dermal connective tissue, joint capsules, bursae, and tendons) forms the target organ. This damaged tissue contains the major proportion of ground substance in the body, and the delayed reconstitution time which we have observed in the skin could be explained by a deficient synthesis of ground substance, due to altered cell nutrition or depressed cell activity.

The role of non-specific serum hyaluronidase inhibitor in the reconstitution of hyaluronic acid in the skin must also be considered. Dorfman (1950) showed this to be raised in acute rheumatic fever, but it was further shown (Dorfman and Moses, 1950) that the inhibitor was decreased by A.C.T.H. in this disease in parallel with clinical improvement. If this inhibitor (by direct action on the enzyme) affected the skin tests described here, a decrease in reconstitution time might be expected in the acute stages, and a prolongation during convalescence—the reverse of the changes actually found. This supports Hechter's (1947) conclusion that plasma anti-hyaluronidases do not significantly affect the spreading activity of the injected enzyme.

Summary

A method for measuring the "reconstitution of the dermal barrier" is described and errors in the technique are analysed.

The normal reconstitution time varies with age and lies between 24 and 42 hours.

We determined 214 reconstitution times—109 in rheumatic fever, 55 in rheumatoid arthritis, 27 in normal adults and children, and 23 in non-rheumatic controls. Reconstitution was prolonged in rheumatoid arthritis and in acute rheumatic fever, but returned to normal levels in the latter during treatment with salicylate, A.C.T.H., and cortisone, and during convalescence.

Non-rheumatic tuberculous controls with raised sedimentation rates gave normal readings.

It is concluded that in both rheumatoid arthritis and rheumatic fever there may well be an alteration in hyaluronic acid metabolism.

We would like to thank Dr. L. E. Glynn for encouragement and constructive criticism; Dr. Frank Fletcher, of Benger's Research Department, for turbidometric studies and a generous supply of hyalase; Dr. L. A. Key, of Heatherwood Orthopaedic Hospital, for permission to test a control series of patients; Dr. R. C. Hallam for assistance; normal volunteers for their co-operation; Mr. P. J. Fisk for the photographs; and the Central Syringe Service staff at this hospital, who rendered this investigation possible.

References

Bywaters, E. G. L., and Dixon, A. St. J. (1951). In press.
Dorfman, A. (1950). *Ann. N.Y. Acad. Sci.*, **52**, 1098.
—— and Moses, F. E. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 667.
Hechter, O. (1947). *J. exp. Med.*, **85**, 77.
—— (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 343.
Holborow, E. J., and Keech, M. K. (1951). *British Medical Journal*, **2**, 1173.
Ragan, C., and Meyer, K. (1949). *J. clin. Invest.*, **28**, 56.

An inquest at Dorchester on September 12 on a 64-year-old woman who died after a wasp sting revealed a most unusual hypersensitivity. Six years ago she was unconscious for some hours after a sting, and on September 10 she was again stung by a wasp. Immediately afterwards she was reported to feel shaky, perspiring, and trembling; she tried to vomit three times, complained of not being able to see, and died in about 15 minutes. At necropsy the heart was found to be contracted and virtually empty of blood, and apart from widespread atheroma no abnormalities were discovered. The cause of death was declared to be syncope.

THE EOSINOPENIC RESPONSE TO CORTISONE AND A.C.T.H. IN NORMAL SUBJECTS

BY

J. H. KELLGREN, F.R.C.P., F.R.C.S.

AND

O. JANUS, M.B., Ch.B., B.Sc., M.R.C.P.

(From the University Department of Rheumatism Research at the Manchester Royal Infirmary)

The eosinopenic response described by Hills, Forsham, and Finch (1948) has been widely used as an index of adrenocortical activity in man, particularly in studies of the response to A.C.T.H. (Forsham *et al.*, 1948), and as a test of adrenocortical function (Thorn *et al.*, 1948). It has also been used to assess the adrenocortical response to various forms of stress, such as surgery, the administration of adrenaline and insulin, and electric-convulsion therapy. The whole subject has been well reviewed by Prunty (1950) and by Sayers (1950), who both consider the eosinophil response in man to be a most useful index of adrenocortical function.

The eosinopenic response to cortisone mentioned by many workers reporting the effects of cortisone therapy has not been submitted to the same detailed study as the response to A.C.T.H., perhaps because much of the cortisone was given by injection and the slow response which follows this method of administration is not readily compared with the rapid response to A.C.T.H.; but Boland and Headley (1951) have noted that the clinical response to oral cortisone is both rapid and of short duration, the result of a single dose being similar to that of a single injection of A.C.T.H. On theoretical grounds the eosinopenic response to oral cortisone might be expected to give valuable information which would be complementary to that obtained from the response to A.C.T.H. We have therefore studied in some detail the eosinopenic response in normal subjects to both A.C.T.H. and oral cortisone in varying dosage.

Method

The number of circulating eosinophils was estimated by a modification of Dunger's (1910) method, using the following technique: blood is obtained from the thumb by a fresh needle prick and drawn up to the usual mark in a standard white-cell pipette. It is then diluted 1 in 10 by a freshly filtered solution of eosin 0.05% and acetone 5% in distilled water. After mixing well by shaking the pipette gently about 20 times, the usual first one-tenth is discarded and a double Fuchs–Rosenthal counting chamber is filled at once from the pipette. The chambers are left to stand for five minutes to allow the cells to settle and stain properly, after which all eosinophil cells in both chambers are counted with a $\frac{2}{3}$-in. objective, using a bright light. To obtain the number of eosinophils per cubic millimetre of blood the number of cells counted is multiplied by a factor of 1.55. As in all blood-cell counting, the statistical error involved depends upon the number of cells counted, and the personal error can be minimized by much practice and careful standardization of technique.

The counts reported here were done by technicians who had been counting eosinophils regularly for periods

of a year or more. The accuracy of the sampling and counting technique was tested from time to time by doing duplicate counts and runs of duplicate sampling at frequent intervals. If two pairs of counting chambers are used for a single pipette the difference between the counts in each pair is in the region of ±5% for counts of 100 or over, though with very low counts the percentage error is of course considerably greater, and Table I

TABLE I.—*Consecutive Eosinophil Counts (Subject 1) During a Control Day and Between the Fourth and Sixth Hour after 50 mg. of Oral Cortisone. Two Pipettes were Filled from Each Needle Prick*

Time	Pipettes		Difference	Time	Pipettes		Difference
	1	2			1	2	
2·10	381	388	+7	1·50	56	34	−22
2·25	366	360	−6	2·5	50	38	−12
2·40	359	369	+10	2·20	59	44	−15
2·55	353	340	−13	2·35	56	41	−15
3·10	375	366	−9	2·50	62	47	−15
3·20	378	362	−16	3·0	69	62	−7
3·35	375	369	−6	3·10	72	59	−13
3·55	362	384	+22	3·20	78	62	−16
4·10	384	372	−12	3·35	84	78	−6
4·25	393	375	−18	3·45	99	72	−27

shows the type of result obtained over a two-hour period by filling two pipettes from one needle prick at intervals of 10 to 15 minutes during periods of average total counts and one of low total counts induced by cortisone. As might be expected, the second sample tends to give a slightly lower count than the first. Apart from this, both counts tend to fall and then rise again during the first experiment and to rise steadily during the second experiment, but there are no wild fluctuations between the consecutive counts or between the first and second pipettes. Since only fluctuations of over 50% in the number of circulating eosinophils are of any significance the technique is clearly adequate for our purposes.

In a monograph by Rud (1947), recently quoted in the *Lancet* (1950), it is claimed that the number of circulating eosinophils fluctuates wildly from one minute to the next, but Rud obtained his samples of blood from a cut in the ear which was reopened from time to time during the day, and indeed on successive days, and the eosinophils in the material obtained from this wound were estimated by counting a small sample and multiplying by a factor of 6.25. In view of the fact that the minute-to-minute differences of eosinophil count observed by Rud were of the same order as he observed at hourly or even daily intervals, it seems likely that many of these differences were due to his methods of sampling and counting, which are clearly open to grave criticism. At all events, we have not observed these rapid fluctuations of eosinophil count, though the count may rise and fall within a wide range over a period of hours, particularly under the influence of stress, cortisone, or A.C.T.H.

Plan of Study

Four male subjects aged 39, 43, 40, and 32 years were selected for the main investigations. They were all working in the department and carried on their usual routine, and except for subject 4, who was 6 ft. 5½ in. (197 cm.) in height, they were in no way remarkable.

Eosinophil counts were done between 9.30 and 10 a.m. on many days, and this is called the "morning" count. On two days each week, usually Monday and Friday, the eosinophils were counted at two-hourly intervals throughout the day. On different days single doses of cortisone or A.C.T.H. were given immediately after the morning count, and in addition a number of control days were interspersed throughout the investigation.

A.C.T.H. was given intramuscularly on the following dosage scale : 2.5, 5, 10, 20, 40, and, in two instances, 100 mg. Cortisone was given as a suspension by mouth on a dosage scale of 6.25, 12.5, 25, 50, and 100 mg. In a few experiments the same dosages of A.C.T.H. and cortisone were repeated, and in another series of experiments other preparations of A.C.T.H. and a number of peptide derivatives were also employed. The number of circulating eosinophils was also investigated during periods of stress produced by T.A.B. vaccine and by incidental disease.

Results

It is well known that the eosinophil count may be greatly influenced by intercurrent disease or unusual stress of any kind. Our studies have therefore been confined to "normal" days, unless otherwise stated, and Table II shows the distribution of "normal" morning

TABLE II.—*Distribution of Morning Eosinophil Counts in Four Subjects on "Normal" Days*

Eosinophils	Subjects			
	1	2	3	4
0–49				
50–99				
100–49				
150–99	1			4
200–49	2	1		6
250–99	10	3	9	9
300–49	5	3	11	2
350–99	7	5	9	2
400–49	1	5	4	
450–99	2	4		
500–49	3	2		
550–99	1	1		
600–49		3		
650–99				
Total counts ..	32	27	33	23

counts in the four subjects under investigation. In subject 1 the counts have a wide range from 150 to 599, centred around the 350 mark. In subject 2 the range is similar, from 200 to 649, but centred a little higher, about 400 or over. In subject 3 the range is small, all counts falling between 250 and 449 ; while in subject 4 there is again a small range from 150 to 399, centred around 250. Thus already in respect of their morning counts these four subjects differ considerably.

It has been customary to express short-term alterations in the eosinophil count as a percentage change, and we have followed this practice by expressing the counts throughout the day as a percentage increase or decrease over the morning count for that day.

During the control days the count increased, decreased, remained unaltered, or fluctuated slightly, the mean change throughout the day of all the counts on any one individual being no more than 10% of the morning count, so that there did not appear to be any consistent diurnal trend. Occasionally there was an increase or decrease of about 40%, and on one occasion an increase of 50%. The changes in the four and six-hour counts are summarized in Fig. 3, and it is clear that in our subjects fluctuations in the eosinophil count of up to 50% may be found during control days and must therefore be regarded as a normal occurrence.

After the administration of cortisone and A.C.T.H. a satisfactory decrease in the number of circulating

eosinophils was observed in subjects 1 and 2. For both substances the decrease began at two hours and was pronounced at four hours. With the higher dosages the count continued to decrease at six hours and was still low at eight hours, while with the lower dosages the count increased again at six hours and tended to return to or surpass the morning count by eight hours. Sample eosinophil depression curves from subject 1 are shown in Fig. 1, and it will be seen that the general shape of the cortisone and A.C.T.H. curves is remarkably similar.

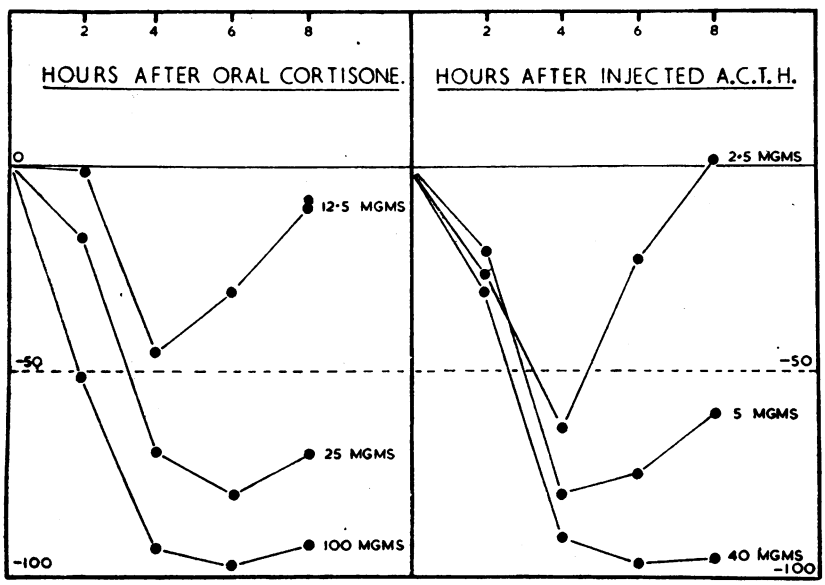

FIG. 1.—Shows percentile change in circulating eosinophils (subject 1) following single doses of oral cortisone and intramuscular A.C.T.H. at different dose levels.

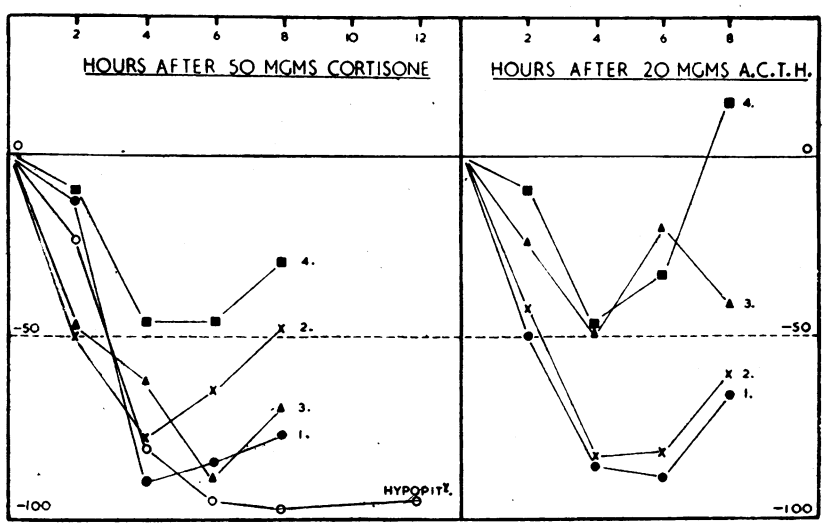

FIG. 2.—Shows percentile change in eosinophils in four subjects following single doses of 50 mg. of cortisone and 20 mg. of A.C.T.H. The clear circles show the effect of 50 mg. of cortisone in a case of severe hypopituitarism.

Fig. 2 shows the eosinophil depression curves produced by 50 mg. of cortisone and 20 mg. of A.C.T.H. in the four subjects under investigation. The responses to cortisone in subjects 1, 2, and 3 are not very dissimilar, but subject 4 fails to give a significant response. With A.C.T.H., subjects 1 and 2 give good depression curves, but subjects 3 and 4 fail to give significant responses.

From the shape of all the curves we have observed it is clear that nearly as much information can be obtained from the four- and six-hour counts as from the whole curve. We have therefore summarized our findings in respect of these counts in Fig. 3.

From this figure it will be seen that subject 1 responds well to both A.C.T.H. and cortisone in low dosage. Subject 2 also responds well to both hormones but at a slightly higher dosage. Subject 3 responds well to cortisone but fails to respond properly to A.C.T.H. even when 100 mg. was given as a single dose. Subject 4, on the other hand, fails to respond significantly to oral cortisone in doses up to 100 mg., but does respond to A.C.T.H. at the higher dose levels.

At first it was hoped that a study of eosinophil depression curves might provide a simple method for assaying the potency of different preparations of A.C.T.H., but repeated injection of the same dosage of the same preparations did not give strictly comparable curves. This is not surprising when we consider that the spontaneous fluctuations in the eosinophil count may occasionally be as great as 50% within the period of the test and that this might have to be either added to or subtracted from the fluctuation induced by the administered A.C.T.H. An accurate assay on these lines therefore seems to be out of the question, but a very rough guide to potency may nevertheless be obtained, particularly if the A.C.T.H. to be tested is given in two dose levels to subjects whose dose-response curves have already been constructed. Too great an accuracy must not, however, be aimed at.

If we consider a partial response to be a fall in the eosinophil count of over 50% at either four or six hours and a full response to be a fall of over 75% it is possible to construct a rough equivalent dosage table. This has been done in Table III for oral cortisone, the A.C.T.H. used in these studies, and two other A.C.T.H. preparations, one stated to be three times more potent than standard and a peptide derivative of this preparation said to be 5 to 15 times more potent than the original by rat assay. Though for assay purposes our results leave much to be desired, they do further illustrate the difference in responsiveness of our four subjects, and they might reasonably be used as a rough guide to the choice of preparation and the dosage likely to be required for therapy in the individuals concerned.

The most valuable result of these studies is, however, the demonstration of the differing responsiveness to cortisone and A.C.T.H. of our four male subjects, at least so far as their eosinopenic response is concerned.

In a previous experiment T.A.B. vaccine (10 ml. intravenously) was used to induce an acute febrile stress

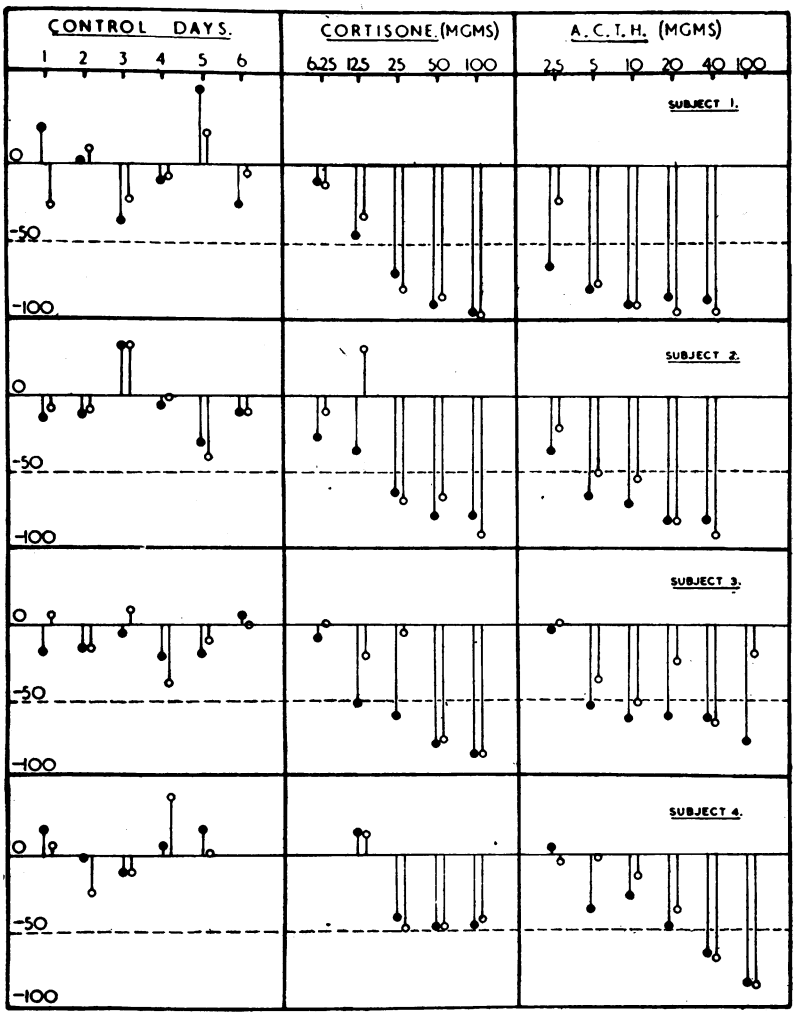

FIG. 3.—Shows the percentile change in eosinophils at four hours (black circles) and six hours (clear circles) in four normal subjects during control days and following varying doses of cortisone and A.C.T.H.

TABLE III.—*Approximate Dosage in mg. Required for Equivalent Response*

Subject	Response	Cortisone	A.C.T.H. J.24410	A.C.T.H. 84–85 H	A.C.T.H. Peptide	Stress
1	Partial	12·5+	2·5	2·5	?	T.A.B. 10 ml. I.V.
	Full	25	5	10	0·25	
2	Partial	25	5·10	?	0·25	
	Full	50	20	2·5	1·0	
3	Partial	25	5–100	2·5–20	2·0	T.A.B. 10 ml. I.V.
	Full	50	?	?	?	Septicaemia
4	Partial	100+	40	2·5	1·0+	
	Full	?	100	10	?	

TABLE IV.—*Comparison of Eosinopenic Response to 25 mg. A.C.T.H. and Febrile Stress Produced by T.A.B. in Four Normal Subjects and Two Patients with Rheumatoid Arthritis*

Subject	No.	A.C.T.H. 25 mg.		T.A.B. 10 ml. I.V.	
		Eosinophil Change	% Fall	Eosinophil Change	% Fall
Normal subjects ..	1	278–7	97	284–15	95
	6	362–7	98	405–23	95
	5	400–144	64	293–96	66
	3	318–137	57	354–118	66
Patients ..	1	59–22	62	59–23	60
	2	200–25	87	225–50	78

in two patients with rheumatoid arthritis and four normal subjects, including subjects 1 and 3 of the present investigation. The resultant eosinopenia was compared with that produced by a single dose of 25 mg. of A.C.T.H. of a different preparation from that used in the rest of this study. The results summarized in Table IV show that for each individual the degree of the eosinopenic response was about equivalent, although it was of longer duration with T.A.B. than with A.C.T.H. In this experiment also the relative unresponsiveness of subject 3 compared with subject 1 is evident. On the other hand, subject 3 is capable of producing a complete eosinopenic response, as his count fell to zero during the onset of a steptococcal septicaemia (Table III).

It was thought that the differing responsiveness of our four subjects might be reflected in differing urinary excretions of formaldehydogenic steroids, but estimations of these steroids by Bassil and Hain's (1950) method in three 24-hour specimens for each of our subjects during control days gave similar results, all of which were within the normal range.

Discussion

Our findings show that even among normal subjects there may be marked differences in the eosinopenic response to single doses of cortisone and A.C.T.H.

It is interesting to note that subjects 1 and 2, who gave satisfactory eosinopenic responses to small doses of both hormones, also showed a wide range of fluctuation in their morning counts, while subjects 3 and 4, who were less responsive to the hormones, also showed less variation in their morning counts. Furthermore, in the six subjects in whom the eosinopenic responses to T.A.B. and A.C.T.H. were compared there appeared to be some correlation between the degree of this response to both stress and injected A.C.T.H. in each individual.

The fact that responsiveness to A.C.T.H. and oral cortisone may be dissociated as in subjects 3 and 4 suggests that the eosinophils themselves cannot be wholly responsible for the difference of responsiveness between one individual and another. A poor response to oral cortisone might result from failure of absorption or destruction of the hormone in the gut, and failure of response to injected A.C.T.H. might result from the development of anti-hormone or the destruction of the A.C.T.H. by tissue enzymes, so that there are clearly many factors to be considered. Nevertheless, the response to A.C.T.H. has been shown to provide useful information in endocrine disorders (Thorn *et al.*, 1948; Prunty, 1950), and in three cases of established panhypopituitarism we have noted a greatly exaggerated and prolonged eosinopenic response to oral cortisone, one example of which is shown in Fig. 2. But to what extent these eosinopenic responses can be more generally applied to assess the state of activity of an individual's

pituitary adrenal mechanism is still a matter for speculation.

At this stage, however, it is sufficient to note that the eosinopenic response to A.C.T.H. and oral cortisone in normal subjects can be usefully compared, and it remains to be seen whether the application of a combined A.C.T.H.–cortisone response test to patients with rheumatic, endocrine, and other diseases will give information of practical value.

Summary

A convenient modification of Dunger's technique for estimating the number of circulating eosinophils has been described and shown to be accurate enough for experimental purposes. By the use of this technique the eosinopenic responses to single doses of oral cortisone and intramuscular A.C.T.H. have been studied in some detail in four normal subjects. It has been shown that the time relations of the responses to these two hormones are remarkably similar, but that there may be considerable individual variations in responsiveness to either cortisone or A.C.T.H., or both.

The eosinopenic response to various A.C.T.H. preparations and to stress have also been compared, and throughout these studies the differences of individual responsiveness of our subjects have been maintained. Three patients with panhypopituitarism showed an eosinopenic response to oral cortisone which was exaggerated and prolonged. In spite of the many factors which may influence eosinopenic responsiveness it is suggested that a study of the eosinopenic response to 50 mg. of oral cortisone, added to the already well-established A.C.T.H. response test, might provide a useful guide to therapy with these hormones in the individual concerned, and might possibly give some indication of the state of activity of the individual's own pituitary–adrenal mechanism.

The cortisone used in this work was provided from a generous gift made jointly to the Nuffield Foundation and the Medical Research Council by Merck and Co., Inc. We are indebted to the Medical Research Council and Organon Laboratories for the A.C.T.H. used; to Dr. Kenner, of the Chemical Laboratories of Cambridge University, for the peptide fraction; and to Mr. D. S. Jackson for the steroid assays.

REFERENCES

Bassil, G. T., and Hain, A. M. (1950). *Nature, Lond.*, **165**, 525.

Boland, E. W., and Headley, N. E. (1951). *J. Amer. med. Ass.*, **145**, 8.

Dunger, R. (1910). *Münch. med. Wschr.*, **57**, 1942.

Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G. (1948). *J. clin. Endocrinol.*, **8**, 15.

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

Lancet, 1950, **1**, 1042.

Prunty, F. T. G. (1950). *J. clin. Path.*, **3**, 87.

Rud, F. (1947). *The Eosinophil Count in Health and Mental Disease*. Johan Grundt, Oslo. Also *Acta psychiat., Kbh.*, Suppl. No. 40.

Sayers, G. (1950). *Physiol. Rev.*, **30**, 241.

Thorn, G. W., Forsham, P. H., Prunty, F. T. G., and Hills, A. G. (1948). *J. Amer. med. Ass.*, **137**, 1005.

Speaking at a meeting of the Society of Public Analysts recently about the chemical quality of milk Dr. J. G. Davis said that it was frequently stated that the overall composition of milk in this country had deteriorated during the last few years. It was difficult, if not impossible, to prove this contention by adequate scientific evidence for the whole country, and the reasons for this were discussed. A review of existing information was given, and data received from a number of public analysts on the composition of milk as sold over the period 1900–51 were presented. Not all records showed a fall in composition over a long period, and yearly and regional variations may be of the same order as the fall which it was claimed has taken place.

EOSINOPHIL COUNTING: A MODIFICATION OF PILOT'S METHOD

BY

J. C. W. MacFARLANE, B.Sc., M.D.

AND

G. W. CECIL, F.I.M.L.T.

(From The Hospital for Sick Children, Great Ormond Street, W.C.1)

The use of A.C.T.H. and cortisone entails the quantitative determination of the circulating eosinophils at frequent intervals as one of the pathological tests employed as a potential measure of the efficacy of the drugs. The method most commonly in use is that originally described by Randolph (1944). The object of this paper is to call attention to the diluting fluid evolved by Pilot (1950) and to the modification of that fluid it has been necessary to make in this laboratory.

Pilot investigated the counting fluids that had already been described. From them he selected as the best those of Randolph (1944) and Rud (1947), and combined the better properties of each in his diluent. Randolph's fluid, a phloxine–methylene-blue stain in propylene glycol, has certain advantages. The glycol base renders the red blood cells relatively non-refractile, and acts as a vehicle for both acid and basic stains, while, being viscous, it does not evaporate quickly. There is no deterioration of the count obtained when the fluid and blood are left in the pipette overnight. All the leucocytes, however, are stained, and a high-power objective is necessary for differentiation of the eosinophils. The methylene blue and phloxine components of the stain must be kept separate until just before use, for after they have been mixed for four hours precipitation of the dyes occurs and with it a loss of differential staining detail. The Rud fluid, a magdala-red stain in acetone, likewise has an advantage. By the use of a small amount of sodium carbonate (0.1%) all leucocytes other than the base-resistant eosinophils are lysed. Its disadvantage is the use of acetone as the vehicle. As this substance is highly volatile, the chambers must be flooded at once on shaking and kept in a moist Petri dish for 15 minutes before making the count.

Pilot decided to use the concentration of base employed by Rud in the propylene glycol vehicle of Randolph. Since the leucocytes other than the eosinophils were lysed, only a single stain was necessary, and no differential count was involved. As stains he used phloxine (0.1%), eosin (0.05%), and magdala red (0.05%), all with equally good results. His diluting fluid he compared with those of Randolph (1944), Rud (1947), and Dunger (1910), employing double counts in a series of 1,000 observations on human blood, with results more nearly constant than those obtained with the other fluids.

The stain he suggested is obtained by mixing the following ingredients: 50 ml. propylene glycol, 40 ml. distilled water, 10 ml. 1% phloxine in water, and 1 ml. 10% sodium carbonate in water. It is necessary to wait for 15 minutes after collection of the count to allow for proper lysis and staining, these occurring in either pipette or chamber.