A QUANTITATIVE STUDY OF THE EFFECTS OF COMPOUND E, COMPOUND F, AND COMPOUND A, UPON THE BONE MARROW OF THE GUINEA-PIG

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INTRODUCTION

The present work is a continuation of earlier attempts to study changes in the bone marrow by quantitative methods (Yoffey & Parnell, 1944; Yoffey, Metcalf, Herdan & Nairn, 1951; Hudson, Herdan & Yoffey, 1952). In a previous communication (Hudson *et al.* 1952) there were described the effects upon the bone marrow of daily injections of ACTH given for 7 successive days. It was thought possible that ACTH might act, either directly or indirectly, as a marrow stimulant, for it appeared to increase the total absolute count of nucleated marrow cells. The effect was evident both in the myeloid and in the erythroid cells, though more so in the latter.

However, in these experiments ACTH was administered to only nine animals. Since the result, if true, might be of fundamental importance in haematological work, it seemed desirable to try and establish the myeloplastic action of ACTH on a firmer foundation, and it was at first decided to repeat on a larger scale the experiments which had already been performed. On further reflexion, however, it was thought that ACTH might introduce too many variables, e.g. the uniformity of the ACTH used, the possibility of contamination, doubts as to whether any effects obtained might be due to ACTH itself or to suprarenal steroids, uncertainty whether the suprarenal cortex itself would always respond in the same way to ACTH stimulation, or whether a response by the output of several steroids would confuse the result unnecessarily. For reasons such as these it was finally thought preferable to use individual steroid hormones, compound E (cortisone), compound F (hydrocortisone) and compound A, although even with these there is always the possibility that any results obtained may be due not to the action of the particular steroid used, but to one or other of its intermediate compounds, or the induced metabolic effects.

MATERIAL AND TECHNIQUE

Animals

The work was done throughout on normal male guinea-pigs, of the Mill Hill strain originated by Dunklin and Hartley. Healthy animals about 2–3 months old were obtained from the University of Bristol Veterinary Field Station at Langford, and were kept in individual cages where they were carefully observed for 14 days before use.

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Compounds injected

The experimental animals were given intraperitoneal injections of compound E, compound F or compound A suspended in a special medium. The greater part of the material used was supplied to us through the courtesy of Dr J. E. Garber of the Research and Development Division of Messrs Merck and Co., Rahway, New Jersey. Additional supplies of compound E and compound F, also prepared by Messrs Merck in an identical manner, were obtained from the Medical Research Council. For compound A we are indebted to Dr Choh Hao Li, of the University of California. The control animals were given daily injections of the suspending medium alone, consisting of: sodium chloride, 0·9 g.; benzyl alcohol, 0·9 ml.; polysorbate USP (Tween 80—Atlas Powder Co.), 0·4 g.; sodium carboxymethylcellulose (low viscosity), 0·4 g.; distilled water to 100·0 ml.

All the steroid compounds were given in single doses of 5 mg. daily for 7 days.

Altogether, seventy-seven experiments were performed, of which thirty were controls, twenty-nine were given compound E, ten compound A, and eight compound F. The compound E experiments were performed by three different groups of observers (groups I–III), each being responsible for ten controls and ten compound E animals, except that in one instance a compound E experiment was so obviously at variance with the entire series that it was discarded.

Experimental procedure

Animals were weighed at the beginning and end of the experimental period, as also were the two adrenal glands when the animals were killed. Peripheral blood, obtained from an ear vein, was examined at the beginning and end of each experiment; absolute counts were made of the total red and white cells and reticulocytes, as well as differential counts of the white cells. No special method, however, was used for the eosinophil counts. Haemoglobin was estimated by the Sahli method.

At the termination of each experiment, the second blood count was performed, after which under ether anaesthesia the abdomen was opened and the animal bled as freely as possible by cutting the lower end of the abdominal aorta. The blood was collected by a funnel into 15 ml. tubes, which were centrifuged immediately at 3500 r.p.m. in order to obtain serum.

Apart from obtaining serum, it appeared desirable, from the point of view of quantitative examination of marrow elements, to drain away from the marrow as much blood as possible, and it was thought that exsanguination might help considerably towards this end.

A small corked glass tube, between $1\frac{1}{2}$ and 2 in. long, internal diameter about $\frac{4}{16}-\frac{5}{16}$ in., is first weighed, then about half filled with autogenous serum, and weighed again. A humerus is then removed and cleaned, its two ends cut off, and the marrow ejected by a blower into the serum-containing tube which is then weighed for a third time. In this way one obtains a known weight of marrow suspended in a known weight of serum. The tube is then shaken in a mechanical shaker of about 6 in. amplitude for 2 min.—occasionally a little longer, if needed—at 400 times per minute. In the majority of cases the marrow disintegrates so that a uniform suspension is obtained, from which counts can be made in the usual manner, and also

dry smears can be prepared and stained. Immediately after shaking the suspension may be somewhat frothy, but the froth soon disappears. Before making a count or smear the tube is shaken by hand, or smartly tapped a few times. If this is not done the larger cells sink to the bottom of the tube, and only the smaller ones are obtained.

Specific gravity

If the specific gravity of both serum and bone marrow were precisely 1·0, the weights could be used to calculate the dilution. However, this is not the case, and so in each series there were calculated conversion factors, based upon specific gravity estimation both of bone marrow and serum. For the maximum degree of accuracy specific gravity determinations should perhaps have been made in each individual experiment. But in view of the large numbers of observations required in each case this was not found feasible; hence in each group of experiments a conversion factor calculated from seven or eight experiments was applied to the remainder of the group. The actual specific gravity estimation was done by a modified pyknometric technique.

Dilution of marrow with residual blood

In the absolute counts a modified Toisson's fluid, without acetic acid, was employed. This enabled absolute counts of both red and white cells to be made. The number of mature red cells in the bone marrow was taken as an indication of the maximum possible contamination with residual blood. The average red cell content in the entire series was approximately 700,000 per cu.mm. of marrow, while the red cells in the circulating blood were approximately 6,000,000 per cu.mm. It will be noted that the figure for marrow erythrocytes is considerably lower than in the previous paper (Hudson *et al.* 1952). This is possibly due to the fact that cutting the abdominal aorta is a more effective means of exsanguination than that formerly employed.

If all the red cells present in the marrow were in fact derived from residual blood, and not newly formed erythrocytes about to be discharged into the circulation, the marrow cells would be diluted with blood by about 1:9 and the calculated absolute counts would be proportionately below their true level. It should, however, be noted that the average marrow reticulocyte count is of the order of 300,000 per cu.mm.; these presumably have just been formed in the marrow and are awaiting discharge into the circulation, so that the marrow red cell count due to residual blood would be about 400,000 per cu.mm.

STAINED SMEARS

Dry smears were prepared on standard microscope slides and stained with Wright's stain, or MacNeal's tetrachrome, which was found particularly useful for the differentiation of cell granules. The preparation of the smears presents some difficulty, for if they are made slowly, the larger cells accumulate at the edges. If, on the other hand, the smears are made quickly, there is an even distribution of cells, but they are rounded off and not sufficiently spread out to facilitate the accurate observation of cytoplasmic and nuclear detail for purposes of cell identification. Furthermore,

in thin smears there is an increased number of damaged cells, which even in successful preparations can reach quite an appreciable figure (Table 2).

Accordingly, in each experiment fifteen to twenty marrow smears were made at different speeds, and then quickly examined unstained; a few slides with good distribution and relatively few damaged cells were then selected and stained. It was thought at one time that a Perspex spreader ($\frac{3}{4}$ in. wide) seemed to produce better smears on the whole, with less damage to the cells, than the ordinary glass type; but while this was undoubtedly true on occasion, it is by no means a constant phenomenon. Counts were made at right angles to the long axis of the smears, as an additional safeguard against minor errors of distribution, and as a rule 1000 cells were counted.

SUPRAVITAL PREPARATIONS

In an attempt to throw light on the nature of the damaged cells, the marrow was studied in a number of cases by the supravital technique. After a number of trials the aqueous method was adopted in preference to the alcoholic, since it was found to give more uniformly reliable results. One or two drops of marrow were mixed with an equal quantity of dilute stain in a small ignition tube. The stain was freshly prepared by mixing solutions of neutral red (1/10 % in Ringer-Locke) and Janus green (1/10 % in Ringer-Locke) in proportions varying from 2:1 to 1:1 to 1:2 according to the concentration of cells in the marrow suspension. The mixture was gently shaken for 5–10 min. at room temperature, then left to stand in an incubator at 37° C. for 30 min. A drop of this mixture was then placed on a slide, and the cover-slip sealed with wax to prevent evaporation; the preparation was examined under oil immersion as rapidly as possible, a minimum of 500 cells being counted.

Teased preparations

In each animal pieces of abdominal and cervical lymph glands and thymus were teased in autogenous serum, and smears made and stained, in order to have undoubted lymphocytes for reference. This was important since one of the cells about whose identification we were particularly concerned was the small lymphocyte. It was also found that if fragments of bone marrow were similarly treated, the reticulum cells were damaged considerably less than in the standard preparations which were shaken for 2 min. Although unfortunately these teased preparations could not be used for quantitative purposes, as they contained numerous cell clumps, they gave valuable morphological information.

THE CLASSIFICATION OF CELLS

(1) *Supravital preparations*

In the supravital preparations, cell identification was based on the description given by Cunningham, Sabin & Doan (1925), and Doan, Cunningham & Sabin (1925). In general, it was found that there was fair agreement between these descriptions and the appearances seen in our preparations. There was one important exception, however, for in our supravital preparations there were a number of large cells with spherical nuclei, and cytoplasm containing only a few large mitochondria. These are not the primitive cells of Cunningham *et al.* (1925), though they may possibly

correspond to the reticulum cells (Pl. 1, figs. 1–7) of the stained smears. However, as this is not certain, the cells have been placed in the unclassified group.

Lymphocytes were identified as such only if they resembled similar cells obtained from a teased lymph gland of the same animal. The nuclei, frequently indented, are typically large in relation to the cytoplasm, which forms a thin rim containing mitochondria and a few small red vacuoles.

(2) *Stained smears*

The reticulum cells, for the reasons already mentioned, could not be studied quantitatively. Apart from these, the classification of cells was into four main groups, myeloid, erythroid, monocytoid and lymphocytic. In addition, there was a large group of damaged cells, a varying number of unclassified cells, and a few miscellaneous cells such as plasma cells and macrophages.

Reticulum cells present a number of difficulties, for the term, though widely used in haematological literature, is usually somewhat loosely defined. The most obvious use of the term is for cells which are actually arranged in a reticulum, and the tearing away of these cells from such a reticulum might account for the fact that they are so readily damaged. It was in this sense apparently that Cunningham *et al.* (1925) used the term (or rather 'reticular'). Pl. 1, figs. 1–7, illustrate what are described as reticulum cells in the present investigation, but we are not altogether satisfied that they are in fact part of a network of cells.

These reticulum cells seem to correspond to what Rohr (1949) calls large reticulum cells (his small reticulum cells being almost impossible to distinguish from the small lymphocyte), and they also in some ways resemble what Ferrata (1918) has termed haemohistoblasts, more especially those found under certain pathological conditions (1918, Vol. II, Tav. XV).

The reticulum cells consist of pale grey-blue cytoplasm, in which there is a characteristic leptochromatic nucleus, with a clearly defined border, chromatin strands of uniform and equal thickness (isochromatic), and several nucleoli. These cells form an appreciable, possibly even the major, part of the damaged cells; and they may often be recognized, even when the cytoplasm is all gone and the nucleus is swollen and disrupted, by the persistence of the more resistant nucleoli. It is not difficult to find transition stages between these cells and the typical myeloblasts, and usually as the cytoplasm becomes more basophilic the nuclear chromatin loses its sharply defined network, while the nucleoli tend to become less conspicuous and disappear.

Lymphocytes (Pl. 1, figs. 8–14) were classified on the basis of their morphology, and as in the supravital preparations, cells in stained smears of lymph glands (Pl. 1, figs. 12–14) were carefully examined in all experiments before performing the differential count of the bone marrow and the identification of marrow lymphocytes (Pl. 1, figs. 8–11). The number of lymphocytes was smaller in the supravital preparations than in the stained smears (Table 3), and this was largely due to the difficulty of distinguishing between lymphocytes and monocytes in the supravital preparations. This is an old problem (see, for example, Cappell, 1929; Wiseman, 1931–2; Hall, 1938; Drinker & Yoffey, 1941), and since the significance of the neutral

red bodies has been so frequently questioned, the stained smear figures are likely to be more reliable.

It should be emphasized that practically all the marrow lymphocytes are small lymphocytes, though a very few were found of medium size (Pl. 1, fig. 10). The nucleus of medium lymphocytes tends to be more leptochromatic than that of the small, though some definite chromatin clumps are still present. No cell with a fully leptochromatic nucleus has been counted as a lymphocyte.

The granulocytes (Pl. 2, figs. 16–23) and the erythroid series (Pl. 2, fig. 24, and possibly fig. 15) call for little comment. The great majority of the myeloid cells belong to the neutrophil—or in the guinea-pig more accurately pseudo-eosinophil—group. Basophil myelocytes (Pl. 2, figs. 19–20) are few in number, but form a very striking group of cells. Though it is quite true that on occasion some of the basophil granules seem to have a quite distinct eosinophil staining component, nothing was seen to indicate the transformation of basophil into eosinophil myelocytes as described by Downey (1915), though very rarely we saw, in an otherwise typical eosinophil cell, one or two deeply basophilic granules.

Monocytes were seen developing from blast cells like those of the myeloid and erythroid series. The term 'metamonocyte' is used for the cell depicted in Pl. 1, fig. 11.

RESULTS

(1) *Specific gravity*

Table 1 gives the specific gravity of serum and bone marrow with the calculated conversion factors, in seven control guinea-pigs, seven treated with compound E, and eight with compound F. The specific gravity of the bone marrow has increased in both the compound E and the compound F experiments, the mean increase in the former case reaching the conventional significance level ($t_{C-E}=2{\cdot}432$, $P<0{\cdot}5$; $t_{C-F}=1{\cdot}74$, $P>0{\cdot}10$).*

(2) *Cellular changes*

(*a*) *Total count*

The compound E experiments, as has already been noted, were performed by three different groups. The total absolute count of nucleated cells showed a marked mean increase in group I ($t_{C-E}=2{\cdot}5$, $P<0{\cdot}025$, the means rising from 1,689,900 per cu.mm. to 2,329,700), and group II ($t_{C-E}=2{\cdot}23$, $P<0{\cdot}05$, the means rising from 1,685,000 to 2,131,600), whereas in group III there was actually a slight fall, not significant ($t_{C-E}=0{\cdot}19$, $P>0{\cdot}50$, mean falling from 1,605,300 to 1,572,000). In the one group of compound F experiments there was also a mean rise in the total count ($t_{C-E}=2{\cdot}10$, $P=0{\cdot}05$, means rising from 1,689,900 to 2,141,130). In the ten compound A experiments, the mean count was 1,438,200. But this may in part be due to the fact that one of the counts at 925,900 was well below the usual range and showed other abnormal features also. The discrepancy between group III and the other groups is one for which no explanation can be offered. The effect of the initial discrepancy in the absolute count is subsequently reflected to a large extent in the

* In giving these '*t*' values, C = control series, A = compound A, E = compound E, F = compound F experiments, *t* = Student's *t*, *P* = probability of a greater *t* value arising on pure chance.

absolute counts of the various cell groups. Table 2 gives the results of group I and illustrates the way in which the mean data of the different groups were arranged for the purposes of analysis.

Table 1. *Specific gravity of serum and bone marrow, with conversion factor, in seven control guinea-pigs, seven treated with compound E, and eight with compound F*

Serial no. of animal	Serum specific gravity	Bone marrow specific gravity	Conversion factor
	Control series		
A7	1·0200	1·0284	1·0078
A9	1·0184	1·0313	1·0130
A11	1·0217	1·0341	1·0118
A13	1·0191	1·0242	1·0050
A15	1·0209	1·0174	0·9966
A17	1·0193	1·0468	1·0271
A19	1·0205	1·0363	1·0158
Arith. mean	1·0200	1·0312	1·0114
S.D.	0·001124	0·00870	0·00847
	Compound E series		
A8	1·0248	1·0450	1·0195
A10	1·0253	1·0651	1·0389
A12	1·0252	1·0548	1·0292
A14	1·0370	1·0829	1·0442
A16	1·0207	1·0507	1·0294
A18	1·0200	1·0442	1·0238
A20	1·0225	1·0420	1·0191
Arith. mean	1·0249	1·0549	1·0320
S.D.	0·00526	0·01355	0·00836
	Compound F series		
A21	1·0139	1·0620	1·0473
A22	1·0220	1·0240	1·0021
A23	1·0194	1·0442	1·0248
A24	1·0223	1·0500	1·0274
A25	1·0120	1·0743	1·0632
A26	1·0099	1·0396	1·0500
A27	1·0211	1·0420	1·0297
A28	1·0194	1·0560	1·0363
Arith. mean	1·0175	1·0490	1·0340
S.D.	0·00453	0·0391	0·0180

(*b*) *Erythroid cells*

The erythroid cells show a mean rise in all three groups (group I mean rises from 358,400 to 659,930, $t_{C-E}=3·82$, $P<0·01$; group II mean rises from 368,720 to 672,000, $t_{C-E}=3·58$, $P<0·01$; group III mean rises slightly from 376,000 to 411,600, $t_{C-E}=0·53$, $P>0·50$; compound F mean rises from 358,440 to 522,080, $t_{C-E}=2·40$, P approx. 0·025). If one combines all these results, then $t_{C-E}=2·9$, $P<0·01$, and the mean rises from 367,700 to 561,000. It will be noted that even in group III, where the increase does not reach the conventional significance level, the trend is, nevertheless, in the same direction. The supravital counts in this latter group showed a somewhat more marked rise, the mean going up from 324,700 to 470,000, but it is difficult to make any direct comparison between these and the counts in the stained preparations.

(*c*) *Myeloid cells*

Though the myeloid cells have increased in all compound E experiments, the mean increase is not as marked as in the case of the erythroid cells. The figures are: group I, 602,330–745,070, $t_{C-E}=1{\cdot}57$, $P>0{\cdot}10$; group II, 579,230–765,600, $t_{C-E}=1{\cdot}85$, $0{\cdot}05<P<0{\cdot}1$; group III, 608,600–625,600, $t_{C-E}=0{\cdot}22$, $P>0{\cdot}50$.

Table 2. *Absolute counts per cu.mm. (group I) of the main groups of nucleated cells in the bone marrow of ten control guinea-pigs, ten after administration of compound E (5 mg. daily for 7 days) and eight after administration of compound F (5 mg. daily for 7 days). (Eosinophils are given in separate column, and also included among 'Myeloid cells'.)*

No. of exp.	Erythroid	Myeloid	Eosino-phils	Lympho-cytes	Monocytes	Damaged	Unclassi-fied	Total abs. count of nu-cleated cells	M:E ratio
				Control					
A1	185,000	476,000	87,050	272,000	142,800	234,000	31,800	1,360,000	2·58
A3	166,500	383,200	38,800	193,900	36,680	221,000	41,890	1,048,000	2·30
A5	322,210	472,200	99,000	432,000	95,350	182,000	79,780	1,596,000	1·46
A7	234,000	418,800	98,100	259,800	62,080	276,200	55,220	1,381,000	1·81
A9	430,800	831,200	72,400	386,800	124,800	231,300	61,400	2,079,000	1·94
A11	524,000	551,800	41,800	349,000	115,600	350,600	35,680	1,995,000	1·05
A13	415,100	609,312	53,661	320,235	100,398	252,726	27,696	1,731,000	1·47
A15	457,090	846,060	108,900	247,000	114,760	243,110	29,174	1,945,000	1·85
A17	255,500	582,100	59,990	260,000	75,420	180,400	34,920	1,396,000	2·28
A19	594,200	820,000	71,100	478,800	65,800	226,800	73,500	2,368,000	1·38
Mean	358,440	599,067	73,076	319,954	93,369	239,814	47,106	1,689,900	1·81
				Compound E (acetate)					
A2	1,015,000	796,000	205,000	468,000	247,900	355,800	94,900	3,060,000	0·78
A4	416,500	556,000	66,000	333,100	86,900	170,600	46,710	1,610,000	1·33
A6	566,800	566,800	115,700	375,000	119,700	309,000	45,970	1,995,000	1·00
A8	554,000	610,000	100,500	398,000	75,800	193,600	66,300	1,898,000	1·10
A10	990,000	1,162,000	147,600	534,900	115,800	570,000	70,400	3,510,000	1·17
A12	490,100	671,800	101,260	451,900	68,400	268,100	46,300	2,010,000	1·37
A14	713,800	1,130,000	109,300	474,900	150,500	330,800	71,986	2,876,000	1·58
A16	515,500	520,000	88,080	278,000	110,200	217,000	47,490	1,695,000	1·01
A18	635,920	686,120	83,234	255,800	79,180	310,600	44,675	2,030,000	1·08
A20	700,800	752,900	36,580	528,600	85,000	392,000	128,000	2,613,000	1·08
Mean	659,842	645,162	105,325	409,820	113,938	311,750	66,271	2,329,700	1·15
				Compound F (free alcohol)					
A21	544,750	450,400	34,506	490,350	54,475	207,010	59,923	1,816,000	0·83
A22	371,500	560,000	91,550	448,300	73,240	291,000	65,880	1,831,000	1·51
A23	302,500	446,900	58,390	259,600	57,180	174,100	54,880	1,298,000	1·48
A24	486,000	706,000	87,220	436,000	122,500	228,200	72,310	2,078,000	1·45
A25	748,000	950,800	55,600	581,800	210,200	423,200	151,500	3,090,000	1·27
A26	603,260	853,890	155,780	340,650	96,983	449,480	10,643	2,460,000	1·42
A27	511,550	771,610	133,350	378,180	109,270	330,140	65,584	2,186,000	1·51
A28	609,120	839,080	105,420	424,220	97,162	317,620	61,616	2,370,000	1·38
Mean	522,150	697,440	90,157	419,890	102,501	302,594	67,796	2,141,000	1·36

(*d*) *Myeloid: erythroid ratio*

The mean ratio dropped markedly in group I (1·83–1·14, $t_{C-E}=4{\cdot}2$, $P<0{\cdot}01$); not so markedly in group II (1·661–1·275, $t_{C-E}=2{\cdot}17$, $P<0{\cdot}05$); and only slightly in group III (1·616–1·520, $t_{C-E}=0{\cdot}22$, $P>0{\cdot}50$). In all three groups, though the total count of myeloid cells had risen, that of the erythroid cells had risen rather more, and hence the myeloid:erythroid ratio has fallen.

(*e*) *Lymphocytes*

The marrow lymphocytes have shown no consistent trend. They rose significantly in group I ($t_{C-E}=2{\cdot}12$, $P<0{\cdot}05$), and slightly in group II ($t_{C-E}=0{\cdot}43$, $P>0{\cdot}50$), whereas they fell in group III ($t_{C-E}=0{\cdot}98$, $0{\cdot}10<P<0{\cdot}50$). Combining all the compound E experiments, the mean lymphocyte count rises from 322,000 to 338,420 ($t_{C-E}=0{\cdot}38$, $P>0{\cdot}50$).

Table 3. *A comparison of the supravital and stained smear techniques. Absolute counts per cu.mm. of the main cell groups in the bone marrow of ten guinea-pigs after the administration of compound A (5 mg. daily for 7 days)*

No. of exp.	Total eryth-roid	Total myeloid	*Eosino-phils	Total lympho-cyte	Mono-cyte	Damaged	Unclassi-fied	Others†	Total nucleated cells	M:E ratio
					Supravital					
H21	413,700	600,100	35,000	113,600	192,300	—	113,600	23,300	1,456,600	1·45
H22	229,600	374,000	16,600	114,800	74,100	—	105,500	27,900	925,900	1·63
H23	278,800	728,600	17,900	137,900	152,900	—	80,900	120,000	1,499,100	2·62
H24	341,000	721,600	53,000	241,600	188,700	—	66,200	95,900	1,655,000	2·12
H25	262,200	957,800	117,700	90,800	141,200	—	77,300	151,100	1,680,400	3·66
H26	301,000	804,800	72,000	266,000	180,000	—	32,600	52,100	1,635,700	2·68
H27	184,800	639,400	102,400	122,400	87,400	5,000	85,000	124,800	1,248,800	3·46
H28	492,000	755,200	84,200	188,000	58,400	9,800	77,800	39,600	1,620,800	1·55
H29	367,200	597,000	35,000	189,400	113,600	—	67,000	123,100	1,457,300	1·63
H30	214,000	490,600	31,200	151,400	141,800	5,000	122,600	77,000	1,202,400	2·29
Averages	308,400	667,000	56,500	161,600	133,000	2,000	82,800	83,400	1,438,200	2·31
					Stained smears					
H21	332,100	606,000	55,000	135,500	182,100	150,000	27,700	23,200	1,456,600	1·83
H22	199,100	369,400	27,700	168,500	43,500	97,300	31,500	16,600	925,900	1·86
H23	379,300	500,700	44,900	319,300	71,900	131,500	34,500	61,900	1,499,100	1·36
H24	448,500	680,200	115,800	147,300	142,300	177,100	15,000	44,600	1,655,000	1·52
H25	327,700	784,700	89,100	201,700	92,400	201,700	16,800	55,400	1,680,400	2·40
H26	340,200	494,000	47,400	616,700	40,900	72,000	37,600	34,300	1,635,700	1·45
H27	332,200	422,100	53,700	144,900	40,000	229,800	15,000	64,800	1,248,800	1·27
H28	518,700	656,400	27,600	273,900	22,700	71,200	21,000	56,900	1,620,800	1·27
H29	304,600	587,300	29,100	347,000	58,300	94,700	18,900	46,500	1,457,300	1·93
H30	221,400	486,900	20,400	282,700	28,900	11,800	30,100	40,600	1,202,400	2·20
Averages	340,400	558,800	51,100	263,700	72,300	133,800	24,800	44,400	1,438,200	1·71

* Eosinophils are also included in the column headed 'total myeloid'.
† 'Others' comprises a small group of miscellaneous cells such as plasma cells, macrophages, reticulum cells.

(*f*) *Eosinophils*

The eosinophils do not show a constant trend. The means and '*t*' values for the three groups are group I, 73,080–105,320, $t_{C-E}=1{\cdot}92$, $0{\cdot}05<P<0{\cdot}10$; group II, 61,330–93,580, $t_{C-E}=1{\cdot}42$, $0{\cdot}10<P<0{\cdot}50$; group III 50,400–56,500, ($t_{C-E}=0{\cdot}67$, $P>0{\cdot}50$). It will be seen that on the whole the tendency is for the marrow eosinophils to rise, but that nowhere does the rise attain the conventional significance level.

(*g*) *Reticulocytes*

There was no marked change in the marrow reticulocytes, though here, too, the mean values seemed to be rising in the experimental animals. Group I, 224,000–292,000, $t_{C-E}=1{\cdot}35$, $0{\cdot}10<P<0{\cdot}50$; group II, 279,030–394,590, $t_{C-E}=1{\cdot}65$, $P=0{\cdot}10$ approx.; group III, 277,780–325,300, $t_{C-E}=0{\cdot}40$, $P>0{\cdot}50$.

DISCUSSION

Dosage and sensitivity

In comparing the present results with those of other workers, it is important to bear a number of factors in mind. One factor is obviously that of dosage, differences in which can probably contribute very materially to differences in results. A second factor is that of sensitivity. Thus it is generally held that the guinea-pig is somewhat insensitive to cortisone. Species differences of sensitivity may be due not only to the substance administered, but also to variations in the metabolism of these substances and the intermediate products of that metabolism.

Specific gravity

The increase in specific gravity is in all probability attributable to an increase in the cellularity of the marrow, with a consequent diminution in its fat content. Mechanik (1926) has shown that fatty marrow has a specific gravity slightly below 1·0, red marrow somewhat above 1·0. In the present experiments similar considerations probably apply, except that all the marrows were 'red' to varying degrees. If this interpretation of the specific gravity changes is correct, it could be regarded as confirmatory of the increase in absolute count obtained in groups I and II. In group III, unfortunately, no specific gravity determinations were made.

However, though it may well be the case that the rise in specific gravity is probably associated with increased cellularity of the marrow, it should be noted that, in the twenty-two experiments in which the specific gravity of the marrow was actually determined, it was not found possible to establish a significant statistical correlation between the specific gravity and the total count of nucleated cells.

Lymphocytes

No significant difference between control and experimental series

As far as lymphocytes are concerned, there is no marked difference between the control and the experimental series. This is equally true whether the bone marrow is examined in stained smears or in supravital preparations. The figures for marrow lymphocytes are of the same order as those in normal guinea-pigs employed in previous experiments, and the injection of the suspending medium in the control experiments does not appear to have materially altered the number of these cells.

Large number of lymphocytes in marrow

Although, therefore, none of the steroid compounds employed seems to have had any obvious effect upon the marrow lymphocytes, the present—and much longer—series of experiments seems to confirm quite unequivocally the previous findings (Yoffey & Parnell, 1944) concerning the large numbers of lymphocytes in the bone marrow. As compared with the earlier—and technically much less satisfactory—results in the rabbit, the present series gives an average marrow lymphocyte content of 322,000 per cu.mm. This is about six times the concentration first noted in the rabbit, and when one compares it with a count of about 4000–5000 lymphocytes per cu.mm. of blood, it becomes clear that the high concentration of marrow lympho-

cytes completely rules out the possibility that their presence is due to blood contamination.

In an earlier paper (Yoffey & Parnell, 1944) attempts were made to compare the total blood and bone marrow lymphocytes, and correlate them with known data concerning thoracic duct lymphocyte output. It was then concluded that even on the basis of 60,000 lymphocytes per cu.mm., the number of lymphocytes in the marrow was sufficient to account for those daily leaving the blood. In the case of the guinea-pig, the present studies seem to make it clear that the number of lymphocytes in the marrow is well in excess of what would be needed to account for those disappearing from the blood.

Marrow lymphocytes occur as scattered cells

It is noteworthy that lymphocytes in the normal guinea-pig marrow occur as scattered single cells. It cannot be emphasized too strongly that in *normal* marrow one rarely sees even small accumulations of lymphocytes, still less organized follicles with or without germinal centres. The literature contains frequent references to the occurrence of lymphoid nodules in human marrow, and the significance of these has been previously discussed (Drinker & Yoffey, 1941; Jordan, 1935). But it is clear from the present material that, in the normal guinea-pig, lymphoid follicles are not a regular constituent of bone marrow, for in not a single one of the seventy-seven animals examined were any follicles found in occasional sections of the marrow. It is further to be noted that mitoses in marrow lymphocytes are singularly infrequent; we have not seen any, nor apparently have other workers seen more than a very few (cf. Leitner, 1949). The bone marrow, therefore, is not to be regarded as a region of lymphocyte production, unless one postulates a constant heteroplastic formation of lymphocytes from reticulum cell or myeloblast. Unlike lymphoid tissue, however (see, for example, Downey & Weidenreich, 1912), the heteroplastic formation of lymphocytes in the bone marrow is difficult to observe.

In fact, despite the large number of lymphocytes occurring in the bone marrow, it is infrequent to see evidence of transition forms which would indicate either the origin of lymphocytes from, or their transformation into, other cells. If such transformation occurs one can only assume that it must take place quite rapidly, so that as a rule it is not detected.

No lymphoclasic action of 11-*oxysteroids*

In the twenty-nine compound E marrows the average concentration of lymphocytes at 338,420 per cu.mm. showed a slight but insignificant increase over the normal. It is evident, then, that if there has been no significant increase in marrow lymphocytes after the administration of compound E (and similarly with compounds F and A), there is equally no obvious decrease. The bone marrow data do not therefore support the concept that the 11-oxysteroids possess a lymphoclasic action.

The identity of the lymphocyte

One further point needs perhaps to be made concerning the lymphocytes. They have been identified as such in the present experiments on morphological grounds; and after repeated comparison of cells in bone marrow and lymph node smears,

the evidence in favour of this identification appears sound. Other views, however, have been expressed. For example, Sabin, Miller, Smithburn, Thomas & Hummel (1936) examined the bone marrow of forty-nine young rabbits, and could not find a single lymphocyte. This finding is somewhat difficult to explain. Even in adult marrow it would be more than surprising that among many thousands of cells a few lymphocytes should not be found, if only as a result of blood contamination. In the marrow of young animals the presence of a fair number of lymphocytes would appear to amount to a certainty, and the fact that none was described suggests that the criteria employed in identification were faulty.

Sabin *et al.* (1936) must in fact be considered to have placed their lymphocytes in the same category as the 'primitive' cell, which they admitted (p. 115, *loc. cit.*) 'looks very much like the small lymphocyte' though it 'lacks certain signs of differentiation'. They emphasize that 'the so-called primitive cell occurs in the bone marrow diffusely scattered and not in the germ centre'. But this latter observation could, with equal validity, be adduced in support of the view that lymphocytes are continually passing into the marrow from the blood. Rohr (1949) does not completely deny the occurrence of small lymphocytes in the bone marrow, but considers that many of the cells which are so described are small reticulum cells, though he admits that the distinction between these cells and the small lymphocytes is one which may be extremely difficult to draw.

It seems to us that the evidence so far available definitely favours the view that the cells in question are small lymphocytes, and that they are continually migrating into the bone marrow from the blood. However, should this view prove to be erroneous, and should these cells belong to a different category, the quantitative data concerning their numbers would still possess great significance, and render it highly probable that they play an important part in the process of blood formation.

In ten compound A experiments an attempt was made to apply Sabin's own criteria, using the supravital technique for the identification of lymphocytes. Though it is true that in this instance (Table 3) there were fewer lymphocytes in the supravital preparations than in the stained smears, the former nevertheless gave the figure of 160,000 per cu.mm., which is still nearly three times as high as the original estimate of lymphocytes in rabbit marrow (Yoffey & Parnell, 1944).

Reticulum cells

In the compound A animals, granules were much more in evidence in the reticulum cells, which also appeared to be more numerous than in normal marrows, though for the reasons already given reliable quantitative data are not available. The granules in the reticulum cells are mainly azurophilic, and sometimes attain quite a large size (See Pl. 1, fig. 6). It would no doubt be quite possible to interpret these as phagocytic reticulo-endothelial cells. The literature contains conflicting views on the phagocytic nature of the early stem cells. Ferrata (1918: see his Tav. XX and XXI, Vol. 1) depicts as 'cellule emoistioblastiche' cells which all look like macrophages or monocytes. In his Tav. XXI the haemohistoblasts have ingested large amounts of lithium carmine. It is of interest, too, that the haemohistoblasts which he chooses for purposes of illustration are mainly taken from spleen, lymph gland,

or connective tissue, but not from bone marrow. Cunningham *et al.* (1925), on the other hand, depict their 'reticular' cell as one which is quite unspecialized and inert, incapable of phagocytic activity. (See also Pappenheim, 1919.)

The myeloid:erythroid ratio

On the whole the effect of compound E has been to increase the erythroid cell content of the marrow and probably the myeloid cells also, but the increase in the former has been greater, with the result that the M:E ratio has fallen. It is all the more interesting therefore to note that the effect of adrenalectomy appears to be the reverse. Thus Gordon, Piliero & Landau (1951), working with mice, found that following adrenalectomy, what they termed the E:M (erythroid: myeloid) ratio diminished, from 1·46 in the controls to 0·91 after 1 week and 0·73 after 2 weeks, but then started to rise somewhat, reaching 0·95 after 3 weeks, and 1·01 after 4 weeks. These changes in the marrow were accompanied by a mild anaemia. On the other hand, the administration of compound E (3 mg. daily for 14 days) appeared to restore the E:M ratio to near the normal level (1·29). This is somewhat difficult to reconcile with the finding that sections of bone marrow displayed 'a considerable reduction in total cellularity due to increased vacuolization'. The experiments just quoted seem to emphasize once again the problems of dosage and species. Gordon *et al.* (1951) gave their mice 3·0 mg. of compound E daily in order to prevent the development of a peripheral anaemia. Doses of 0·5 and 1·0 mg. were not effective, even though in relation to body weight they represent a much bigger dose than the 5 mg. per day given to the guinea-pigs in the present series of experiments.

Appearances suggesting degenerative changes in the marrow were also observed by Baker & Ingle in the rat (1948) after the administration of ACTH. Gordon *et al.* (1951) suggest that high doses of compound E—and presumably of ACTH also—induce an excessive deposition of fat, as previously noted also by Winter, Silber & Stoerk (1950). However, in the present experiments there was no sign whatever of increased deposition of fat in the bone marrow, and the dosage presumably is approximating more to the physiological levels.

Finally, as far as the bone marrow is concerned, the present findings seem to accord with the clinical conclusions of Wintrobe, Cartwright, Palmer, Kuhns & Samuels (1951) that '...the principal alterations in the blood seen in the diseases other than leukemia, were those of increased bone marrow activity'.

Blood changes

Few significant changes were observed in the peripheral blood. Lymphocytes showed no change of note in the compound E experiments, but in the compound A series there was an actual increase, the mean in ten experiments rising from 3890 to 5450 ($t_{C-A}=2·11$, $P=0·5$). It may be that one is dealing here with too small a series (10) of animals on which to base a really firm conclusion, but the result is so much at variance with what is usually thought to be the response of blood lymphocytes to 11-oxysteroids that it seemed worthy of note, and even of further investigation in a larger series. The blood eosinophils were not examined by a chamber technique, and no valid conclusions can be drawn from the ordinary method of counting.

There was also an increase in the blood reticulocytes in the twenty-nine compound E experiments, the mean count rising from 69,600 to 114,000, $t_{C-E}=2{\cdot}96$, $P<0{\cdot}01$. Despite the fact that only 1000 cells were counted in each case (see, for example, Marcussen, 1939) this increase is probably a reflexion of increased activity in the erythroid cells of the bone marrow, and may be regarded as affording confirmatory evidence of the stimulating action of compound E upon erythropoiesis. The rise in the blood reticulocytes appears also to argue against the view put forward by Quittner *et al.* (1951), to the effect that cortisone acts by producing a block to the escape of cells from the bone marrow.

The response to different steroids

One of the more interesting general problems in connexion with the steroid hormones is that of the differences in action between a number of closely related substances. The view seems to be gaining ground that compound F is more potent than compound E. Recently, Hungerford, Reinhardt & Li (1952) have reported that 'at comparable dose levels hydrocortisone is demonstrably and significantly more effective in producing a thoracic duct lymphopenia than is cortisone'. However, as far as the bone marrow is concerned, the present experiments have afforded no evidence of such a difference. It is true that only eight experiments were performed with compound F (see Table 2), but it seems difficult to believe that had a marked difference really existed, these experiments would have given no indication of it.

Although no very clear-cut differences could be established between compounds E and F, the compound A experiments appeared to reveal two distinctive features, to both of which reference has already been made. In the bone marrow there was noticed an effect upon granulopoiesis; granule formation seemed to occur with marked frequency in reticulum cells, which matured to a myelocyte stage rapidly, with little sign of typical myeloblast formation. If this observation is correct, more prolonged administration of compound A should result in an increase in myeloid cells which can be measured quantitatively. It would be interesting at the same time to note whether the more prolonged administration of compound A gives rise to an enhanced lymphocytosis.

SUMMARY

1. In seventy-eight male guinea-pigs, 2–3 months old, the action upon the bone marrow of a number of steroid hormones has been studied by a quantitative technique.

2. The administration of compound E, in doses of 5 mg. per day for 7 days, appears to stimulate increased red cell formation in the bone marrow. There may be a concomitant increase in the myeloid cells, but this is not so marked, and thus there is a fall in the myeloid:erythroid ratio.

3. Compound A given to ten animals (5 mg. daily for 7 days) gave rise to a moderate but definite lymphocytosis in the blood, while in the marrow it seemed to stimulate granulocyte formation.

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EXPLANATION OF PLATES

(Figs. 1–24. The figures are all untouched photomicrographs of stained smears, taken at a magnification of × 1330, of guinea-pig bone marrow or lymph node.)

PLATE 1

Fig. 1. Reticulum cell, without granules, from the bone marrow of a guinea-pig which has been given compound A. Note blue-grey somewhat mottled cytoplasm, with irregular edge. Nucleus possesses sharp nuclear membrane, and several nucleoli.

Fig. 2. Reticulum cell from bone marrow of a normal guinea-pig. A somewhat larger cell than no. 1.

Fig. 3. Reticulum cell from bone marrow of normal guinea-pig. Cytoplasm showing more obvious signs of damage than figs. 1 and 2.

Fig. 4. Reticulum cell—from bone marrow of normal guinea-pig—somewhat more damaged than fig. 3.

Fig. 5. From bone marrow of normal guinea-pig. Still more advanced stage of reticulum cell damage. The nucleoli still persist.

Fig. 6. From bone marrow of guinea-pig after treatment with compound A. Reticulum cell with granules, mainly azurophilic.

Fig. 7. From bone marrow of normal guinea-pig. Later stage than fig. 6 of transformation of reticulum cell to granulocyte. This type of cell has much in common with Pappenheim's leucoblast (1919).

Fig. 8. Small lymphocyte from bone marrow of normal guinea-pig. Typically pachychromatic nucleus, moderate cytoplasmic basophilia.

Fig. 9. Two small lymphocytes from bone marrow of normal guinea-pig.

Fig. 10. Small and medium lymphocyte from bone marrow of normal guinea-pig. The larger of the two cells has a somewhat more leptochromatic nucleus, and in some ways resembles a blast cell.

Fig. 11. Small lymphocyte and metamonocyte from bone marrow of normal guinea-pig.

Fig. 12. Two small lymphocytes from lymph gland (teased) of normal guinea-pig.

Fig. 13. Group of small lymphocytes from teased lymph gland of normal guinea-pig.

Fig. 14. One medium lymphocyte and two small lymphocytes from teased lymph gland of normal guinea-pig. Compare the medium lymphocyte with the similar cell in fig. 10.

PLATE 2

Fig. 15. From normal guinea-pig bone marrow ?myeloblast ?proerythroblast. The nucleus suggests a proerythroblast but the cytoplasm showed precisely the same staining as other cells which were undoubtedly myeloblasts. This cell then could be a myeloblast in early prophase or later telophase.

Fig. 16. From bone marrow of normal guinea-pig. Myeloblast with nucleus resembling that of the reticulum cell.

Fig. 17. Normal guinea-pig bone marrow. Very early promyelocyte with only a few granules. Swollen and pale nucleoli. Sharp nuclear border. Also one pseudo-eosinophil myelocyte and two metamyelocytes. The granules in the myelocyte give the impression of being more eosinophilic than those in the metamyelocytes.

Fig. 18. Normal guinea-pig bone marrow. Large early myelocyte.

Fig. 19. Normal guinea-pig bone marrow. Basophil myelocyte, two eosinophil metamyelocytes and one pseudo-eosinophil metamyelocyte.

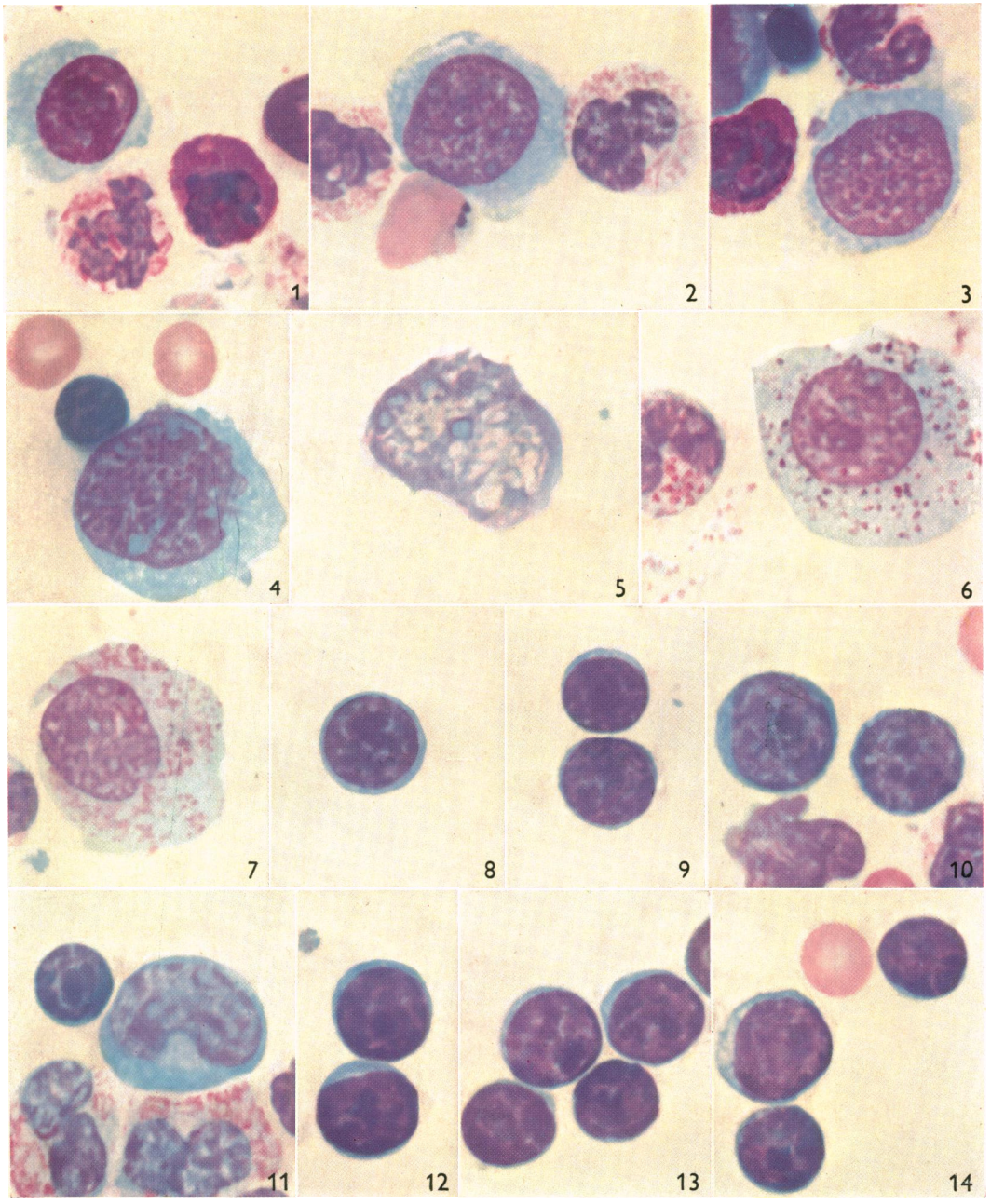
Fig. 20. Normal guinea-pig bone marrow. Basophil metamyelocyte.

Fig. 21. Normal marrow. Mature pseudo-eosinophil and eosinophil granulocyte.

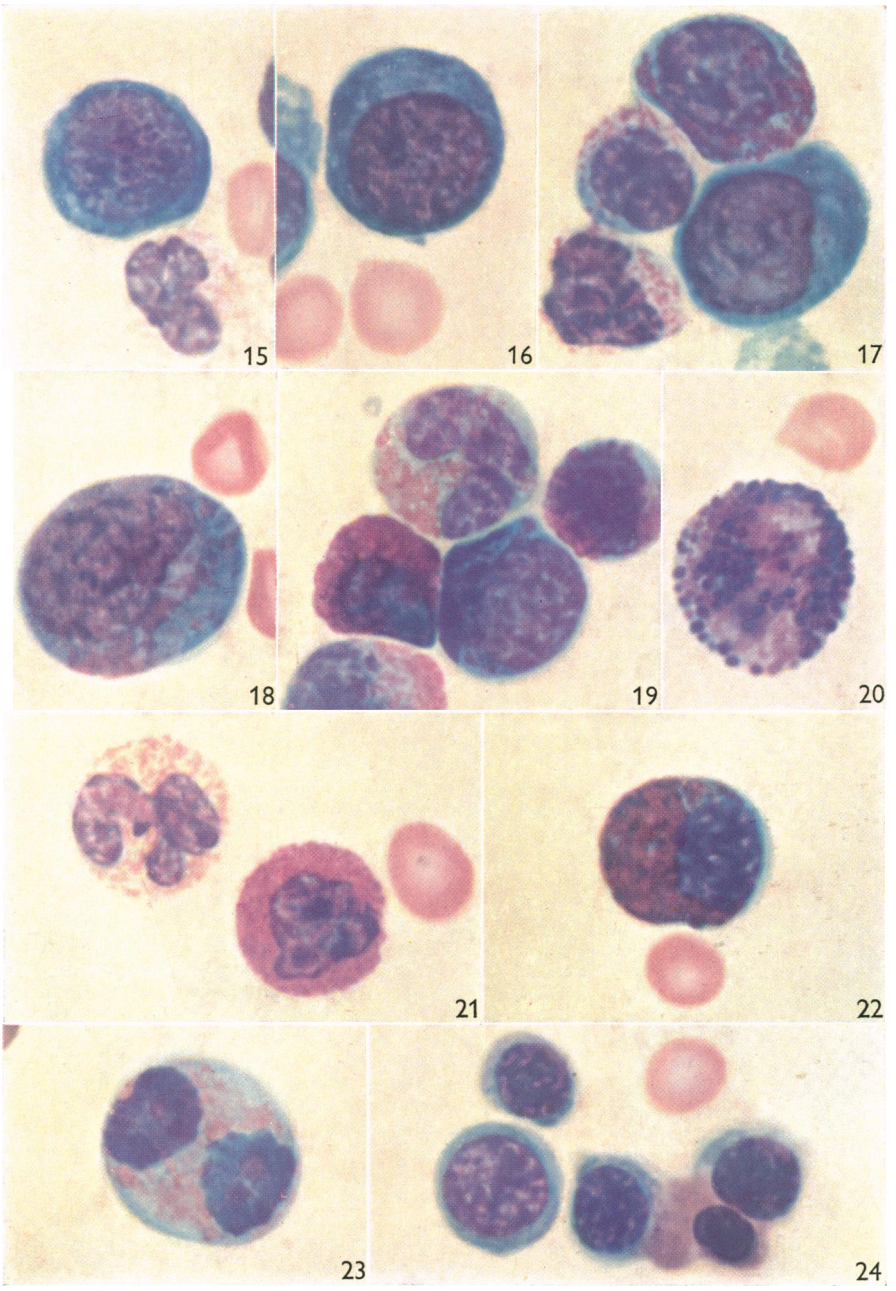
Fig. 22. Normal marrow. Eosinophil myelocyte.

Fig. 23. Pseudo-eosinophil myelocyte or metamyelocyte in mitosis. Normal marrow.

Fig. 24. Normal marrow. Red cells in different stages of maturation.



YOFFEY AND OTHERS—EFFECTS OF COMPOUNDS E, F AND A ON GUINEA-PIG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