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NORMAL VARIATION IN THE COUNT OF CIRCULATING EOSINOPHILS IN MAN

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Since Forsham, Thorn, Prunty & Hills (1948) observed that administration of 11, 17-oxysteroids caused a fall in circulating eosinophils, many physiological and clinical studies on human subjects have included eosinophil counts among tests of adrenal cortical function. For instance, the degree of eosinopenia resulting from exposure to environmental stress has been taken to be one measure of its severity (Stein, Bader, Eliot & Bass, 1949; Hale & Keator, 1952). Use of the eosinophil count for this purpose is dependent on obtaining adequate control values, and many workers have carried out serial investigations to determine how far normal rhythmic or random variations might interfere with this test. The results they have obtained do not, however, agree completely. Rud (1947), Tatai & Ogawa (1951) and Donato & Strumia (1952) observed a tendency for the mean eosinophil count of a group of subjects to decrease until about noon and to increase steadily for the rest of the day. Swanson, Bauer & Ropes (1952) further observed that a spontaneous eosinopenia of over 50% was more likely to occur in a subject during the morning than during the afternoon. Fisher & Fisher (1951), however, failed to obtain clear-cut evidence for a trend of this kind, while Mann & Lehmann (1952) observed three different patterns in different individuals. The first was identical with that described above, showing a minimum at noon. In the second, the count decreased steadily from 9 a.m. to 5 p.m., while in the third a steady increase occurred over the same period. Besides this rhythmic variation, eosinophil counts are subject also to the random error of the counting procedure, to random biological variations (Donato & Strumia, 1952; Rud, 1947) and to alterations in the basal level from day to day (Rud, 1947). The present investigation was designed to estimate the relative importance of these four sources of variability. None of the published data was in a form which could be used satisfactorily in the type of statistical analysis which was contemplated, so a new series of eosinophil counts was obtained as described below.

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MATERIALS AND METHODS

A group of twenty healthy male laboratory and office workers acted as subjects during normal working days. These men were leading sedentary lives and their degree of activity was judged to remain approximately the same from day to day. Samples (0·1 ml.) of finger blood were taken from each of them at 10 a.m., 11 a.m., 12 noon, 2 p.m., 3 p.m., and 4 p.m. for three consecutive days, not more than two subjects being tested in any one three-day period. The blood samples were drawn into a straight form pipette calibrated 'to contain' and were delivered into ½ oz. screw-cap bottles containing 0·9 ml. of eosinophil diluting fluid made up as follows (Henneman, Wexler & Westenhaver, 1949): phyloxine, 0·05 g; propylene glycol, 50 ml.; distilled water, 50 ml. The diluted blood

TABLE 1. Variations in eosinophil count (cells/mm³ blood) during 3 successive days

			Hour									Hour					
Subject	Age	Day	10	11	12	2	3	4	Subject	Age	Day	10	11	12	2	3	4
J.D.A.	29	1	122	170	207	116	151	133	G.A.L.	49	1	394	355	407	366	436	462
		2	193	163	184	153	153	140			2	478	427	484	413	446	538
		3	144	140	177	160	160	140			3	463	412	471	472	545	534
T.L.P.	23	1	479	372	379	379	374	453	F.J.N.	38	1	318	298	311	327	330	296
		2	407	400	474	512	344	400			2	262	280	258	288	295	217
		3	226	288	335	300	265	347			3	345	263	298	330	285	313
J.W.T.R.	32	1	63	77	81	119	79	74	R.S.B.	29	1	203	235	210	203	231	245
		2	51	86	104	67	74	86			2	174	137	169	253	222	202
		3	77	91	91	126	100	95			3	179	193	193	240	242	212
R.F.C.	28	1	195	205	191	165	165	244	P.F.G.	34	1	110	91	71	65	81	84
		2	163	209	170	189	202	165			2	85	112	91	70	87	91
		3	176	149	230	121	179	167			3	84	79	108	63	76	89
J.R.R.	39	1	149	188	102	170	230	309	J.D.O.	30	1	180	229	241	159	197	171
		2	209	116	142	140	204	172			2	180	142	158	195	193	149
		3	165	137	130	181	281	267			3	179	152	196	187	181	194
A.H.G.	32	1	258	195	172	247	242	197	D.N.	34	1	35	41	47	42	61	66
		2	333	181	270	209	174	195			2	42	57	61	49	66	72
		3	223	235	316	279	277	230			3	41	63	40	62	64	54
E.E.T.	35	1	163	144	105	67	84	135	S.C.H.M.	42	1	61	59	65	69	80	72
		2	126	63	67	88	91	114			2	76	93	100	84	109	99
		3	77	47	49	28	37	47			3	74	80	84	120	108	87
M.P.K.	32	1	133	202	119	142	165	176	D.H.I.	33	1	364	296	270	218	257	266
		2	195	186	158	200	156	153			2	359	268	247	380	362	397
		3	225	230	165	200	170	149			3	308	329	314	313	427	378
B.M.	25	1	533	570	402	235	251	251	L.J.M.	25	1	196	141	130	111	125	138
		2	381	409	349	274	288	386			2	162	170	185	163	163	113
		3	484	421	421	281	340	293			3	148	148	132	159	170	181
R.W.S.	30	1	128	190	212	189	178	178	B.T.F.	39	1	92	79	85	69	78	103
		2	156	183	234	174	185	252			2	99	117	129	143	109	154
		3	148	193	240	239	175	198			3	134	116	109	115	120	136

was mixed for at least 10 min in a rotary cell suspension mixer rotating at 25 rev/min, similar to that described by Dacie (1950). Four Fuchs-Rosenthal chambers were filled from each bottle by means of a Pasteur pipette and all the eosinophils in the ruled areas counted. Identification of these cells was made easier by using a blue-green filter in conjunction with the microscope. The number of eosinophils in each of the four chambers was recorded separately and the sum of the four counts was then divided by 1·32 to express the results as cells/mm³ blood. For reasons of space the four separate values or replicates from which each count is derived are not given in Table 1, although they form the basis of the statistical analysis. Individual counts ranged from 28 to 570 cells/mm³ blood. Fig. 1 is a graphical representation of the mean values for the eosinophil count at different times of day.

STATISTICAL ANALYSIS

The data and sources of variation in the data

The fundamental random variate arising in the data is not the eosinophil count for a subject at any time but the replicate, four of which are summed to give the count. Differences between the replicates in any one count may be caused by inherent variation in the distribution of eosinophils within the chamber or by random errors in the procedures used for mixing the blood sample and counting the cells. Any difference between actual counts would be compounded of the basic random variation arising as described above, together with biological variation, which may be random or may be in accordance with some systematic pattern. In the present series the use of only one puncture and one pipette for each count tends to increase the component of random sampling error which arises in conjunction with the random biological variation. It was felt, however, that the use of a 0·1 ml. blood sample would minimize the former source of variation. Such sampling errors are, in any case, small provided care is taken in obtaining the blood sample (Biggs, 1951).

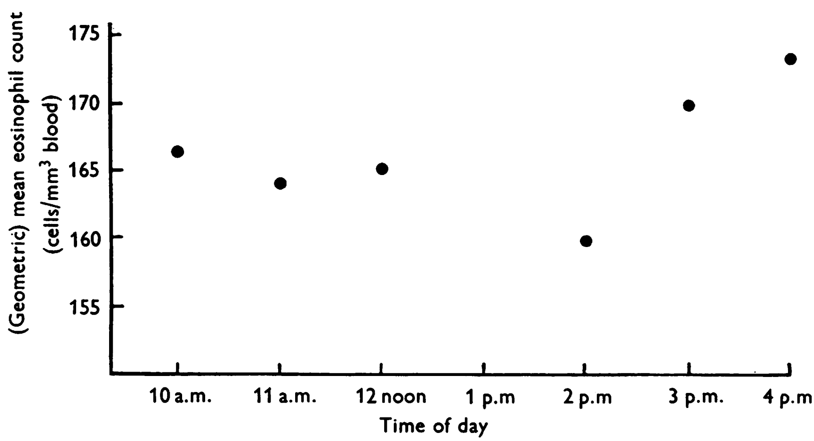

Fig. 1. Mean values during the day for the count of circulating eosinophils: average of twenty subjects. The geometric rather than the arithmetic mean was used because variations in eosinophil count had been shown statistically to be proportionate in character. To obtain the points, the figures in Table 1 were converted to logarithms. The log counts for each hour were then averaged and converted back to absolute numbers.

The statistical model adopted

The model adopted in the analysis to introduce all the probable elements of variation was then

$$x_{ijkt} = K + A_i + B_j + C_k + I_{ij} + u_{ik} + v_{jk} + w_{ijk} + z_{ijkt}. \tag{1}$$

In this relation the symbols have the following significance:

i denotes the individual subject ($i = 1, 2, \ldots, 20$).

j denotes the individual hour ($j = 1, 2, \ldots, 6$).

k denotes the individual day ($k = 1, 2, 3$).

t denotes the individual replication ($t = 1, 2, 3, 4$).

Then

x_{ijkt} denotes the variate value corresponding to the tth replication on the kth day at the jth hour for the ith subject.

K is a constant.

A_i denotes the basic level for the ith subject.

B_j denotes the basic level for the jth hour.

C_k denotes the basic level for the kth day.

I_{ij} denotes the difference superimposed on the jth hour basic level in respect of the ith man.
u_{ik} denotes the variation in the kth day level in respect of the ith subject.
v_{jk} denotes the variation in the jth hour level on the kth day.
w_{ijk} denotes the random biological variation.
z_{ijkt} denotes the random variation between replicates.

In the model (1) the 'man-hour' interaction I_{ij} is considered as a difference superimposed on the 'basic diurnal pattern', whilst the 'man-day' and the 'hour-day' interactions u_{ik} and v_{jk} are considered as random variations. The reason for the difference in the mathematical nature of these interactions is that it has been assumed, following Mann & Lehmann (1952), that the diurnal pattern of each individual subject follows one of a limited number, three, of distinct patterns. In this case the basic diurnal pattern is then in fact the average of all the individual patterns and man-to-man differences within each of the three groups are caused by the random biological variation. The man-day interaction, on the other hand, represents a difference in the subject's level from day to day and there is no reason to believe that this difference follows any fundamental pattern or is anything but random from day to day; a similar argument holds for the hour-day interaction, if it exists.

The analysis of the data

Before proceeding to the actual analysis of variance, it was necessary to examine the distribution of the dependent variate, x_{ijkt}, to see whether this was best expressed in terms of absolute numbers of cells or of percentage variations about a basic level. In the first case, the random component is normally distributed, while in the second, the logarithm of the random component is normally distributed. The model relating to the latter hypothesis is in fact equivalent to (1), but with x_{ijkt} now representing the logarithm of the count instead of its absolute value. The second model is multiplicative, the effect of an element of variation now being represented by a multiplication of the variance and not by an addition to it.

Considering first the absolute values of all replicates, the distribution of the random errors was obtained and the values

$$\beta_1 = 0\cdot121, \quad \beta_2 = 6\cdot159. \tag{2}$$

were calculated. From the logarithms of the readings the values

$$\beta_1 = 0\cdot069, \quad \beta_2 = 3\cdot829, \tag{3}$$

were obtained. For a normal curve, based on 1440 observations, the values would be $\beta_1 = 0$, $\beta_2 = 3\cdot0$, with standard errors of 0·065 and 0·129 respectively.

It can be seen that whilst neither distribution is significantly skew, as measured by the magnitude of β_1, both curves show a significant departure from normality in the magnitude of β_2. Of the two, the logarithmic transform approaches much nearer to normality than the absolute values. It therefore seems advisable to reject the first hypothesis, that the model is additive and based on absolute values, and accept the second, that the model is multiplicative and based on the logarithmic transform, even though this latter results in a distribution of random errors which still departs from normality.

Examining now not the individual replicates but the 360 separate counts, considering the absolute values, the parameters

$$\beta_1 = 0\cdot007, \quad \beta_2 = 5\cdot89 \tag{4}$$

were calculated. For the logarithmic transform the values

$$\beta_1 = 0\cdot0, \quad \beta_2 = 3\cdot117 \tag{5}$$

were obtained. For a normal curve with 360 observations the values of $\beta_1 = 0$, $\beta_2 = 3\cdot0$ have standard errors of 0·129 and 0·259 respectively.

It can be seen that whereas the distribution of the absolute values of the counts still departs significantly from normal, the distribution of the logarithms of the counts can be accepted as normal.

It appears from the above that the biological variations in eosinophil counts are best expressed in terms of percentages and not of absolute values. In considering not the counts but the four individual replicates which make up each count, it is still preferable to consider the logarithmic values, even though the distribution of these values is not strictly normal.

From the analysis of variance of the 1440 separate readings, the estimated value of the residual variance is

$$\sigma^2 = 0{\cdot}008955 \quad \text{or} \quad \sigma = 0{\cdot}0946. \tag{6}$$

This value of σ is a logarithm and corresponds to an absolute value of 1·243. Thus there is a standard deviation of 24·3 % in the value of any replicate. On a count which is the sum of four replicates this would give rise to a variance of 0·002239 and a standard deviation of 0·0473, equivalent to 11·5 % due to random error alone.

Analysis of variance of the logarithms (to base 10) of the individual replicates gave the results shown in Table 2.

TABLE 2. Analysis of variance of the logarithms of individual replicates, four of which comprise a single eosinophil count

Source of variance	Factor	Sum of squares	Degrees of freedom	Mean square and significance of effect when tested against item shown in brackets
Between men	M	92·3429	19	4·8602†
Between hours (diurnal pattern)	H	0·2001	5	0·0400†
Between days	D	0·0945	2	0·0473 N.S. [M × D]
Individual day-to-day variation	M × D interaction	4·0821	38	0·1074* [M × H × D]
Individual diurnal pattern	M × H interaction	4·7612	95	0·0501* [M × H × D]
Changes in mean hourly values from day to day	H × D interaction	0·2873	10	0·0287 N.S. [M × H × D]
Random biological variation	M × H × D interaction	3·2515	190	0·0171* [R]
Random variation between replicates	R residual	9·6714	1080	0·008955

* = very highly significant ($P \ll 0{\cdot}001$). N.S. = not significant. † = Significance of these effects discussed in text.

CONCLUSIONS FROM SIGNIFICANCE TESTS

Comparison of the random biological variation (variance 0·0171, with 190 degrees of freedom) with the random counting error (variance 0·008955 with 1080 degrees of freedom) shows a very highly significant effect, i.e. it can safely be accepted that there is some random biological variation over and above the random counting error. Accordingly, all comparisons made in Table 2 and the inferences drawn are based on this random biological variation as the fundamental variation. The magnitude of the random component of the variation of a single count is found to be

$$\sigma^2 = \frac{0{\cdot}0171}{4} = 0{\cdot}004278$$

or $$\sigma = 0{\cdot}0654, \text{ equivalent to a standard deviation of } 16{\cdot}2\,\%. \tag{7}$$

From the analysis of variance shown in Table 2, it is seen that the between-men sum of squares is by far the largest component and is very highly significant. It is further obvious that the significance of this difference is in no way affected by considerations of the random or systematic nature of the man-hour interaction. This value of the mean square can therefore be reasonably accepted as a basis for the estimation of the population between-men variance for predictive purposes in the future, provided that there are no long-term trends in eosinophil counts which would bias this difference.

The 'between-days' effect is not significant when tested against the man-day interaction, which is the appropriate error term to use in this case. This indicates that taking blood samples from a subject on any one day does not affect the eosinophil count on subsequent days.

The decision as to the existence of a 'between hours' mean square, that is, of an average diurnal pattern, must be viewed against the assumption made in the model regarding the form of the man-hour interaction. The assumption that there is only a small limited number of different man-hour patterns (in this case three) and that the individual subjects in our group of twenty must fall into one or other of these three patterns, implies that if this group of twenty is considered in isolation, it is possible to judge the existence of an average diurnal pattern for this group by comparing the expected mean-square for the between-hours variance with the random hour-day interaction if it exists, or with the random biological variation if it does not exist. As from this analysis it is concluded that the hour-day interaction does not exist, the criterion is then a comparison of the between-hour variance with the random biological variation.

Testing for the existence of the average diurnal pattern on this basis, a difference is found which is significant at the 5 % level. There are thus reasonable grounds for believing that *for this group of twenty subjects* there is an average diurnal pattern. The existence of this diurnal pattern is established, however, only for the group considered and in no way establishes any property of the general population.

For an extension of this average effect to the whole population, it would be necessary to postulate that the twenty subjects in the group fall into the three distinct diurnal patterns in exactly the same proportion as the whole population. There is no obvious justification for this statement. It would have been of great value had it been possible from the data to confirm the existence of the three different patterns or to have found the mode of separation of the subjects into these three patterns, but, by reason of the comparatively large random biological variation relative to the small number of days' experience for each subject, this has not been possible.

Of the interactions, the hour-day interaction does not appear to exist, that is, there is no suggestion that the mean hourly count varies from day to day irrespective of the subject. Both the other interactions are found to be very highly significant. The existence of the highly significant man-hour interaction confirms that certain different diurnal patterns exist, although as has been stated earlier it is not possible to determine how many such patterns there are. The diurnal pattern shown in Fig. 1 is then the average of such distinct patterns taken over the twenty subjects in the trial group. The man-day interaction, implying that for any subject a different level is set each day, is also very highly significant. The extent to which this level varies is effectively random and from the results of Table 2 the calculated magnitude of its variance is

$$\sigma^2 = 0{\cdot}015052 \text{ or } \sigma = 0{\cdot}1227, \text{ equivalent to a standard deviation of } 32{\cdot}6\,\%. \quad (10)$$

The values of the variances for random counting, random biological variation and day-to-day difference in a specified subject can be combined to give the following results:

(*a*) The count for a given subject at a fixed time of day has a day-to-day variance of

$$(0{\cdot}004278 + 0{\cdot}015052) = 0{\cdot}019330, \text{ equivalent to a standard deviation of } 37{\cdot}8\,\%. \quad (11)$$

(*b*) The 95 % confidence limits for any count in one subject at a given time of day are 54–186 % of the mean value for the subject at that time.

(*c*) The 95 % confidence limits for the ratio of *two counts* for the same subject at the same time of day but *on different days* are 41–243 %.

(*d*) *On the average*, for the twenty subjects studied in the present investigation, the 95 % confidence limits for the ratio of *two counts* at different times *on the same day* are 67–150 % multiplied by the appropriate ratio shown in Fig. 1 for the times in question.

It should be noted that (*a*), (*b*) and (*c*) above are statements of general validity. On the other hand (*d*) was obtained by treating the man-hour difference as systematic, based on the assumption that a subject's diurnal pattern falls into one of three distinct groups. The result shown here thus holds only in respect of the average of the twenty subjects in one group and cannot be applied to any other subjects. It provides some indication, however, of the likely range of variation.

DISCUSSION

The statistical analysis shows that in normal subjects, proportionate, but not absolute changes in eosinophil count can be treated as if they were normally distributed. The eosinopenic response to adrenocorticotropin (ACTH) has also been shown to be proportionate (Hills, Forsham & Finch, 1948). This suggests that the pituitary-adrenal mechanism might be causing normal variations. Both the differences in the mean level of the eosinophil count from day to day and the slow periodic variations during the day might result from alterations in the rate of secretion of adrenocortical hormones (Halberg, Flink & Visscher, 1951). In Addison's disease, random variations in eosinophil count are still present, though limited in size (Halberg *et al.* 1951; Flink & Halberg, 1952; Bonner, 1952), but, as the last-named author points out, remnants of the adrenals may remain active in this condition. However, Swingle, Eisler, Ben, Maxwell, Baker & Le Brie (1954) have observed a profound eosinopenia in adrenalectomized dogs exposed to stress. In a later publication, Swingle, Eisler, Baker, Le Brie & Brannick (1955) showed that prior treatment of such animals with the antihistaminic drug tripelennamine hydrochloride (Pyribenzamine, Ciba) abolished the eosinopenic effects of histamine itself, posterior pituitary extract (Pituitrin, Parke, Davis), epinephrine, phentolamine (Regitine, Ciba) and compound 48–80. The fall in eosinophils caused by cortisone was not affected, however. In view of these findings it seems probable that noxious agents may cause eosinopenia both through the pituitary-adrenal mechanism and directly as a result of the release of histamine from tissues. It must be concluded, therefore, that the precise physiological basis of observed changes in the eosinophil count of an intact animal or human subject is not easily defined. It is possible that further work on the ingestion of eosinophils by macrophages, which is stimulated by cortisone (Padawer & Gordon, 1952) and on the release of eosinophils from the bone marrow, which is inhibited by glucocorticoids (Essellier, Jeanneret & Morandi, 1954) might elucidate the origin of all these types of variation.

The results of the present investigation confirm the existence of individual diurnal patterns. However, owing to the large size of the random biological variation, the data in Table 1 do not permit discrimination between Mann & Lehmann's (1952) view that there are only three types of diurnal pattern and any other hypothesis about the number of different types.

The curves of group means given by Rud (1947), Tatai & Ogawa (1951) and Donato & Strumia (1952), which show a minimum count in the middle of the day, resemble Fig. 1, while similar results led Swanson *et al.* (1952) to recommend that tests involving the measurement of a fall in eosinophils should be carried out in the afternoon. It seems likely, therefore, that a diurnal pattern of this type is a feature of group means. However, on examining Table 1, it is found that twenty-two out of the sixty records or, taking geometrical means, eight out of the twenty subjects, show a fall between 2 and 4 p.m. in spite of the general trend in the opposite direction as exemplified by the curve in Fig. 1. Consequently the essential condition for comparative studies is that measurements on the same subject should always be made at the same time of day, not necessarily only in the afternoon.

The ranges of variation in the eosinophil count of an individual subject, given in the statistical section of this communication, are similar to those observed by Rud (1947) and by Fisher & Fisher (1951), although the latter workers do not express their results in terms of confidence limits, thus making detailed comparison impossible. Rud (1947) derived his results from scatter diagrams in which the greatest positive and negative deviations from the mean count of a number of individuals were plotted against the mean count. Two straight lines were drawn on the diagram to represent the upper and lower limits respectively of individual counts. These lines, which did not, however, enclose all the points in the scatter diagram, were used subsequently to calculate maximum and minimum counts for the same subject as percentages of the mean count. His limits varied from 28 % and 200 % for an average count of 25 cells/mm^3 to 33 % and 179 % for an average count of 350 cells/mm^3. Comparable figures for the present series are 54–186 %. The small effect due to diurnal periodicity shown by the latter group of subjects would, if its maximum influence were exerted, reduce the lower value to 50 % and increase the higher to 201 %. The upper limits of the two series are in fairly good agreement but the lower limits given by Rud (1947) are smaller than those obtained in the present investigation. It is possible that this worker's use of only one counting chamber and a 1:20 dilution for each count might increase the likelihood of obtaining a low result, as might his use of a dilution fluid containing acetone. Fluids containing the latter solvent have been shown by Henneman *et al.* (1949) to cause eosinophils to fragment and disappear, giving low counts after the first 15 min of contact with the solution.

Normal variations in the eosinophil count are important since they might interfere with tests of adrenocortical reserves, such as that of Thorn, Forsham, Prunty & Hills (1948). These authors state that in normal subjects 25 mg of ACTH injected intramuscularly causes a fall in eosinophil count to at least 50 % after 4 hr. Others have found that occasional patients suffering from Addison's disease may show in this test an eosinopenia of more than 50 % (de Mowbray & Bishop, 1953) and that occasional normal individuals show a response of less than 50 %, although the ACTH preparations used retain full activity (Best, Muehrcke & Kark, 1952). These anomalous results might well have been caused by normal variation, within the limits suggested above, superimposed on the effect of added ACTH. Thorn, Goetz, Streeter, Dingman & Arons (1953) have devised an intravenous ACTH test and use the original intramuscular test only for 'screening'. In normal subjects an intravenous infusion of 20 U.S.P. units of ACTH during an 8 hr period is claimed to cause a fall in eosinophils of 70–100 %, measured 8–10 hr after commencing the infusion, while patients with Addison's disease show a change of +40 to −30 % in the same conditions. This intravenous test should eliminate false negative responses in healthy individuals but might not eliminate false positives in Addison's disease.

Thorn *et al.* (1953) consider that the response to ACTH is best followed by the increase in 17-hydroxycorticosteroid excretion and by the increased ratio of uric acid to creatinine in the urine. However, it is likely that eosinophil counts will continue to be used in some investigations as they are relatively simple to carry out. The performance of duplicate tests for clinical purposes as suggested by Best *et al.* (1952) should eliminate most of the unsatisfactory responses, while due attention to the normal level of eosinophil variation in planning physiological experiments should enable valid results to be obtained. The analysis of variance given above provides data which can be used for the latter purpose. It is felt, furthermore, that the method adopted in the present investigation could be applied to studies on normal changes in other physiological variables.

SUMMARY

1. Considerable hourly changes in eosinophil count were observed in twenty subjects during three successive days.
2. Individuals showed definite diurnal patterns. There was no evidence that one pattern was common to all subjects nor could the total number of basic patterns be determined.
3. A diurnal pattern was present in the group mean, statistically significant *only for the twenty subjects studied.*
4. Significant (random) day-to-day differences within the same subject were found.
5. Normal variability in eosinophil count is discussed in relation to physiological and clinical investigations.

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