, a necessary condition for most studies on their cytochemistry and physiology. With this aim in view, some methods have been elaborated[1]. These imply rather rough treatments of the cells which must be considered to suffer considerable damage. It was therefore felt important to investigate the possibility of concentrating eosinophile cells with the aid of a counter-streaming centrifuge, constructed by the senior author[2]. This centrifuge, which will soon be described[3], is constructed with the aim of separating differently sized particles of the same density. Liquid holding the particles to be separated is forced through a conical centrifuge tube against the centrifugal force. Here the larger particles down to a certain limit are accumulated whereas the smaller ones are carried off to a second tube where they are collected.

Because of the eosinophils being extremely large and the sedimentation rate of the erythrocytes high in the horse, blood of this species was used. As is well known, leucocytes have a tendency to agglutinate. This was counteracted by the use of versene which also served as an anticoagulant.

Blood was drawn from the jugular vein, mixed with isotonic phosphate buffer (pH 7·4) containing versene as to give a final concentration of 0·1% versene. The erythrocytes settled down on standing, and the supernatant, containing the leucocytes, was drawn off, sedimented at 170 g for 3 min and washed several times with Ca^{++}-free Tyrode solution, containing 0·05% versene. At this stage, we speak of the material as the "original suspension". The separation in the counter-streaming centrifuge was carried on for 60 min with 300–450 R.P.M. and a streaming velocity of 0·28 ml/sec. The flowing medium in each experiment is given in the Table. The Tyrode solution was Ca^{++}-free and contained versene as above. The greatest diameter of the conical tube was 1·60 cm, and the distance between this and the theoretical point of the cone 18·0 cm, the tube being mounted so that this point was 24·0 cm from the centre of rotation[4]. In some experiments the cells were given two runs in the counter-streaming centrifuge.

After this treatment, the state of the material was examined under the microscope at 38°C. The cells appeared morphologically intact and showed normal amoeboid motility. The eosinophile count was determined with the aid of a Fuchs-Rosenthal chamber after staining according to RANDOLPH[5]. In the original suspension, this count varied between 2 and 8% of the total leucocytes.

Eosinophile counts (per cent eosinophils of total leucocytes) in the original suspension and in the conical tube after running in the counter-streaming centrifuge. In Experiment No. 3 and 4 the same material was given two runs.

Experiment No.	Medium	Original suspension	Fraction in conical tube
1	Isotonic Tyrode	6	21
2	Isotonic Tyrode + saccarose (specific density 1·114)	8	34
3 First run	Hypotonic Tyrode (corresponding to 0·85% NaCl)	8	27
3 Second run	Hypertonic Tyrode (corresponding to 0·95% NaCl)	27	50
4 First run	Hypotonic Tyrode (corresponding to 0·85% NaCl)	2	25
4 Second run	Hypertonic Tyrode (corresponding to 0·95% NaCl)	25	50

As seen from the Table, the separating-out of eosinophils is better the lower the count of the original suspension, the concentrating effect being 3–4 times in the lower, and 2 times in the higher concentration range. By using the counter-streaming centrifuge, samples with eosinophile counts of 20–50% were obtained. On account of individual variations among the horses, it appeared difficult to standardize the procedure.

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Zusammenfassung

Eine Methode zur Anreicherung lebender eosinophiler Leukocyten des Pferdes mit Hilfe einer Gegenstromzentrifuge wird ausgearbeitet. In den angereicherten Suspensionen wurde eine Konzentration von 20 bis 50% erreicht.

[1] R. VERCAUTEREN and G. PEETERS, Arch. Int. Pharm. *89*, 10 (1952). – M. BEHRENS und H. R. MARTI, Exper. *10*, 315 (1954). – K. M. LINDAHL, *6*, 569 (1954).

[2] P. E. LINDAHL, Nature *161*, 648 (1948).

[3] I.V.A.'s Tidskrift för Tekniskt-vetenskaplig Forskning (Stockholm).

[4] P. E. LINDAHL, Nature *161*, 648 (1948).

[5] T. G. RANDOLPH, Proc. Soc. Exper. Biol. et Med. *52*, 20 (1943).

The Effect of Protamine on the Action of ACTH

The problem of ACTH-heparin antagonism has been extensively dealt with in recent literature, since a number of authors attributed a physiological role to heparin in the inactivation of ACTH[1]. We have been unable to confirm this view since it was found that not even pharmacological doses of heparin had any influence on the adrenal ascorbic acid depleting effect of ACTH[2].

In view of the antagonism existing between heparin and protamine in the mechanism of blood clotting, we have examined protamine for possible effects thought to be specific for ACTH, starting from the assumption that although exogenous heparin cannot enhance the action of physiological heparin, it may be possible that, by neutralising heparin circulating under normal conditions, the effects of ACTH are increased.

[1] Z. Z. GODLOWSKY, Brit. Med. J. *1951*, Bd. 4711, 854. – L. WEISSBECKER and A. SCHRÖTER, Klin. Wschr. *31*, 288 (1953). – F. KOLLER and W. FRITSCHY, Helv. med. Acta *14*, 263 (1953).

[2] GY. FEKETE, Acta med. Acad. Sci. Hung. (in press).

Methods

(1) Adrenal ascorbic acid depletion was estimated by the method of GYERMEK[1]. Essentially this method consists of a comparison of the ascorbic acid contents of the right and left adrenals, following the intravenous administration of ACTH in the decapitated rat. Protamine sulphate was administered immediately prior to ACTH, also by the intravenous route.

(2) The thymus involution test was carried out by the method of BRUCE *et al.*[2] as modified by FEKETE and GYERMEK[3]. The test substances were given in a ratio of 1:3, on one occasion, using $Zn_3(PO_4)_2$ as a vehicle. At 48 h after treatment, the animals were sacrificed and examined for thymus involution, comparing the glands with those of the controls. Protamine sulphate and albumin, respectively, were added to the pecipitate formed and the subcutanous route of administration was used.

(3) Melanophore expanding activity was studied in the frog by GYERMEK's method[4]. The frog was given adrenalin (to induce decolorisation of the skin) ACTH and protamine sulphate simultaneously into the abdominal lymph sac.

Results

The results of the experiments are tabulated.

According to the data in Table I, 2,0 mg/100 g protamine inhibited almost completely the adrenal ascorbic acid depleting effect of 0,5 milliunits/100 g ACTH. When given alone, this dose of ACTH caused a depletion of 66·4 ± 5·8 mg%, while after protamine the depletion was only 14·1 ± 8·3 mg%, the difference being significant ($P < 0{\cdot}01$).

Table I

No.	No. of rats	ACTH dose	Protamine dose	Ascorbic acid depletion
1	18	0·5 μg/100 g	1·5 mg/100 g	14·1 ± 8·3 mg%*
2	30	0·5 μg/100 g	–	66·4 ± 5·8 mg%*

* S.E.

Table II shows that 0·6 Units of ACTH in Zinc phosphate caused a considerable involution of the thymus gland, and even a dose of 0·2 Units/animal produced a more marked atrophy than a larger dose of ACTH in case 1 mg/animal protamine sulphate was added to the precipitate. It might be claimed that the cause of this phenomenon lies in the fact that protamine sulphate exhausts a considerable proportion of the adsorptive capacity of the precipitate at the expense of ACTH. This, however, is not the case, since the addition of the same amount of albumin to the precipitate increases rather than decreases the effect of ACTH under this condition, presumably by virtue of a protective protein action.

On the other hand, protamine was found to be ineffective on the melanophore expanding activity in the frog, since ACTH combined with protamine sulphate had the same effect as ACTH alone.

Table II

No.	No. of rats	Treatment	Thym. involution	P
1	9	600 μg ACTH	53%	1–2 < 0·01
2	11	600 μg ACTH + 1 mg Protam.	15%	
3	9	200 μg ACTH	22%	3–4 < 0·02
4	11	200 μg ACTH + 0·3 mg Protamine	–	1–5 > 0·05
5	9	600 μg ACTH + 1 mg Alb.	58%	2–5 < 0·01

For the explanation of the phenomenon described, two hypotheses have arisen. First it was suggested that the two basic proteins would compete for the receptors in the adrenal cortex and protamine, being present in greater concentration, would gain ground at the expense of ACTH, while in the chromatophore effect, in which the adrenal cortex is not involved, this competition is absent. However, paper chromatographic studies[1], have revealed that protamine sulphate adsorbed ACTH and the presumably resulting complex possessed no more ACTH activity. The recent report by KREBS[2] and MADSEN[3] that phosphorylase is inhibited by protamine in a similar way, lends substantial support to the validity of this explanation. This explains also the negative effects in the chromatophore test which is thought to be further evidence indicating that ACTH and chromatophore effects are bound to the presence of two different pituitary factors, as it was emphasized in the recent convincing work by LERNER and SHIZUME[4].

The ACTH-protamine complex is being studied by paper electrophoresis and chromatography and investigations are in progress to elucidate the possible role played by basic proteins in the physiological inactivation of ACTH. The results of this work will be published elsewhere.

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Zusammenfassung

Bei Ratten verursachen 0,5 μg ACTH/100 g keine Verminderung des Ascorbinsäuregehaltes der Nebenniere, wenn gleichzeitig 20 mg/kg Protaminsulfat gegeben werden.

Ebenfalls bei Ratten vermag ACTH bei gleichzeitiger Verabreichung mit Protaminsulfat keine Thymusatrophie hervorzurufen. Die Beeinflussung der Chromatophoren-Aktivität durch dasselbe ACTH-Präparat bei *Rana esculenta* wird dagegen durch Protaminsulfat nicht geändert.

[1] L. GYERMEK, Exper. *8*, 438 (1952).

[2] H. M. BRUCE, A. S. PARKES and W. L. M. PERRY, Lancet Apr. *19*, 790 (1952).

[3] Gy. FEKETE and L. GYERMEK, Acta Pharmaceutica *25*, 74 (1955).

[4] L. GYERMEK, Orvosi Hetilap (Hung.) *4*, 122 (1954).

[1] GY. FEKETE and A. HEGYELI, unpublished results.

[2] E. G. KREBS, Biochim. et Biophys. Acta *15*, 508 (1954).

[3] N. B. MADSEN and C. F. CORI, Biochim. et Biophys. Acta *15*, 516 (1954).

[4] A. B. LERNER, K. SHIZUME, and I. BUNDING, J. Clin. Endocrin. *14*, 1463 (1954).

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