A STUDY ON EOSINOPHILS IN RELATION TO STRESS

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ABSTRACT

A review has been prepared of the present state of knowledge concerning the eosinophil cells of blood in relation to stress. Eosinophil test which is being widely used as an index of adrenal activation or stress has been discussed particularly with reference to its scope and limitation. As the counting of circulating eosinophils forms an integral part of this test, a critical evaluation of the various methods for determining the eosinophil count in blood has also been made.

Introduction

The object of this report is (*i*) to review the present state of knowledge concerning the eosinophil cells of blood with particular reference to the significance of eosinophil test in studies on physiological stress and (*ii*) to make an appraisal of the various methods used in the determination of eosinophil count.

Some Characteristics of Eosinophils

(*a*) *Morphological and Histological Properties*—In 1865 Max Schultze recognised eosinophil cells as a separate entity[1]. The cells are distinguished by certain morphological and histological properties[2], [3], [4]. For instance, their cytoplasm is filled by large, round or oval, pink staining granules. The cellular protein is basic and hence stains with such acidic dyes as eosin, phloxine, etc. They have a diameter of 8—12 microns and their life span is reported to be 8—12 days as compared with 2—4 days in the case of neutrophils[5]. They originate in the bone marrow and normally constitute about 1—4 percent of the total circulating leucocytes.

(*b*) *Factors Stimulating the Formation and Liberation of the Cells*—The stimulus to formation and release of the cells from bone marrow seems to be under the influence of four possible factors, (*i*) chemotaxis, (*ii*) a leucocytosis promoting factor, (*iii*) the spleen and (*iv*) the endocrine system. Conditions favouring eosinophilia (an increase in the number of circulating eosinophils) relate to certain clinical states of the body such as infestation, infections, allergy and some other diseases and the eosinophil test has a clinical value in such cases.

(*c*) *Functional Properties*—In the case of human subjects it has been shown that these cells possess less phagocytosing power than the neutrophil leucocytes[6] The eosinophils carry histamine or histamine like substances from the marrow to the tissues for inactivation [7]. It would be interesting to see if these cells contain any histaminase. However, one may assume that they serve as a second line of defence in the system.

Mechanism of Response to Stress

In order to understand now the significance of blood eosinophil count when the body is in a state of internal or external stress, it is necessary to explain the mechanism of response to stress. When a stressor acts upon some part of the body, a stimulus travels through some unknown pathway from the injured ed area to the anterior pituitary which is induced thereby to discharge adrenocorticotrophic hormone (ACTH). To most stressors the first response may possibly be the secretion of adrenaline by the adrenal medulla. The adrenaline stimulates the pituitary to secrete more ACTH, which in turn acts on the adrenal cortex, causing an increased secretion of adrenocortical hormones. It is through these corticoids that different biochemical processes are set in motion to enable the animal to resist the action of a wide variety of stressors. A few salient points relating to systemic stress have been discussed eleswhere[8]. A comprehensive treatment of the subject has been given in several reports[9—12].

The importance of adrenaline in the regulation of the activity of the pituitary-adrenal axis has been the subject of many studies[13—17]. While it has been reported that adrenaline may play a primary role in the activation of the pituitary-adrenal axis, more recent data have raised doubts regarding the role of adrenaline[17]. It has been suggested that adrenaline and other stressors may increase the inactivation or utilisation of adrenal cortical hormones[15, 16]. As a result of this suggested process, a diminished level of circulating corticosteroids has been postulated. Consequent to this diminished circulating titer the secretion of ACTH by the pituitary is thought to occur. The data collected by Bachus[18] suggest that adrenaline in large doses fails to elevate the circulating levels of 17-hydroxy corticosteroids and fails to detectably alter the levels of these substances following the injection of cortisone into adrenalectomized rats, or of ACTH into intact rats. In vitro experiments fail to demonstrate any effect of adrenaline on the metabolism of cortisone.

With regard to eosinophils, the most spectacular effect is the fall in the number of these cells following the action of a stressor. In other words, the action of cortical hormones on eosinophils is an important point to study, if the eosinophil test is to be used as an index of stress.

In the light of several reports[19—23] it will be equally important to study also the factors other than the adrenal steroids which can cause eosinopenia. For example, adrenalectomized dogs maintained on desoxycorticosterone (DCA) were subjected to stress procedures of various kinds (*e.g.*, muscular exertion, electrical stimulation, injection of anti-adrenergic drug such as regitine, and of anticholinergic drug such as antrenyl) and all showed eosinopenia 2—7 hours following stress. In order to remove doubts regarding the absence of adrenal accessories, a few dogs which had already shown eosinopenia as a result of stress were permitted to develop adrenal insufficiency by withholding DCA.

Probable Clauses of Eosinopenia

Quantitative aspect of the action of corticoids on eosinophils will be discussed later. Here it may be stated that eosinopenia (fall in the number of circulating eosinophils) has been observed not only with one kind of stress factor, but with various types such as emotional stress[24, 25], surgical operations[26, 27] coronary occlusions[28], electroshock[29], heat and cold[30].

low oxygen tension[31], secretion of adrenaline[32], etc. Though the exact nature of the reaction leading to eosinopenia is not clearly known, the question has been raised whether eosinopenia may be due to decreased production, sequestration in blood vessels of large organs, movements into tissue beds, or increased destruction[33—46]. Some idea as to the probable causes may, however, be obtained from the following observations—

(*i*) At the time of maximum eosinopenia there was no decrease in the eosinophils in the bone marrow[33]. Further, an increase in immature eosinophils was noted[34], a finding which suggested an inhibition of maturation.

(*ii*) The evidence for sequestration in the spleen is conflicting. Although some workers[35] found that eosinophils accumulate in spleens of mice during the period of eosinopenia, the majority of investigators[36—38] were unable to demonstrate eosinophil sequestration in rat spleens. In fact eosinopenia has been reported to occur in splenectomised animals[39].

(*iii*) There has been a suggestion that eosinopenia may result in part from movement of eosinophils from the blood into the extravascular loose connective tissues[40]. Pycnosis and fragmentation of blood eosinophils have been observed by some[41—43].

(*iv*) Destruction and subsequent phagocytosis of eosinophils in peritoneal fluid of epinephrine-treated rats were found to take place[42]. Heparin appears to prevent the in vitro and in vivo eosinopenic effect of cortisone[41, 44].

Although the evidence thus far expressed tends to favour the concept that adreno-cortical hormones act on the eosinophils in a similar manner as on lymphocytes, that is by eosinolytic action[46], the necessity of further work in this direction is quite clear.

Now the question may be asked as to how the eosinophil count will help in assessing the degree of stress. One has therefore to turn to such studies which have aimed to find a quantitative relationship between the degree of stress and eosinophil fall.

Relationship between Hormone Dosage and the Fall in Eosinophil Count

In order to ascertain the relationship between eosinophil test and stress it is necessary to estimate the amounts of corticoids secreted in stress and the corresponding fall in eosinophil count. As quantitative estimation of the corticoids secreted is rather a problem, the stress is indirectly measured by determining the dose of ACTH or cortisone, which produces an equivalent change in the eosinophils. How far such a procedure gives a true picture is difficult to state. That exogenous supply of ACTH, cortisone, etc. at frequent intervals may affect the normal activities of the glands cannot be ruled out.

It has been reported that quantitative relationship exists between hormone dosage (ACTH, cortisone and compound F) and the degree of eosinopenia[11, 47—49]. Compound F and cortisone are apparently the most effective eosinopenic agents whereas compound A, sex steroids and DCA are relatively inactive[48].

The latter compound and compound A are effective in producing eosinopenia when massive doses are given to adrenalectomized animals[48, 50]. The observations of Thorn et al[51] are worth quoting here—

(*i*) Almost complete disappearance of circulating eosinophils may occur long before the maximum secretory response of the gland has been attained. Therefore, quantitative evaluation of the degree of adrenal cortical activation produced by either endogenous or exogenous ACTH is valid only within the range of 30—90 per cent eosinophil fall.

Whereas the eosinopenia observed with ACTH almost certainly reflects increased adrenal cortical secretion, adrenaline may induce eosinopenia in completely bilaterally adrenalectomized patients, who are unresponsive to ACTH. Interpretation of the eosinophil fall in severe stress may be difficult.

(*ii*) A change in the total 17-hydroxycorticoid excretion appears to be a more sensitive indicator of adrenal activation following ACTH administration than is the alteration in urinary 17-ketosteroid excretion.

(*iii*) The intravenous infusion of ACTH provides a more reproducible means of investigating lower levels of adrenal activation than does the intramuscular administration of a single dose of ACTH.

(*iv*) In evaluating the quantitative response of the adrenal cortex to intravenously administered ACTH, the duration of the infusion assumes an importance equal to or even greater than the total dose of material administered.

Using a 4-hour period of intravenous infusion of ACTH, titration curves were constructed for several normal subjects, employing dosages of ACTH ranging from 12·5 to 0·125 units per hour. With quantities as small as 0·25 uuit per hour over a 4-hour period an increase in 17-hydroxycorticoids and a fall in eosinophils was consistently observed. It was of interest to note that in no instance was a significant fall in circulating eosinophils observed without a significant rise in 17-hydroxycorticoid excretion.

The intravenous infusion of 1 unit of ACTH over an 8-hour period resulted in a much more intensive adrenal cortical stimulation than that produced by the same dose administered during four hours.

The intravenous infusion of 20 to 25 units of ACTH over a period of 8 hours or more is capable of inducing a near-maximal response of the adrenal cortex.

(*v*) Continuous intravenous infusion of relatively large quantities of compound F produced similar effects as observed with ACTH administration in short term experiment. A delay of 1 to 2 hours occurred in the appearance of increased 17-hydroxycorticoids in the urine with hydrocortisone, as well as with ACTH infused intravenously.

The interval between the activation of the pituitary-adrenal axis and the excretion of 17-hydroxycorticoids in urine might therefore be expected to be 1 to 2 hours.

(*vi*) Following the infusion of 1 mg. of adrenaline over a 4-hour period in normal subjects, there occurred a significant fall in the level of circulating eosinophils. With 0·3 mg. infused intravenously over a 4 hour period an insifignicant change in circulating eosinophils was observed, whereas when a total dose of 0·3 mg. was administered in a shorter time, *i.e.* 30 seconds, intravenously, a significant decrease in circulating eosinophils was noted. In none of these experiments was there any indication of increased adrenal cortical activation as reflected by a significant rise in the excretion of 17-hydroxycorticoids. Similar results were obtained by stimulating endogenous secretion of adrenaline. Simultaneous administration of small quantities of adrenaline and ACTH results in a greater eosinophil fall than that produced by ACTH alone, without significant increase in 17-hydroxycorticoid excretion. It appears therefore that the eosinopenic effect of adrenaline does not involve either an increase d secretion of ACTH, or an enhanced effect of ACTH upon the adrenal cortex.

The above findings are of great value in differentiating the effects of adrenaline from those of ACTH, cortisone and compound F and hence urinary analysis of 17-hydroxy corticoids is a sensitive index of the fall of eosinophil count due to adrenocortical response to stress.

It appears also that a comparison of the changes in 17-hydroxycorticoid excretion provides a means of assessing the extent to which activation of the adrenal cortex contributes to the total stress response of man.

As an exposure to a changed environment means a kind of stress, it would also be interesting to study the relationship between the process of acclimatization and the activation of the pituitary-adrenal system in human subjects.

With regard to acclimation in rats, the recent works of Heroux and Hart [52], [53] and of others [54], [55] may be mentioned here.

It has been shown that under adrenalectomized condition the amount of adrenal cortical hormone (ACH) required to maintain body weight is no greater in cold-acclimated than in warm-acclimated animals. In other words, the suggestion has been made that the initial rise in ACH secretion during cold acclimation may be followed by a reduced secretion in fully acclimated animals. Similar trend has been observed during adaptation to other kinds of chronic stress [54], [55].

Various indices have been used in assessing adrenal cortical activity during development of acclimation to cold and warm environments. For example, the thymus weight, adrenal ascorbic acid concentration and liver alkaline phosphatase activity were maintained at similar levels in some rats acclimated to warm and cold environments [56]. Application of absolute eosinophil counts and change in eosinophil level 4 hours after injection of ACTH (Thron index, 11) as further indices has led to the following results [53].

Absolute eosinophil level did not indicate an increased rate of ACH secretion during development of cold acclimation and did not parallel eosinophil trends found during adaptation to traumatic shock[54] and muscular work[55]. Eosinophils of rats exposed to 10°C and 6°C remained at the initial level throughout the entire period of cold exposure, though there was a fall in absolute eosinophil level during acclimation to 30°C. The latter fall has not, however been adequately explained by the authors.

Usefulness of this index is recognised under shock conditions when a sudden discharge of ACH rapidly decreases the eosinophil level. But in a chronic stress, such as prolonged cold exposure, production of new eosinophils might balance any increased disappearance caused by adrenal steroids.

ACTH, injected at different interval during acclimation to 6°C, produced an increasing fall in eosinophils up to the 20th day, followed by a gradually decreasing response up to the 53rd day. The observed trend in eosinophil response to ACTH indicates that the higher-adrenal activity, known to occur during the first few days of exposure to cold, gradually reaches a maximum in about 20 days and then returns to its pre-acclimation level. The Thorn index further shows that the adrenals of fully acclimated rats, although hypertrophied, are not hyperactive. While acclimation would reduce the ACTH requirement, it would not affect the postulated adrenal weight factor[57] secretion which would persist as long as the animal is exposed to a cold environment. The disappearance of hypertrophy when the cold acclimated animal is returned to a normal environment was supposed to be related to a decrease in this secretion[58].

It has been known that ACTH administration produces by an unknown mechanism an increase in eosinophil level in adrenalectomized animals[59—64] or inorganisms that have deficient adrenals[11]. In other words, in adrenalectomized or adrenal deficient animals, the eosinophilic elect would naturally predominate. The balance between the action of ACTH (*i.e.* ACTH secretion) and the activation of adrenal gland (*i.e.* cortical secretion) will determine the level of eosinophil. This subtle mechanism may play a great role in explaining the variations in eosinophil count under different conditions.

Clinical Value of Eosinophil Test

The eosinophil response is also used to assess the functional state of the anterior pituitary and the adrenal cortex in patients[65]. Some of the tests for these glands are carried out as follows :—

(*i*) Adrenaline test—A dose of 0·2 mg. of adrenaline in 200 cc of saline is infused intravenously in one hour. In normals the eosinophil count 4 hours later falls on an average by 60 per cent. In patients with severe hypopituitarism or Addison's disease no change occurs. With the above dose the total white cell count after 4 hours is increased owing to a rise in the neutrophils; there is an irregular rise in the lymphocytes followed by a fall.

(*ii*) ACTH test—A dose of 25 mg. of ACTH is injected intramuscularly. In normals the average fall in the eosinophils 4 hours later is 75

per cent. In Addison's disease there is no response. In hypopituitarism the response is smaller than normal owing to the atrophic condition of the adrenal cortex.

(*iii*) Corticoid test—A positive response with this test proves that unknown peripheral meachanism which lowers the eosinophil bount are not reactive. The adrenal amorphous fraction, cortisone and compound F may be used for this purpose, whereas desoxycorticosterone (DCA) is ineffective.

In Cushing's syndrome which is associated with hypercorticalism, the eosinophil count, as expected, is low. The value of the eosinopenic response as compared to the lymphopenic response as a clinical test for adrenal insufficiency has been expounded by Thron et al[11] and Roche et al[27]. Many investigators have cautioned that such a test must be better standardized and that more than one determination must be made on each subject to draw valid conclusion[66—69]. Roninger[69] found that in infants the lymphopenic response is more reliable following ACTH administration than is the eosinopenic response.

However, certain substances besides C_{11}—oxysteroids induce eosinopenia in the Addisonian patient for, according to Recant et al[63], large doses of adrenaline are effective in this respect.

Knowlton[70] has reported similar observations on the salt maintained patient, and other investigators state that both the cortisone maintained Addisonian and the bilaterally adrenalectomized human subject[19, 71] also showed a pronounced fall in eosinophils when injected with adrenaline.

Variation in Eosinophil count under Normal Condition

Apart from the induced eosinopenia discussed above and the eosinophilia occurring in certain clinical states of the body, there is variation in the eosinophil count of individuals even under normal condition. It has been noted, for instance, that there may be (*i*) individual variation, (*ii*) diurnal variation, (*iii*) daily variation, (*iv*) variation due to food ingestion or inanition and (*v*) variations due to other ill-defined factors.

It is necessary to consider these variations while asses ing the effect of an external or internal stress factor.

(*i*) *Individul variation*—This is noticeable even when observations are taken under strictly controlled conditions. If the necessary details of the subjects under investigation prior to observations are not available, it becomes very difficult some times to draw any conclusion. One may even discard the eosinophil test as an index of cortical response.

(*ii*) *Diurnal vriation*—This has been observed by a large number of workers[45, 72—81]. Rud[72] in a very elaborate statistical analysis of the behaviour of normal eosinophils showed that in may instances there were hourly variations of eosinophil levels with a drop in the forenoon and a rise in the afternoon and evening, and this variation was independent of food intake, but in a sufficient

number of cases fasting caused the number of eosinophils to drop. However, the eosinophil counts were reproducible within a normal range in the same subject from day to day and season to season, regardless of body position, sex, age and constitutional type.

Thorn et al[73] and Bonner[74] also noticed the difference in the counts between morning and evening. The latter author pointed out further that the daily pattern of eosinophil count might vary widely.

Donato and Strumia[75] noticed a diurnal cycle with a low point at noon and a higher one at night.

Halberg et al[76] made a study in the case of eleven individuals in a controlled environment and six with unlimited activity and diet. There was a regular diurnal rhythm in the eosinophil count of circulating venous blood with an average morning drop of 430 to 231 cells per c.mm. during 6·30 to 9·30 a.m. in the eleven controlled individuals. A rough inverse relation between oral temperature and eosinophil level was observed in the controlled subjects. In seven patients with Addison's disease, bilateral adrenalectomy and hypopituitarism failed to show normal diurnal eosinophil rhythm. It seemed to the authors that the adrenal cortical hormones might play a role in the fluctuations of the eosinophil level in normal subjects.

Observations in this laboratory in the case of limited number of subjects remaining in air conditioned room also pointed to a tendency of the eosinophil level to rise during the afternoon[77].

Halberg et al[79] reported that intact dogs of both sexes showed an early afternoon fall in numbers of circulating eosinophils. Dogs with bilateral arteriovenous anastomoses showed a rise in number of eosinophils during the morning. Superposition of adrenalectomy upon arteriovenous anastomoses had no observable effect upon this morning rise.

That physiological short term and diurnal variation occur in eosinophil levels has been considered by Best et al[45] while performing clinical eosinophil test.

According to Godlowski[80] sleep itself tends to speed up the recovery of normal levels of eosinophils after an eosinopenia produced in his case by insulin shock and the consequent release of adrenaline. The above author and also others[81] independently have suggested that parasympathetic stimulation, for example, pilocarpin stimulates an eosinophilia, while sympathetic stimulation produced an eosinopenia.

(*iii*) *State of Nutrition and Eosinophil Level*—Adequate information in the case of human subject is lacking. That fasting causes eosinopenia in many cases has been noted by Rud[72], Bonner[74] and others.

Butler and Morgan[82] reported that blood eosinophil levels of pyridoxine-deficient and paired-weighed young and adult male rats were similar and significantly lower than those of full-fed normal controls. This indicated that inanition alone was the cause

of the chronic eosinopenia. When healthy volunteers swallowed test meals of casein and tyrosine, the number of eosinophils circulating in their blood dropped. The effect was much quicker when the test meal consisted of gelatin. It has been suggested that proteins and amino acids in foods might produce changes similar to those caused by epinephrine and that proteins could be considered as stimulants of the sympathetic nervous system[82a].

The effect of prolonged fasting and realimentation on the capillary resistance and circulating eosinophils of dogs and rats has been studied by Kramar et al[83]. When dogs were subjected to a prolonged fast there was a progressive rise in capillary resistance and a fall in circulating eosinophils. Realimentation of fasted dogs with luxus consumption diets high in carbohydrate tended to maintain the fasting levels of capillary resistance and eosinophils. Realimentation of fasted dogs with luxus consumption diets high in protein, either initially or subsequent to a diet high in carbohydrate, caused a rapid return of capillary resistance to control level. The rise of the eosinophils was slower, often with marked initial overshooting. In the albino rat capillary resistance increased during the fasting period, dropped to pathologically low levels upon realimentation, remained there for various lengths of time, and finally returned to normal. The number of eosinophils changed inversely with the capillary resistance. These changes are similar to those found previously following various other forms of stress in the rat. In contrast to the dog the realimenting diet (high in protein or in carbohydrates) did not influence the described capillary response in the rat. Similar investigation in the case of human subjects remains to be carried out.

Application of Eosinophil Test in Aviation Medicine

Though eosinophil count has been widely used in recent years in viation medicine [84—88], it should not be construed that the test is free from any shortcoming. Gofton et al[84] reported that no consistent relationship could be shown between the drop in the eosinophil count and the length of flight nor were there consistently lower levels of eosinophils on flying days. The crew members who consistently showed the lowest eosinophil count seemed to be most subject to fatigue. If the test is valid within 30—90 per cent fall in eosinophil count, as pointed out by Thorn et al[51] a greater fall would be difficult to interpret and hence it would be futile to establish a correlation between the degree of stress above a certain limit and the eosinophil fall.

The eosinophil response to exercise in 21 R.C.A.F. ground crews is reported by Wake et al[86]. The subjects were given exercise in a gymnasium for 12 hours from 8 a.m. to 8 p.m. On another day they were given rest for a similar period. On each occasion eosinophil counts were obtained every two hours. Statistical evaluation of the data indicated that the counts other than the first and second were significantly lower on the day of exercise illustrating the fact that exercise of this order causes activation of the adrenal cortex.

Domenski and Nuttall[87] made use of the blood eosinophil count as a possible aid in the subjective recognition of excessive strain in flying personnel and in the evaluation of physiological toll associated with specific duties and assignments relative to both training and combat air operations.

However, the problem of differentiating the effect of mental activity from that of physical exertion remains to be studied more, although in flying personnel eosinopenia is believed to be associated with a mental in contrast to muscular activity. From the magnitude of diurnal variation and of variation caused by other causes, it appears that a decrease in count of more than 50 per cent may be regarded as eosinopenia for the purpose of studying stress. The above authors[87] suggest that the occurrence of a count of the order of 40 cells per cmm. of blood constitutes an eosinopenia. In this connection one may refer to the work of Thorn et al[51] for more detail.

Before the eosinophil test could be applied to yield reliable results in the case of flying personnel, preliminary experiments with a few subjects should show a definite relationship between the fall in eosinophil count and urinary 17-hydroxy-corticoids. This will enable one to know that the stress has caused activation of the adrenal cortex. If the drop in eosinophil count follows excessive secretion of adrenaline as might be encountered in emotion, the various biochemical and physiological changes associated with adrenaline have to be studied simultaneously. But a direct approach will be to determine adrenaline concentration in blood at frequent intervals. This is not, however, going to be convenient at the present moment. A few other problems of this nature have been indicated elsewhere [8].

Standardisation of Eosinophil Test

Standardisation of eosinophil test which is an essential step in its application must take into account all the salient facts stated above. If proper conditions are not met with regard to the control of subjects, planning of the experiment and the method of determining eosinophil count, the results will be simply erroneous. This also has been evident from the works of several authors[25, 51, 67, 84, 85, 87, 89]. It would be desirable, however, to do a good deal of preliminary work in this direction. Facts emerging from such studies may throw further light on the reliability and limitation of eosinophil test as a measure of stress or responsiveness of the adrenal cortex. In other words, one will be in a better position to understand whether or not the eosinophil count can constitute a general method for the above purpose.

In the course of an investigation on stress, it would be desirable to study the following points as well. (*i*) At what time of the day the test should be carried out, (*ii*) what is the time lag between the application or action of a stress factor and initiation of reaction leading to eosinopenia, (*iii*) how long the reaction persists after a stimulus is applied and hence how many samples are to be collected and finally (*iv*) whether the test should be conducted on fasting or non-fasting subjects.

The usual Thorn test[11] for examining adrenal response in patients is performed with fasting subjects at 8 a.m. and 12 noon or at 9 a.m. and 1. p.m. Working with both patients and normal subjects, Bonner[74] points out taht the test should be carried out under non-fasting condition from 1 p.m. to 5 p.m. Similar view has also been expressed by Swanson et al[89].

The next question relates to the determination of circulating eosinophil count. This has been discussed below.

Determination of Eosinophil Count

It has been quoted by Bonner[74] that by 1888 observers were studying differential counts and in the same year Mayet described his eosin-glycerine method. In the next few years methods requiring the use of a counting chamber were devised by many authors, but none was successful; these methods were superseded by Dunger's method in 1910[90], which met with competition from exponents of the smear technique.

In view of the relationship between adrenal function or stress and change in eosinophil count, increasing interest in the development of methods for eosinophil count has been displayed in recent years. An attempt has been made to evaluate the various methods as follows:

(A) *Comparison of Direct and Indirect Methods*—Determination of eosinophil count can be done in two ways, either by an indirect method or a direct method. The former is used commonly in clinical practice and has been described by several authors[91, 92]. For this, it is necessary to do white cell differential count on stained blood film and also to make white cell count independently. From a knowledge of the proportions of eosinophils in a definite number of white cells, eosinophil count per c.mm. of blood is calculated. The blood film procedure is reported to involve a great variation in cell counts and is impractical especially when the eosinophil counts are low[48, 72]. The entire procedure is quite time consuming and laborious. On the other hand, the direct method does not involve an additional step of determining the proportion of eosinophils in a definite number of total white cells and hence is preferred by many workers. Even if the proportion is wanted, the entire procedure will still be less time consuming than the differential count method.

Though the direct method is subject to many sources of error, several modifications of it for counting these cells have been developed[74, 93—98]. The advantages, disadvantages and problems encountered with the technique now in use have been discussed very recently by Bonner[74] and Speirs[98]. In spite of the various limitations, direct methods have gained much favour in most of the recent studies on eosinophil counts in connection with stress.

In an exhaustive work to evaluate the methods of counting eosinophils in the case of 550 subjects, Rud[72] concluded that the chamber method, using as a diluent 0·1 cc of 10 per cent magdola red, 6 cc. of acetone, 14 to 20 drops of 10 per cent sodium carbonate solution, and 45 cc of water was superior to the smear method and that the eosinophil counts were reproducible within a normal range in the same subject.

An evaluation of the methods described by Rud[72] and by Dunger[90] shows they are reliable only when 500 or more cells are counted[99].

Bonner [74] also has made a similar study quite recently. In the direct method he used propylene glycol as the base and a mixture of pholoxine and methylene blue as the staining material. The method will, however, be described later. In the indirect method, blood smear was on a glass slide as

usual and stained with Wright's solution. The two methods paralleled each other, provided that 4 chambers were counted and averaged or 800 cells were counted on the smear. The choice of method depends on the local circumstances. In a laboratory where many eosinophil counts are done by the same personnel, the chamber method is probably preferable because it is more rapid. A technician will develop the proficiency required for good results with this method. For those who do only occasional eosinophil counts, it is perhaps advisable to use the smear method, because most technicians are experienced in the preparation of blood smears and in doing differential white blood cell counts. The results obtained from freely flowing finger blood or from oxalated venous blood do not differ significantly, but either one or the other should be used consistently. .. ..

With regard to the relative accuracy it has been stated that provided proper precautions are taken, the direct counting procedure is more accurate[98]. However, it would be desirable to verify this point before one decides to apply the direct procedure.

It has been proposed to deal here with the direct counting procedure in a more elaborate way.

(B) *Direct Method*—In contrast to the examination of a stained blood film on a slide which is a part of the indirect method, the direct procedure employs pipetting whole blood into a solution called diluent and transferring subsequently a part of the diluted mixture to a counting chamber having a definite area and depth. The eosinophil cells get selectively stained and are counted under the microscope.

What seems to be most important in this connection is the choice of diluent. The various eosinophil diluents have been outlined and the action of each component discussed by Speirs[98]. Depending on their composition, they may be classified into different categories as follows:

(*a*) *Eosinophil Diluting Fluids*
Acetone Diluents—

(*i*) Dunger's original solution (90)

Aqueous eosin	0·1 gm.
Acetone	10·0 cc.
Distilled water	100·0 cc.

(*ii*) Modification A (Thorn, 11)

Aqueous eosin	0·1 gm.
Acetone	5·0 cc.
Distilled water	95·0 cc.

(*iii*) Modification B (Rud, 72)

Magdala Red .. .. (possibly phloxine)	0·02 gm.
Acetone	12·0 cc.
Sodium carbonate (10%)	1·2 cc.
Distilled water	90·0 cc.

(*iv*) Modification C (Speirs, 98)

Phloxine B	0·02 gm.
Acetone	15·0 cc.
Distilled water	85·0 cc.
Detergent (Alconox)	0·02 gm

Propylene Glycol Diluent

(*i*) *Modified Randolph Diluent* (93—97)

Phloxine	0·05 gm.
Propylene glycol	50·0 cc.
Distilled water	50·0 cc.

Urea Diluent (*Manners, 100*)

Urea	50·0 gm.
Trisodium citrate	0·06 gm.
Phloxine	0·1 gm.
Distilled water to	100·0 ml.

The composition of an eosinophil diluent is based upon the specific staining properties of the eosinophil granules and the relatively increased resistance to lysis of eosinophil cells over other white and red blood cells.

With the above diluents, 1 part of blood is mixed with 20 parts of diluent, usually in a white blood cell pipette. If proper precautions are taken other proportions such as 1 to 10 or 1 to 100 may be used.

The functions of the various components making up each class of diluent may be explained as follows:

Eosin or phloxine—Each is an acid dye and stains the eosinophil granules which contain basic proteins.

Water—It causes the blood cell to swell and aids in rupturing the blood cell membranes. Eosinophils are more resistant to lysis than other blood cells.

Acetone—It possesses inhibiting effect on the lytic action of water and the effect is proportional to the concentration used. A 15 per cent solution is optimal for normal human blood in a 1 : 20 dilution.

Detergent—The use of detergent helps in reducing the surface tension of water and ultimately hastens mixing of blood and diluent. The rate of staining of the eosinophils is also increased.

Alkali—Small amounts of alkali solution increase the lytic action of water especially on the red blood cells and neutrophils. It also increases the staining rate of eosinophil granules, possibly by affecting the permeability of cell memberanes.

Diethyl Glycol or Propylene Glycol—It increases the viscosity of the diluent and offers an advantage in situations where the acetone may evaporate rapidly. It is also isotonic and does not rupture cell membranes. It is particularly suitable when speed is not important or when citrated blood is sampled over prolonged periods.

Urea—This compound has also been used in place of acetone or propylene glycol. But the difference is that the staining is more delayed than when acetone or propylene glycol is used. In the presence of acetone staining is more rapid.

The criterion of a good eosinophil diluent is a solution which will produce the following:

(*i*) The eosinophil cells will stand out as distinct units with unruptured membranes and granules stained for easy identification.

(*ii*) The white blood cells except the eosinophils should be seen only as ghost cells or pale cells with wrinkled or broken cell membranes.

(*iii*) There should be no red blood cells or precipitated dye present.

Actual procedures followed in using some of the above diluents may be described as follows:

(*b*) *Randolph's Technique*[95]—This represents a typical example utilizing propylene glycol as a base and phloxine as the dye. Two stock solutions are prepared and placed in dropper bottles. Solution No. 1 consists of 0·1 per cent methylene blue in propylene glycol. Solution No. 2 is a 0·1 per cent solution of phloxine in propylene glycol. For use, each is diluted with an equal volume of distilled water. To obtain the final stain, an equal number of drops of the two diluted solutions are mixed. The stain mixture remains usable for approximately 4 hours.

The final mixture is used on freshly drawn oxalated blood in the same manner as the diluting fluid for leucocytes. About 15 minutes are required to obtain maximum staining of eosinophils. Counts are made in the standard counting chamber after allowing cells to settle for about 5 minutes.

The eosinophilic granules stain red and stand out in contrast to the green colour of the nuclear elements. The eosinophils in all 9 large squares are counted and the total is multiplied by 22·2 to obtain the eosinophil count per c.mm. of blood.

The method is relatively accurate but is said to have an inherent error of about 18 per cent. This technique has been adopted with minor changes by several workers and has constituted in principle a general method.

(*i*) *Procedure followed by Domanski et al* [101]—In this procedure, the eosinophil counts were made on peripheral blood (finger puncture) using the Randolph technique, except that the pipettes were allowed to stand for 20 minutes after filling, rotated for 10 seconds and shaken for 30 seconds prior to transfer to a Fuchs-Rosenthal chamber (0·2 mm. depth). It was furthermore found desirable to delay the counting procedure for 15 minutes after the chamber had been filled, in order to achieve an adequate fading of the red blood cells and a settling of the white blood cells.

Blood specimens (finger puncture) were obtained in triplicate for each count using certified or calibrated pipettes. Each of the counts reported was arithmetic mean of at least two separate counts, using different pipettes.

(*ii*) *Procedure followed by Bonner*[74]—Bonner whose work has already been mentioned also used a similar procedure but employed a different concentration of the dyes in the fluid base. Equal parts of 0·1 per cent phloxine and 0·1 per cent methylene blue in propylene glycol were mixed and used as the staining solution. Blood from a finger

puncture was drawn into a standard white blood cell count pipette in the usual manner, using the fluid as a diluent. The pipette was shaken by hand for 2 minutes and then 4 counting chambers were charged. The chambers were allowed to stand for 15 minutes, and the eosinophils seen in the 9 squares of each chamber were counted and averaged. The figure obtained was multiplied by the factor 22·2 to obtain the final eosinophil count. If the number in one chamber varied greatly from the results in the other three, the figure was not used in calculating the average.

The time factor appears to be important in connection with staining and counting the eosinophils. The differences in the procedures based on Randolph's technique are summarized as follows:

Author	Conc. of dye in diluent	Time required in various operations	Total time excluding the time of counting
Randolph (95) ..	Phloxine 0·025% .. Methylene blue 0·025%	15 min. in staining or after filling the pipette, and 5 min. allowed in the chamber before counting.	20 min.
Domanski (101) ..	As above	20 min. after filling the pipette, 10 sec., for rotation of the pipette, 30 sec. for shaking and 15 min. allowed in the chamber before counting.	35 min. 40 sec.
Bonner (74) ..	Phloxine 0·05% .. Methylene blue 0·05%	2 min. for shaking immediately after filling the pipette, and 15 min. allowed in the chamber before counting.	17 min.
Henneman (97) ..	Phloxine 0·025% .. (dilution of blood—1 : 10)	2 min. for shaking immediately after filling the pipette, and 15 min. for complete staining.	17 min.

(*iii*) *Procedure Followed by Pilot*[102]—A counting fluid that has been used incorporates 50 per cent aqueous propylene glycol solution which renders the red cells invisible and 0·1 per cent sodium carbonate which lyses all leucocytes except eosinophils. Phloxine at a level of 0·1 per cent is used as staining material. A modification of Pilot's method has been reported by MacFarlane and Cecil[103].

(*iv*) *Procedure followed by Donato and Strumia*[75]—These authors claim to work out an exact method for the chamber count of eosinophils in capillary blood. Oxalated blood is hemolysed with saponin and the eosinophils are stained with eosin Y. The eosinophils are then counted in a counting chamber.

(v) *Procedure followed by Ferrington and Jetter*[104]—These authors have described an improved staining solution for counting eosinophils in dogs. The solution employs eosin Y (0·25%) and phloxine B (0·25%) in an aqueous propylene glycol base to which are added 0·5 ml. each of concentrated formalin and phenol. It is claimed to be particularly adaptable for counting dog's eosinophils and may also be used satisfactorily in a 0·1 per cent dye concentration for eosinophil counts in man.

(c) *Discombe's Technique*[105]—This represents a technique employing acetone as the base and eosin Y as the dye. Blood is diluted 1 in 20, with the fluid (1% aqueous eosin Y and acetone 5 vols. each, distilled water to 100 vols.), which keeps some weeks, and after mixing allowed to stand 5 minutes. After this, the pipette is shaken vigorously for 2-3 minutes by hand; a Fuchs-Rosenthal chamber filled and when the cells have settled (1-2 min.), the preparation is examined with a 2/3 in. objective and x 10 eyepiece under the most brilliant illumination tolerable. Under these conditions unstained leucocytes cannot be seen at all, and the stained eosinophils appear as deep red particles and can easily be counted; in case of doubt one may examine individual cells with the 1/6 in. objective. It is stated that with this technique there can be no confusion between eosinophils and other cells. Clumping is unusual; if it develops, the preparation must be rejected.

Henneman et al[97] have made a comparative study of eosin-acetone and phloxine-propylene glycol diluents. The concentration of eosin employed was 2 per cent as against 1 per cent in the formula of Discombe[105]. Speirs[98] has made a suggestion as to how the results obtained by eosin-acetone diluent could be improved.

In spite of the fact that so many diluents have been worked out so far for direct counting of eosinophils, comparative results based on direct and indirect procedures are not frequently reported. More information along this line will be desirable.

The criterion of a good diluent has already been mentioned above. Not only should the staining be specific but the base must not also volatilise quickly within the time of observation. In addition, the diluent should preferably be simple in composition. Needless to mention that clumping of the cells in the diluent should always be avoided. The diluent should not be unusually unstable. The importance of time factor has already been pointed out. Optimum time for staining of eosinophils, settling and fading of red blood corpusles and the time to be allowed for actual counting and obtaining reproducible results need to be worked out.

(d) *Improved Chamber*—In most of the studies, Fusch-Rosenthal chamber with a depth of 0·2 mm. has been recommended. Recently an improved eosinophil counting slide has been described by Speirs[106]. Specification for a four chamber slide with ten 1 sq. mm. ruled area per chamber and 0·2 mm. chamber depth are given. It is claimed that this chamber helps in the counting quite promptly and accurately.

(*e*) *Sources of Error*—According to Kolmer et al[91], the following constitute some of the common sources of error in erythrocyte counts. This would give an idea as to how error can be minimized in counting eosinophils as well—

(*i*) Inaccurate dilution due to faulty pipette or technique.

(*ii*) Too slow manipulation, allowing some of the blood to coagulate.

(*iii*) Inaccuracy in the counting chamber and especially its depth due to an inaccurate cover glass of faulty manufacture.

(*iv*) Presence of yeasts or other artefacts in the diluting fluid.

(*v*) Delay in filling the counting chamber after shaking pipettes.

(*vi*) Improper filling of the counting chamber with too large or too small a drop.

(*vii*) Not allowing sufficient time for the cells to settle before counting.

Some of these errors can be avoided or minimised. A considerable source of error is inherent in the method itself.

Berkson[107] has made a satistical study of the error in blood cell count. The error of the blood count as made with the haemocytometer has been analysed into three components, the field error, the chamber error, and the pipette error. Total error of the count is given as follows:

For a count of 5,000,000 erythrocytes per c.mm. obtained from a single specimen sample counted in 80 small squares, the coefficient of variation is 7·8 per cent. Following the usual practice of taking twice the standard error as significant limits, such a count is, therefore, determined significantly within ± 16 per cent.

Applying the same statistical procedures it was found that for a leucocyte count of 7,000 per c.mm. obtained by counting 4 sq. mm. the coefficient of variation was 10·7 per cent, and count was determined significantly within ± 21 per cent.

It is apparent, therefore, that wide variations may occur in successive counts, even if the counting is done precisely and accurately. Consequently, the need for care in avoiding the introduction of additional factors, which would extend the range of probable error, cannot be over emphasized.

As in normal condition eosinophil count is about 1 to 4 per cent of the total leucocyte count, it would be desirable to find out the above type of error in the case of eosinophil count by the direct counting procedure.

Summary and conclusion

1. The present state of knowledge concerning circulating eosinophils in relation to stress has been reviewed. A number of fundamental problems regarding the scope and limitation of eosinophil test have been indicated.

2. The eosinophil test as an index of cortical activation in normal persons appears to be valid within 30 to 90 per cent fall. Interpretation of eosinophil fall in severe stress is difficult.

3. Though the eosinopenic effect of corticoids and adrenaline is well established, other probable causes of eosinopenia during stress should be explored.

4. It is also necessary to work out methods for differentiating the eosinopenic effect of several stress factors. Such investigation may help in the proper interpretation of eosinophil data in the case of flying personnel.

5. It will be desirable at the same time to correlate changes in eosinophil count with other bio-chemical changes occurring in the body under the influence of a particular stress agent.

6. A critical evaluation of the various methods for determining eosinophil count has been made. The nature of a few analytical problems requiring further investigation has been explained.

7. Studies on the application of eosinophil test as a measure of stress in the case of Indian subjects have so far been rare, and hence it would be worth undertaking some problems in this direction.

References

1. Max Schultze. Cited by Bonner, C.D., J. Am. Med. Assoc. 148: 634 (1952).

2. Whitby, S.E.H. and Britton, C.J.C., Disorders of the blood (1947). London, J. & A. Churchill.

3. Wintrobe, M.M., Clinical Haematology. 3rd Ed. (1951). London. Henry Kimpton.

4. Cape, R.D.T., Edinburgh Med. J. 59: 374 (1952).

5. Osgood, E.E., J. Am. Med. Assoc. 109: 933 (1937).

6. Karki, N., Seppala, T., and Halonen, P.I., Ann. Med. Exptl. et Biol. Fenn. 29: 197 (1951). Biol. Abstracts. 26: 5609 (1952).

7. Vaughn, J., Blood. 8 : 1 (1953).

8. Ray Sarkar, B.C., Some Aspects of Systemic Stress., Def. Sci. J. 5: 5 (1955).

9. Selye, H., Text Book of Endocrinology. Acta Endocrinologica Inc. Montreal, Canada. 2nd Ed. (1949).

10. Hills, A.G., Forsham, P.H., and Finch, C.A., Blood. 3: 755 (1948).

11. Thorn, G.W., Forsham, P.H., Prunty, F.T. and Hills, A.G., J. Am. Med. Assoc. 137: 1005 (1948).

12. Hume, D.M., J. Clin. Investigation. 28: 790 (1949).

13. Gerschberg, H., Fry, E.G., Broveck, J.R., and Long, C.N.H., Yale J. Biol. Med. 23/32 (1950).

14. McDermott, W.V., Fry, E.G., Broveck, J.R., and Long, C.N.H., Yale J. Biol. Med. 23 : 52 (1950).

15. Sayers, G. and Sayers, M.A. Endocrinology. 40: 265 (1947).

16. Sayers, G., Physiol. Rev. 30: 241 (1950).

17. Sandberg, A.A., Nelson, D.H., Palmer, J.G., Samuels, L.T. and Tyler, F.H., J. Clin. Endocrinol. Metab. 13: 629 1953).

18. Bacchus, H., Am. J. Physiol. 178: 437 (1954).

19. Henry, W.L., Oliver, L., and Ramey, E.R., Am. J. Physiol. 174: 455 (1953).

20. Bibile, S.W., J. Endorcrinol. 9: 357 (1953).

21. Soylemezoglu, B. and Wells, J.A., Proc. Soc. Exptl. Biol. Med. 77: 43 (1951).

22. Swingle, W.W., Eisler, M., Max. Ben., Maxwell, R., Baker, C., and Le Brie, S.J., Am. J. Physiol. 178: 341 (1954).

23. Dumn, M.E., and Ralli, E.P., Endocrinology. 54: 71 (1954)

24. Humphreys, R.J., and Raab, W., Proc. Soc. Exptl. Biol. Med. 74: 302 (1950).

25. Renold, A.E., Quigley, T.B., Kennard, H.E., and Thorn, G.W., New Engl. J. Med. 244: 754 (1951).

26. Laragh, J.H., and Almy, T.P., Proc. Soc. Exptl. Biol. Med. 69: 499 (1948).

27. Roche, M., Thorn, G.W., and Hills, A.G., New Engl. J. Med. 242: 307 (1950).

28. Gabrilove, J.L., J. Clin. Endocrinol. 10: 637 (1950).

29. Altschule, M.D., Parkhurst, B.H., and Tillotson, K. J, J. Clin. Endocrinol. 9: 440 (1949).

30. Brown, H.E., Master's thesis. Univ. of Utah., Salt Lake City, Utah. 70 pp (1951). Quoted by Dougherty, T. F. and Dougherty, G.J., Ann. Rev. Physiol. 15: 200 (1953).

31. Penneys, R., Thomas, C.B., and Lewis, R.A., Bull. Johns Hopkins Hosp. 86: 102 (1950).

32. Dury, A., Endocrinology. 47: 387 (1950).

33. Rosenthal, R.L., Wald, N., Yager, A., and Litwins, J., Proc. Soc. Exptl. Biol. Med. 75: 740 (1950).

34. Durgan, M.L. and Meyer, R.K., Endocrinology. 48: 518 (1951).

35. Spain, D.M. and Thalhimer, W., Proc. Soc. Exptl. Biol. Med. 76: 320 (1951).

36. Best, W.R., Muehrcke, R.C., and Kark, R.M., J. Clin. Investigation 30: 629 (1951).

37. Solomon, D.H. and Humphreys, S.R., Blood, 6: 824 (1951).

38. Fruhman, G.J. and Gordon, A.S., Fed. Proc. 2: 51 (1952).

39. Dury, A., Am. J. Physiol. 163: 96 (1950).

40. Dougherty, T.F., Pituitary Adrenal Function Symposium, 79—87 (Am. Assoc. Advancement Sci. Washington, D.C. 1950).

41. Godlowski, Z.Z., J. Endocrinol. 8: 102 (1952).

42. Padawer, J. and Gordon, A.S. Abstract Proc. Endocrine. Soc. p. 27 (1952).

43. Pool, W.E. and Becken, M., Am. J. Physiol. 169: 453 (1952).

44. Lewis, J.L. and Kark, R.M., Science. 115 : 377 (1952).

45. Best, W.R., Kark, R.M., Muehrcke, R.C. and Samter, M, J. Am. Med. Assoc. 151: 702 (1953).

46. Dougherty, T.F. and Dougherty, G.J., Ann. Rev. Physiol. 15: 200 (1953).

47. Rosenberg, E. and Lewis, R.A., J. Appl. Physiol. 3: 164 (1950).

48. Speirs, R.S. and Meyer, R.K., Endocrinology, 48: 316 (1951).

49. Halberg, F. and Visscher, M.B., Abstracts Proc. Endocrine Soc 40, (1952).

50. Scott, J.K., Proc. Soc. Exptl. Biol. Med. 57: 502 (1950).

51. Thorn, G.W., Jenkins, D. and Laidlaw, J.C., Recent Progress in Hormone Research 8: 171 (1953).

52. Heroux, O. and Hart, J.S., Am. J. Physiol. 178: 449 (1954).

53. Heroux, O. and Hart, J.S., Am. J. Physiol. 178: 453 (1954).

54. Langley, S.S. and Clarke, B.W., Yale J. Biol. Med. 14: 529 (1942).

55. Bojovic, Lj., and Kostial-Zwanovic., Arch. Internat. Physiol. 60: 457 (1952).

56. Heroux, O., and Hart, J.S., Am. J. Physiol. 178 : 445 (1954).

57. Stack-Dunne, M. and Young, F.G., J. Endocrinol. 7, Suppl. 66 (1951).

58. Heroux, O., Laval Med.16: 1 (1951).

59. Speirs, R.S. and Meyer, R.K., Endocrinology 45 : 403 (1949).

60. Rosenberg, E., Cornfield, J., and Anderson, E., Abstracts of 34th meeting of the Endocrine Society, 40 (1952). Cited by Heroux, O. and Hart, J.S., Am. J. Physiol. 178: 453 (1954).

61. Dury, A., Am. J. Physiol. 72: 82 (1950).

62. Coste, F., Laurent, F., and Delabarre, F., Compt. Rend. Soc. Biol. 145: 838 (1951). Cited by Heroux, O. and Hart J.S., Am. J. Physiol. 178: 453 (1954).

63. Recant, L., Hume, D.M., Forsham, P.H. and Thorn, G.W., J. Clin. Endorcrinol. 10: 187 (1950).

64. Speirs, R.S., and Sullivan. J, Am. J. Physiol. 173: 282 (1953).

65. Wright, S., Applied Physiology, p. 952. 9th Ed. (1952). Oxford University Press, Amen House, London E.C. 4.

66. Best, W.R. and Samter, M., Blood, 6: 61 (1951).

67. Fisher, B. and Fisher, E.R., Am. J. Med. Sci. 221: 124 (1951).

68. Flink, E.B., and Halberg, F., Abstracts Proc. Endocrine Soc. 26 (1952).

69. Roninger, E., Schweiz Med. Wochscher. 82: 411 (1952). Cited by Dougherty T.F. and Dougherty A.J., Ann. Rev. Physiol. 15: 200 (1953).

70. Knowlton, A.M., Clin. North America. 36: 721 (1952) Cited by Swingle et. al., Am. J. Physiol. 178: 341 (1954).

71. Muehrcke, R.C., Stople, T.W. and Kark, K.M., J. Lab. Clin. Med. 40: 169 (1952).

72. Rud., F., Acta Psychiat et neural suppl. 40, 443 pp. (1947). Cited by Leader., Lancet. 1: 1042 (1950) and by Bonner, C.D., J. Am. Med. Assoc. 148: 634 (1952).

73. Thorn et al (1950). J. Clin. Endocrinol. 10: 198 (1950). Quoted from Applied Physiology, p. 950 (1952 by Wright, S.

74. Bonner, C.D, J. Am. Med. Assoc. 148: 634 (1952).

75. Donato, R.A., and Strumia, M.M., Blood. 7: 1020 (1952).

76. Halberg, F., Visscher, M.B., Flink, E.B., Berge, K., and Bock, F. Lancet, 71: 312 (1951).

77. Mookerjee, M.K. and Ray Sarkar, B.C., A Study on Stress in Flying During Tropical Summer. In Communication for publication in Def. Sc. J. 5: 313 (1955).

78. Muehrcke, R.C., Eckert, E.L., and Kark, R.M., J. Lab. Clin. Med. 40: 161 (1952).

79. Halberg, F., Halberg, E., Wargo, D.C., and Visscher, M.B., Am. J. Physiol. 174: 313 (1953).

80. Godlowski, Z.Z., Brit. Med. J. 1: 46 (1948).

81. Eppinger, H., Hess, L., and Ztschy, F. Klin. Med. 67: 345 (1909) Cited by Best, W.R. and Samjer, M., Blood 6 : 61 (1951). and Godlowski, Z.Z., Brit. Med. J. 1: 46 (1948).

82. Butler, L.C. and Morgan, A.F., Proc. Soc. Exptl. Biol. Med. 85: 139 (1954).

82(*a*) Science News letter 65(4): 56 (Jan. 23, 1954); J. Clin. Endocrinol Metab. (Dec. 1953).

83. Krammer, J., Wilhelmj, C.M., Meyers, V. W., Milani, D.P., Gunderson, D.E., Shuput, D., Racher, E.M., and Mahoney, P.S., Am. J. Physiol. 178: 486 (1954).

84. Gofton, J.P., and McGrath, S.D., J. Aviation Med. 24: 107 (1953).

85. Gofton, J.P., Graham, B.F., McGrath, S.D., and Cleghorn, R.A., J. Aviation Med. 24: 123 (1953).

86. Wake, R.F., Grahman, B.F., and McGrath, S.D., J. Aviation Med. 24 : 127 (1953).

87. Domanski, T.J. and Nuttall, J.B., J. Aviation Med. 24: 441 (1953).

88. Murphy, C.W., Gofton, J.P., and Cleghorn, R.A., J. Aviation Med. 25: 242 (1954).

89. Swanson, J.N., Bauer, W. and Ropes, H., Lancet. 262 (1) : 129 (1952).

90. Danger, R., Munchen Med. Wchnschr 57: 1942 (1910). Cited by Speirs, R.S., Blood 7: 550 (1952).

91. Kolmer, J.A., Spaulding, E.H. and Robinson, H.W. Approved Laboratory Technic. 5th Edn. (1951). Appleton-Century-Crafts, Inc. New York.

92. Napier, L.E., and Das Gupta, C.R., Haematological Technique. 3rd Edn. (1945). U.N. Dhur & Sons Ltd., Calcutta.

93. Randolph, T.G., Proc. Soc. Exptl. Biol. Med. 52: 20 (1943)

94 Randolph, T.G., Am. J. Clin. Path. *14*: 48 (1944).

95. Randolph, T.G., J. Immunol. 15: 89 (1944).

96. Randolph, T.G., J. Lab. Clin. Med. 34: 1696 (1949).

97. Henneman, P.H., Waxler, H. and Westenhaver, M.M., J. Lab. Clin. Med. 34 (B): 1017 (1949.)

98. Speirs, R.S., Blood, 7: 550 (1952).

99. Hanne, U., Acta Haematol. 9: 121 (1953).

100. Manners, T., Brit. Med. J. 2: 1429 (1951). Cited by Speirs, R.S., Blood 7: 550 (1952).

101. Domanski, T.J., Swan, A. G., Wells, J.G., and Hughes, L.B., Project No. 21-32-025. Report No. 1, USAF School of Aviation Medicine (1951).

102. Pilot, M.L., Am. J. Clin. Path. 20: 870 (1950).

103. MacFarlane, J.C.W., and Cecil, G.W., Brit. Med. J. 2: 1187 (1951).

104. Ferrington, E.M. and Jetter, W.W., Am. J. Clin. Path. 23: 836 (1953).

105. Discombe, G., Lancet, 250 (1): 195 (1946).

106. Speirs, R.S., J. Lab. Clin. Med. 39: 963 (1952).

107. Berkson, J., Medical Physics. p. 110 (1947).