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Genetical studies on the skeleton of the mouse

XXXII. THE DEVELOPMENT OF SHAKER WITH SYNDACTYLISM

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THIS PAPER IS DEDICATED TO PROFESSOR L. C. DUNN IN RECOGNITION OF HIS LONG AND DISTINGUISHED CAREER

INTRODUCTION

In a previous paper (Grüneberg, 1956), the anatomy of the cartilaginous and osseous skeleton of three genes for syndactylism in the mouse has been described. For two of these genes, syndactylism (*sm*/*sm*) and Oligosyndactylism (*Os*/+), this has since been supplemented by a description of the muscular anatomy (Kadam, 1962), and both genes have been studied by standard embryological methods (Grüneberg, 1960*a*, 1961). An embryological investigation by means of histochemical techniques (Milaire, 1962) will be published in the near future. The present paper rounds off the work on these three mutants by a description of the development of the third of them, shaker with syndactylism (*sy*/*sy*). With the exception just mentioned, it also brings to an end this long series of investigations on the skeleton of the mouse in which this laboratory has been engaged for the past 15 years. Important contributions have also come out of other laboratories. An assessment of all these facts from the genetical, from the embryological and from the medical angle will, it is hoped, be presented in due course in the form of a book.

ADULT ANATOMY

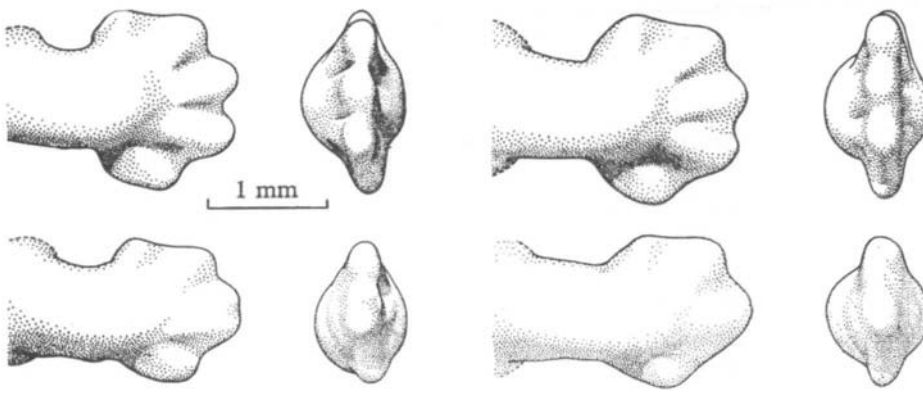
As described in more detail previously (Grüneberg, 1956), the mutants *sm*/*sm*, *Os*/+ and *sy*/*sy* differ from each other in the pattern of digital fusions. In both *sm*/*sm* and *Os*/+, all four feet are regularly affected, and a single primary type of fusion can be identified. In *sm*/*sm*, digits 3 and 4 are always involved, with digit 2 often also affected; but fusion between digits 2 and 3 alone was never observed. In *Os*/+, the primary fusion is between digits 2 and 3, with digit 4 occasionally also affected; but fusion between digits 3 and 4 alone was never observed. In *sy*/*sy*, by contrast, normal overlapping is common in the fore-limbs and not rare in the hind-limbs. As in the other two mutants, the expressivity is stronger in the hind-limbs than in the fore-limbs. There is no single primary type of fusion: syndactylism may thus involve digits 2 and 3 alone, or digits 3 and 4 alone, or all three of them simultaneously. Fusions generally involve all three phalanges or the two distal phalanges and are apparently always primary. Metacarpals and

metatarsals are not affected. There are, however, extensive secondary fusions between carpals and between tarsals respectively. In addition to these anomalies of the hands and feet, there are certain deviations in the shape of various bones which are not, or not seriously, pathological in nature.

The development of syndactylism will be described first. This will be followed by some observations on the remainder of the skeleton. The labyrinthine pathology of this mutant is outside the scope of this paper.

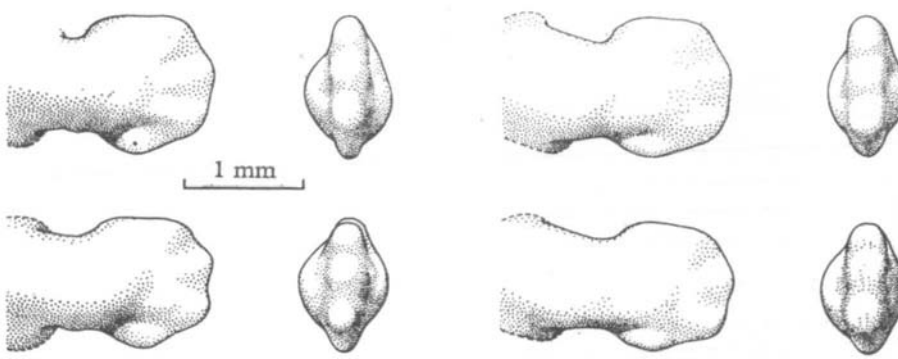
THE DEVELOPMENT OF THE FOOT ANOMALIES

From the age of 13 days onwards, *sy/sy* embryos can easily be identified by the shape of their hand and foot plates. The peripheral contour clearly shows that



Text-fig. 1. The right fore- and hind-limbs of a normal (top row) and an *sy/sy* embryo. Litter-mates, nearly 14 days old. Camera lucida drawings.

some digits are much closer together than normal (see, e.g., Grüneberg, 1956, text-figs. 15 and 16, p. 133). In Text-fig. 1, digits 3 and 4 of the right fore-limb are joined, but still distinguishable by outline and surface moulding as separate

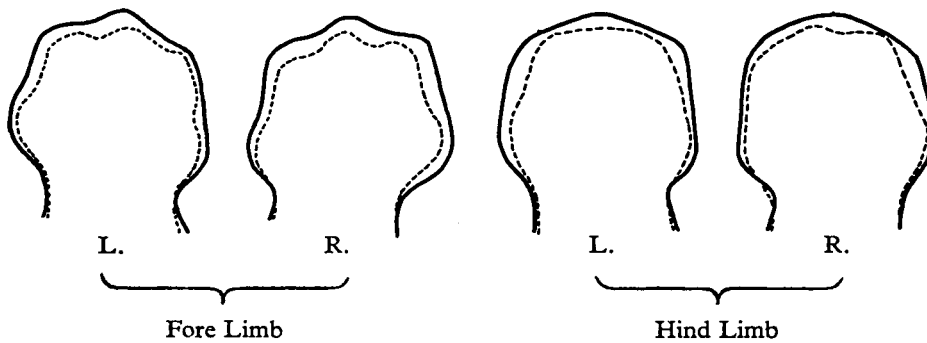


Text-fig. 2. The right fore- and hind-limbs of a normal (top row) and an *sy/sy* embryo, 12½-day-old litter-mates. Camera lucida drawings.

entities; such digits are syndactylous by skin and soft tissues, but have separate phalanges (Fig. 3, Plate 1). Whereas the fore-limbs are often not syndactylous at all or only mildly abnormal, the hind-limbs tend to be more severely affected;

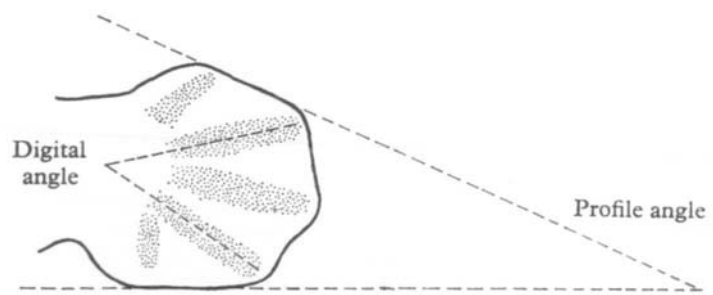
that shown in Text-fig. 1 betrays neither by outline nor by surface moulding that the element corresponding to digits 3 and 4 is in fact composite.

At the 12½-day stage *sy/sy* embryos can still be recognized with confidence. At this stage (Text-fig. 2), the foot plates of normal embryos have just become



Text-fig. 3. Outlines of normal (solid lines) and *sy/sy* limbs (broken lines) superimposed. Embryo No. 1480 (normal, C.R.L. 8·4 mm.) and embryo No. 1481 (*sy/sy*, C.R.L. 8·3 mm.), 12½-day-old litter-mates. Tracings from photographs. Magnification approximately 19 times.

pentagonal in outline and shallow surface impressions indicate the developing digits. In the fore-limbs there is the very beginning of marginal indentations. At this stage, the limbs of *sy/sy* embryos are pentagonal like those of normals, but the segment of the periphery corresponding to digits 2, 3 and 4 is narrower than



Text-fig. 4. The 'profile angle' as measured in photographs, and the 'digital angle' as measured in projection drawings of sections.

normal. This is best seen by superimposing the outlines of normal and abnormal limbs (Text-fig. 3). The whole periphery seems to be shortened, both preaxially and postaxially. In this respect *sy/sy* differs from *Os/+* in which the reduction is virtually confined to the preaxial margin of the foot plates.

The comparison between normal and *sy/sy* limbs at this stage can be made more objective and quantitative by measuring the 'profile angle' as defined in Text-fig. 4. Figures for four normal and four *sy/sy* embryos (two 'quartets' from

separate litters) are given in the upper half of Table 1. Both in the fore-limbs and in the hind-limbs, the profile angle of *sy/sy* embryos is markedly greater than that of their normal sibs. The distributions overlap, but the means are clearly different. Moreover, in agreement with the ultimate phenotypic results, the hind-limbs deviate more from normality than the fore-limbs: the ratio Abnormal/Normal A/N) being 1·55 for the hind-limbs and 1·24 for the fore-limbs.

As the marginal segment corresponding to digits 2, 3 and 4 is narrower in *sy/sy* than in normal embryos, the corresponding metacarpals and metatarsals may be expected to include a smaller angle than in normal embryos. This is easily seen in sections (Figs. 1 and 2, Plate 1), particularly of the hind-limbs. More critical than simple observation is the measurement of the 'digital angle' as defined in Text-fig. 4. As shown in the lower half of Table 1, the digital angle of 12½-day-old

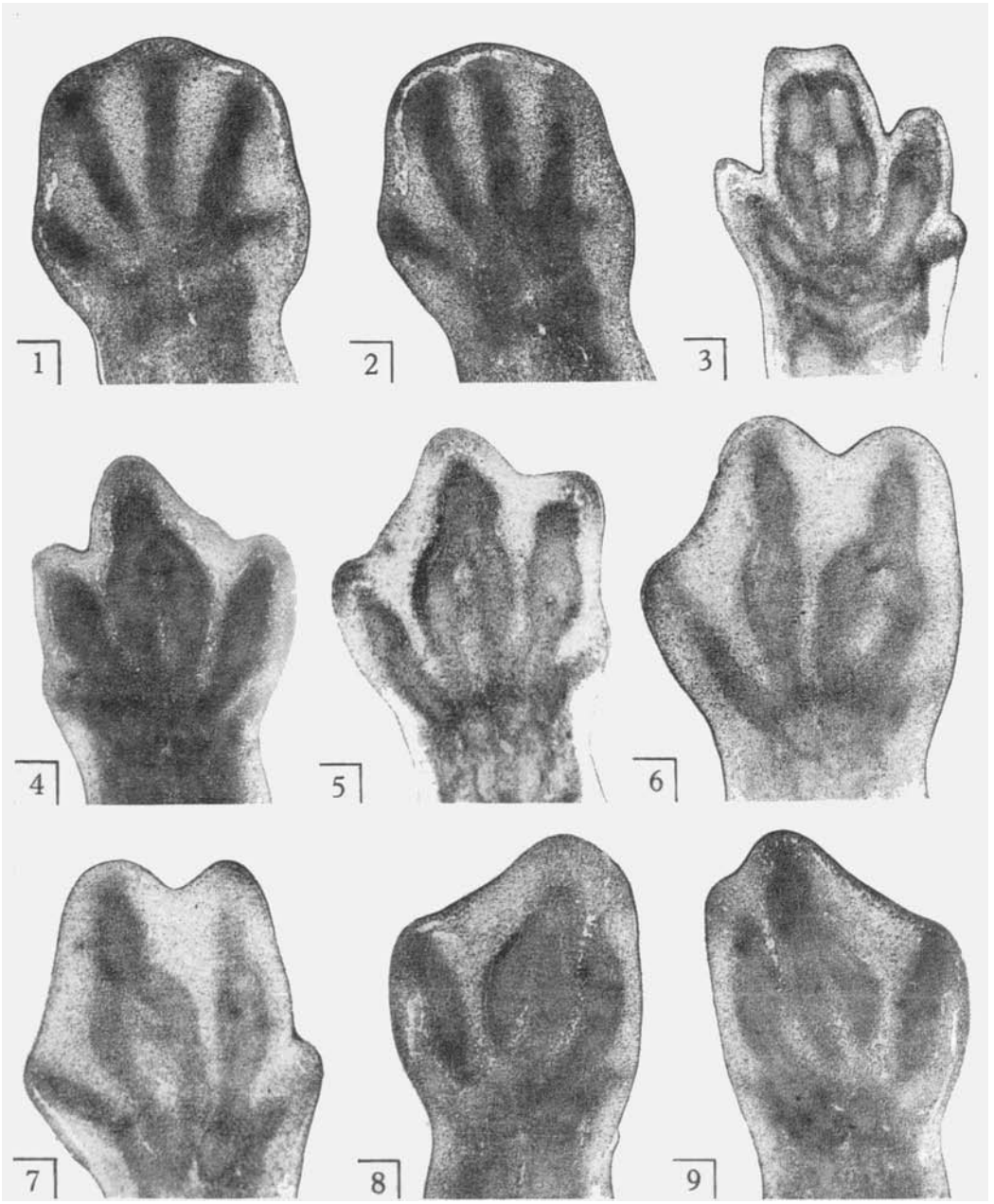
Table 1. *The 'Profile Angle' (upper half) and the 'Digital Angle' (lower half of table) of 12½-day-old embryos (N = normal, A = abnormal). Mean values based on the original (ungrouped) data. The digital angle of one normal fore-limb could not be determined on account of faulty orientation in sectioning.*

		12° to 14°	15° to 17°	18° to 20°	21° to 23°	24° to 26°	27° to 29°	30° to 32°	33° to 35°	36° to 38°	39° to 41°	42° to 44°	45° to 47°	Mean
Fore-limb	N	.	.	.	1	1	6	.	.	.	.	.	.	26·6°
	A	.	.	.	.	.	1	2	3	1	1	.	.	33·1°
Hind-limb	N	2	2	3	.	1	.	.	.	.	.	.	.	17·4°
	A	.	.	1	1	1	3	1	1	.	.	.	.	27·0°
Fore-limb	N	.	.	.	.	.	.	.	.	.	3	2	2	42·9°
	A	.	.	.	.	.	.	1	1	3	1	2	.	37·3°
Hind-limb	N	.	.	.	.	.	.	.	.	3	1	4	.	40·4°
	A	.	.	.	2	1	1	1	2	1	.	.	.	29·0°

sy/sy embryos is smaller than in normals. The distributions overlap, and once again the hind-limb deviates more from normality (A/N = 0·72) than the fore-limb (A/N = 0·87). It will be noticed that the profile angle of *sy/sy* embryos is more strongly abnormal than the digital angle.

At the 12½-day stage, the digital blastemata in fore-limbs and hind-limbs correspond mainly, if not entirely, to metacarpals and metatarsals respectively. In sections of 12½-day embryos, the digital blastemata of *sy/sy* individuals are still distinct throughout their length without any sign of syndactylism. Soon afterwards, however, in the 13-day stage, blastemata which are close together tend to coalesce distally, and once they have run together, they generally continue as a single composite blastema to the end. Examples of increasing degrees of syndactylism are shown in Figs. 3–9, Plate 1.

Once coalescence of neighbouring blastemata has taken place, the outgrowth of composite phalanges distorts the shape of the foot plate, and the anomaly



1. Normal embryo, left hind-limb (No. 1465, C.R.L. 8·9 mm., 12½ days).
2. *sy/sy* embryo, left hind-limb (No. 1466, C.R.L. 8·9 mm., 12½ days, litter-mate of No. 1465).
3. *sy/sy* embryo No. 561, right fore-limb (13 days old).
4. Right hind-limb of same embryo.
5. Left hind-limb of same embryo.
6. *sy/sy* embryo, right hind-limb (No. 1462, C.R.L. 9·3 mm., 13 days).
7. Left hind-limb of same embryo.
8. *sy/sy* embryo No. 560 (litter-mate of No. 561, 13 days old), left hind-limb.
9. Right hind-limb of same embryo.

Embryos 560 and 561 fixed in acetic Zenker, embryos 1462, 1465 and 1466 in Bouin's solution. Imbedded by Peterfi's method. Sections 7·5 μ thick. Haematoxylin and eosin. Photographs × 26, enlarged to × 35.

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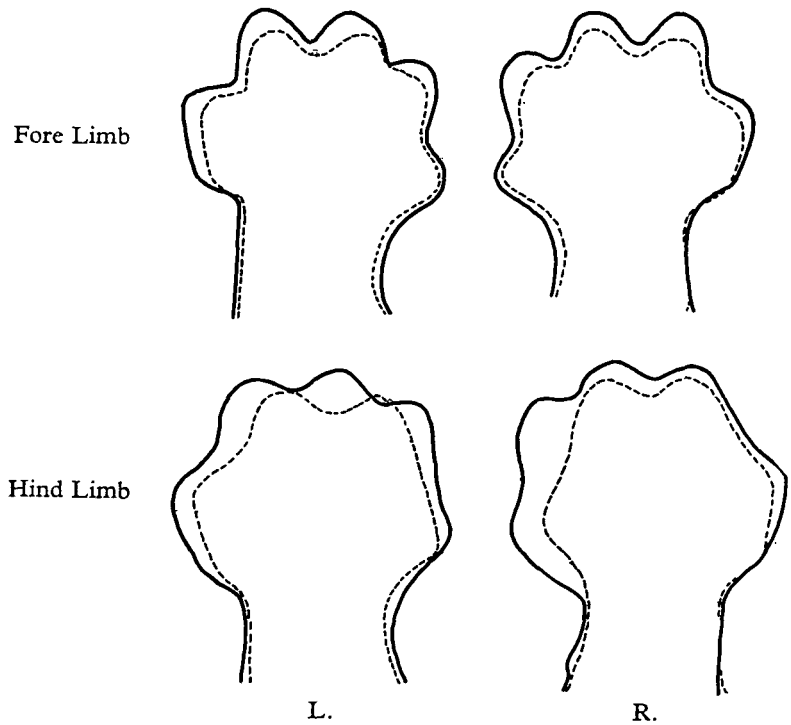
immediately becomes very conspicuous (see, e.g., Text-fig. 5). This prompts the question of whether the condition has in fact become more extreme between the 12½- and the 14-day stage, or whether it has only become more striking to the eye. The foot plates in Text-figs. 3 and 5 are roughly circular in outline. Their size can be determined approximately by superimposing a series of circles of increasing diameter. If this is done, it is found that the A/N value for the four feet of the 12½-day embryos is 0·91 while that for the 14-day embryos is 0·90. There is thus no evidence that the anomaly of *sy/sy* embryos has become any more extreme between the 12½- and the 14-day stage, though, of course, it has become much more conspicuous. Evidently, the damage has already been done in the 12½-day stage.



Text-fig. 5. Outlines of normal (solid lines) and *sy/sy* limbs (broken lines) superimposed. Embryo No. 1461 (normal, C.R.L. 9·5 mm.) and embryo No. 1462 (*sy/sy*, C.R.L. 9·3 mm.), littermates, about 14 days old. Tracings from photographs. Magnification approximately 19 times.

I have not been able to identify *sy/sy* embryos with any confidence at the 12-day stage. At that age, normal foot plates are circular in outline, and no digits can yet be recognized either by surface moulding of the foot plates or as blastemata in sections. The features by which *sy/sy* embryos can be recognized at the 12½-day stage are thus not yet in existence. Whether they can be identified by other criteria is, of course, an open question. An attempt to distinguish *sy/sy* from normal embryos at the 12-day stage by more refined measurements would have been practicable if (as in *sm/sm*) whole litters of known *sy/sy* embryos could have been

compared with known normals; it might even have been tried if (as in *Os*/+) most litters could have been relied upon to contain both types of embryos side by side. But in the present case, more than one-third of the litters will not contain any abnormal embryos at all; moreover, differences due to genotype if present would be confounded with differences due to stage of development which are always present in litters of embryos of this age; finally, there is the variable manifestation of this gene with many normal overlaps on individual feet. Hence, in the absence of a lead to be followed up, no attempt has been made to trace the effects of the *sy* gene beyond the 12½-day stage.

No gross pathological features have been noticed in sectioned feet of 12½-day *sy*/*sy* embryos. This may be due to limitations of the technique or of the observer. On the other hand, the difference between normal and *sy*/*sy* embryos at 12½ days is comparatively slight and may well come into being in a rather unspectacular fashion. A reduction in mitotic rate near the free margin of the foot plates acting over 12 hours or so might be sufficient to account for it.

SYSTEMIC ANOMALIES OF THE OSSEOUS SKELETON

As described in more detail previously (Grüneberg, 1956), *sy*/*sy* mice in post-embryonic life show a characteristic array of comparatively minor deviations in shape of the osseous skeleton. The most typical manifestation is a reduction in calibre of the shafts of the long bones whereas the epiphyseal ends are much less affected. Two or three incidental breakages of bones macerated by the papain method (which rarely happen in normal specimens) led to the suspicion that perhaps *sy*/*sy* bones are structurally abnormal in addition to their shape anomalies. X-ray photographs showed that this is indeed the case. All the long bones of the limbs and the girdles as well as the vertebrae show a fairly uniformly reduced density of bone structure. There is thus clearly a systemic involvement of the osseous skeleton.

The appearance of the *sy*/*sy* bones in X-ray photographs might be due to a reduction in size of the bones, or to relative reduction of their wall thickness, or to incomplete mineralization, or to a combination of these causes. To distinguish between these possibilities, transverse sections through the tibia and fibula were studied; litter-mate pairs of the ages of 7 and 16 days were used (Suza fixation; imbedding by Peterfi's method; sections at 12·5 μ; H. & E. staining), and projection drawings of the tibia were made at magnification × 132. The region studied was that just proximal to the fork of tibia and fibula, and in each case twelve sections (as far as possible every tenth section) were drawn. In this neighbourhood, the cross-section of the tibia is approximately circular as is the inner contour of the marrow cavity. It is thus easy to determine approximately the dimensions of this ring of bone by superimposing on the projection drawings a series of circles of graded sizes. From the radius of the shaft and its mean thickness, the relative thickness of the wall in per cent can be calculated; this eliminates the fact that the bones of the *sy*/*sy* mice (which are always much retarded) are smaller than those of their normal litter-mates. As both calibre and relative thickness of the wall of

the tibia change in proximo-distal direction, three groups of four neighbouring sections each have been averaged (*a*, *b* and *c* in Table 2, where *a* is the most distal and *c* the most proximal group of sections); an average of the whole series of twelve sections is also given. For the relative thickness of the wall, A/N values are included in Table 2.

The tibia of *sy/sy* mice is not only smaller, but it also has a relatively thinner wall. The A/N values are in the neighbourhood of 0·91, corresponding to a reduction of 9% at both ages; there is thus no change in severity during the time interval covered. The findings in the tibia can presumably be taken as representative for the skeleton as a whole.

Table 2. *Dimensions of the tibia in* sy/sy *and normal mice in the region just proximal to the fork of tibia and fibula. Further explanations in the text.*

		Normal (N)			*sy/sy* (A)			A/N,
Age		Radius (μ)	Wall (μ)	Wall (%)	Radius (μ)	Wall (μ)	Wall (%)	Wall (%)
7	*a*	278	66·3	23·8	215	49·2	22·9	0·959
	b	292	70·1	24·0	213	44·5	20·9	0·870
	c	307	56·8	18·5	243	39·8	16·4	0·885
	Mean	292	63·8	21·8	224	44·5	19·9	0·909
16	*a*	337	104·2	30·9	278	78·6	28·3	0·914
	b	332	90·0	27·1	284	71·1	25·0	0·924
	c	348	85·2	24·5	301	68·2	22·7	0·926
	Mean	339	93·1	27·5	288	72·6	25·2	0·918

It is less certain whether these metrical differences account for the whole of the lowered density of the *sy/sy* bones as seen in X-ray photographs. The absolute wall thickness of the *sy/sy* tibia is reduced by 30% and 22% respectively in the two age-groups. As judged by eye, the reduction of density in X-ray photographs appears to be roughly of this order. But imperfect mineralization of the bone as an additional feature is not excluded; particularly in the younger *sy/sy* mouse, there are extensive areas of eosinophil 'osteoid' which may be incompletely calcified. However, as the papain skeletons which were X-rayed came from much older mice than those which were available for histological study, it must remain an open question whether the *sy/sy* skeleton is incompletely mineralized.

It remains to mention a structural difference. The osseous shaft of the tibia, in both age-groups, shows more blood vessels in the normal than in the *sy/sy* mouse.

The mode of origin of the systemic skeletal effects has not been completely elucidated. As mentioned previously (Grüneberg, 1956), some, at least, of the shape anomalies of the osseous skeleton are preformed in cartilage. But it has become certain that this does not apply to all of them, or, more accurately, that some of the shape anomalies are not yet detectable in the cartilage model when this is first laid down; the possibility remains that some anomalies may arise during cartilage growth, or indeed during ossification.

The main example studied is the distal region of radius and ulna. As shown in Text-fig. 20, p. 136 (1956), the proximal half of the (adult) ulna is not greatly different from the normal, but the distal half is strikingly thinner. In 13-day embryos, radius and ulna have just chondrified. But there is no detectable difference in calibre as seen in projection drawings; the same applies to the distal half of tibia and fibula (No. 1459, normal, C.R.L. 9·2 mm., and No. 1460, *sy/sy*, C.R.L. 9·3 mm.; litter-mates). Seven days after birth, the anomaly is strikingly present in its osseous form (same animals as in Table 2), but in the 16-day mice sectioned, the *sy/sy* individual failed to show this anomaly virtually altogether. There is, indeed, a certain lack of uniformity as regards these systemic effects which will require a more detailed study.

As mentioned previously (Grüneberg, 1956), *sy/sy* mice tend to grind down their molars to an extent not usually encountered in normal mice. Serial sections through the teeth of *sy/sy* mice and normal litter-mates aged 6 days (Nos. 1478 and 1479) and 16 days (same animals as in Table 2) respectively have been studied. No structural differences have been discovered. The enamel and dentine layers of *sy/sy* teeth did not differ significantly in thickness from those of normal mice, and the degree of calcification of the dentine was about normal. The structure of the enamel is recognizable in decalcified sections of 6-day-old mice, but showed no obvious abnormalities; at the 16-day stage, enamel is dissolved virtually completely by the process of decalcification. In view of the limited value of such negative findings, the reason for the dental anomaly of *sy/sy* mice remains obscure.

DISCUSSION

The effect of the *sy* gene can be traced with confidence to the $12\frac{1}{2}$-day stage, that is to say to a stage before actual syndactylism has made its appearance. The first effect so far discovered is a reduction in width of the periphery of the foot plates when these are assuming a pentagonal outline and when digital blastemata have just been formed. Unlike *Os/+* where a similar reduction of the foot plates is confined to the preaxial border, that in *sy/sy* is both preaxial and postaxial. It is obvious that the narrowing of the foot plates is the factor which forces digits 2, 3 and 4 to be formed more nearly parallel to each other, and similarly that it is the closer proximity of the blastemata which tends to lead to their coalescence distally. The fact that the shape of the foot plates deviates less from normality in the fore-limbs than in the hind-limbs tallies with the greater expressivity of the gene posteriorly; and the fact that the distributions of limb-bud measurements overlap with those of the normal embryo is in agreement with the incomplete penetrance of the *sy* gene as regards its effects on the feet. The more uniform reduction of the periphery of the foot plates is also in agreement with the fact that, unlike *Os/+* and *sm/sm*, there is no primary type of fusion either between digits 2 and 3, or between digits 3 and 4. It may be suggested, then, that the digital anomalies (and presumably the secondary fusions between carpals and tarsals) are direct consequences of the shape anomalies of the foot plates. As in *Os/+*, the reason for these shape anomalies remains to be discovered.

However, if both in *Os*/+ and in *sy*/*sy* the structural defects are a simple consequence of a reduction of foot plate material, why are many digital fusions in *Os*/+ secondary whereas those in *sy*/*sy* (and in *sm*/*sm*) seem to be always primary? Whereas the shape of the foot plates, both in *Os*/+ and in *sy*/*sy*, satisfactorily accounts for the digital fusions, does it also convincingly explain the extensive fusions in carpus and tarsus which, in *Os*/+ at any rate, are by no means confined to the preaxial side? Similarly, why do most of the secondary carpal and tarsal fusions in *Os*/+ happen in foetal life whereas those in *sy*/*sy* are mainly (? entirely) established after birth? The suspicion thus arises that perhaps the purely quantitative interpretation may not be the whole story. The shape of the foot plates both in *Os*/+ and in *sy*/*sy* is an obvious condition for the development of syndactylism. But perhaps the shape of the foot plates is itself the consequence of other events which are different for the two mutants and which, in their different ways, also enter into the structural anomalies of hands and feet. If so, the phenotype of *Os*/+ would not be fully explained by absence of material on the preaxial side of the limb buds, nor that of *sy*/*sy* by absence of material both preaxially and postaxially. It may be mentioned that Kadam (1962) has recently expressed similar doubts in the case of *Os*/+ where extensive muscular anomalies occur in the peroneus group, i.e. postaxially, whereas the reduction of material visible on the outside of the foot plates is virtually all preaxial.

Disregarding in this context the labyrinth, the *sy* gene has two major pleiotropic effects, the localized shape anomalies of the foot plates which lead to syndactylism and which can be traced to the 12½-day stage, and the systemic skeletal effects which arise much later in development. The relationship between these effects is still obscure.

On the assumption that *sy* is a point mutation and not a small structural rearrangement, its pleiotropic effects may be expected to be traceable to a common root cause in conformity with the postulate of the Unity of Primary Gene Action. That principle as first clearly stated by the present author in 1938 and amplified in 1943 excluded multiple primary gene effects. However, while cases of spurious pleiotropism (i.e. with a single primary effect) can be demonstrated, there is no direct way to distinguish genuine pleiotropism (if it should exist) from cases which have a common, though unknown, root cause. The situation has since changed as the result of progress in the field of chemical and molecular genetics. It is now widely accepted that gene (point) mutations represent highly specific and strictly localized changes in DNA which in turn lead to similarly specific and localized changes in protein (mainly enzyme) molecules. If so, it is difficult to see how a gene could have more than one primary effect. The question to be answered is thus not so much of *whether*, but of *how* syndactylism and the systemic skeletal effects are physiologically connected. Does syndactylism itself lead to the systemic changes which arise later or, more probably, do both owe their existence to the action of the same protein (? enzyme) acting in different localities and at different times? This question cannot be answered at present. But it can be answered in principle. If the two effects are co-ordinated in the sense that a single anomalous

protein (enzyme) is responsible for both, it may be identifiable by suitable techniques in the localities, and at the times, at which the various anomalies come into being.

The question has recently been discussed (Grüneberg, 1960*b*) of whether genes with obvious effects during development may not sometimes have less obvious functions in the physiology of the adult organism. This question of the genic control of adult physiology is obviously a fundamental one. For that reason a case like *sy*/*sy* which may supply relevant information is probably worthy of more detailed study.

The possibility should, however, not be lost sight of that *sy* (which was probably induced by X-rays) may be a small structural rearrangement (or loss of material) rather than a true point mutation. If so, two effects without obvious physiological relationship might in fact be independent of each other.

SUMMARY

1. The first known effect of the gene for shaker with syndactylism (*sy*) in the mouse is a reduction, both preaxial and postaxial, of the foot plates at the 12½-day stage. This reduction forces the blastemata of digits 2, 3 and 4 to be laid down more nearly parallel to each other, with a subsequent tendency for adjacent blastemata to coalesce with each other. The possibility is considered that this essentially quantitative reduction of the foot plate material may itself be the consequence of some more specific and qualitative (but hitherto undiscovered) change in the limb buds.

2. The physiological connexion between syndactylism and the systemic changes in the cartilaginous and osseous skeleton which arise later in *sy*/*sy* mice remains obscure.

The microscopical sections used in this investigation were made by Mrs H. Deol and by Miss June Denny, who also took the photomicrographs. Text-figs. 1 and 2 were made by Mr A. J. Lee. X-ray photographs were taken by Mr. P. Venning. To all of them I would like to express my appreciation. The work was partly supported by a grant from the Rockefeller Foundation which is gratefully acknowledged.

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