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Regulation of deficiencies along the proximal distal axis of the chick wing-bud: a quantitative analysis

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SUMMARY

The wing-bud shows zero regulation following removal of a whole slice of the proximal-distal axis from stage 22 or later. Prior to this it is possible that it may show some regulation. Some of the apparent regulation may be explained by the way in which the limb grows. The bud never shows perfect size regulation or morphallaxis. The proximal-distal axis of the bud between shoulder and wrist expands uniformly from stage 20.

INTRODUCTION

A developmental process in which an abnormal complement of cells in an embryo or part of an embryo gives rise to a normal embryo or part of an embryo is termed regulative (from Driesch, 1891). There is conflicting evidence about the ability of the developing chick limb-bud to regulate. To my knowledge no one disputes the experiments of Barasa (1964) or Stark & Searles (1974) who obtained good regulation having removed portions of the limb-bud passing right through the entire dorsal–ventral axis. These experiments, for short, I call 'holes'. There is controversy about the ability of the bud to regulate when a piece of tissue containing both the entire dorso-ventral and antero-posterior axes is removed. These experiments, for short, I call 'slices'. The difference between holes and slices is very obvious if the operated limb-bud is viewed from the dorsal surface (see Fig. 1*b*). Using slices-type experiments, together with a modification to produce an excess of tissue along the proximal–distal axis, Hampé (1959) and Kieny (1964*a*, *b* and *c*, 1967) have emphasized the regulative capacity; while Amprino & Camosso (1965) and Summerbell & Lewis (1975) have considered the limb-bud to be relatively mosaic. In this paper I consider only regulation along the proximal–distal axis and only experiments in which

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slices containing the entire anterior–posterior and dorsal–ventral axes are removed from the limb-bud, i.e. deficiencies.

In a typical slices experiments all the protagonists have *mentally superimposed a fate map* on their experimental limb-buds. Then have either removed a slice; or have replaced the tip of the host limb-bud, by grafting a new tip containing more tissue from a donor bud so as to produce an excess of tissue in the host. They then guess, *by reference to their superimposed fate map*, which bits of limb-bud are present in their experimental limb-bud. The embryo, and in some cases the fragment removed (Kieny, 1964*a*, *b*, *c*, 1967), are then allowed to continue development (the fragments by way of CAM grafts) until they possess well formed skeletons. They are then fixed and stained for examination. If the limbs contain the correct number of skeletal elements in the correct sequence and assignable to those adjudged to be present from the fate map then it is considered *not* to have regulated. If it has a complement of skeletal elements closer to normal than was predicted *from the fate map* it is said to have regulated. This method of analysis has two major drawbacks.

(1) It relies heavily upon fate maps which may themselves be inaccurate (see Summerbell & Lewis, 1975; Lewis, 1975; Summerbell, 1976 for a discussion).

(2) The data obtained are only qualitative or at best only semi-quantitative. Even Summerbell & Lewis (1975) who accurately measured their results, only estimated the original composition of the grafted limb-buds.

To try and avoid these two problems and to gain an estimate of the extent of regulation I have used a rather different technique which avoids reference to a fate map and gives a measure of the ability of the limb-bud to regulate in well defined circumstances. These being the regulation of deficiencies following removal of *whole slices* of the proximal–distal axis of one limb-bud of an embryo. The contralateral limb I keep as a control. I reduce the deficiency in the bud to a single variable namely the proportion of the proximal–distal axis remaining immediately following the operation (see results for details). This I call the predicted result assuming zero regulation. The outcome of the operation in the 10-day wing I similarly reduce to a single variable by measuring the total length of humerus (stylopod), ulna/radius (zeugopod), wrist and hand (autopod), in the operated and control limb, I then calculate the proportion of the normal limb length by comparing operated and control side limbs (see Summerbell and Wolpert, 1972; Summerbell, 1976). This I call the observed result. I then relate the observed result to the expected result.

This method of analysis suffers from two small flaws. (1) It is uncertain at early stages how much of the presumptive limb lies in the flank and how much in the bud so there is always a slight uncertainty of the absolute percentage of limb tissue removed. (2) It is extremely complicated if one attempts to take into account differential growth rates for different parts of the limb. Fortunately if the deletion is restricted to proximal levels the uncertainty due to this factor is very slight as I shall show below.

METHODS

Fertilized White Leghorn eggs were incubated at 38 °C and windowed on the third or fourth day of development. The embryos were staged according to Hamburger and Hamilton (1951), the window sealed over with sellotape and the egg returned to the incubator. Appropriately staged eggs were selected for each operation. The length of the proximal–distal axis was measured from the base of the limb-bud to the most distal point as shown in Fig. 1. The tip of the limb bud was cut off in a plane parallel to the base of the bud, then a second slice of tissue proximal to the tip was similarly removed. The tip was then returned to the proximal stump and fixed in place in its normal anterior–posterior and dorsal–ventral orientation using platinum wire pins.

The length of the stump, the discarded middle slice and the tip were all measured using an eyepiece graticule calibrated at 50 μm per division in a Zeiss stereo IV microscope. Although there were some difficulties in measuring (it was not easy to keep the dorso-ventral axis of the amptuation plane perpendicular) it was probably correct to $\pm 50\,\mu$m and certainly to within $\pm 100\,\mu$m. At each stage sham control operations were performed, the tip was removed then immediately pinned straight back on to the stump so that there was no deficiency. The excision was normally aimed at about the level of the elbow so that any deficiency due to the primary effect of its removal would be at the level of the stylopod and/or zeugopod. The eggs were then returned to the incubator.

On the tenth day of incubation the embryos were sacrificed and operated (right) and control (left) wing were fixed in 5 % TCA stained in 0.1 % alcian green 2GX in 70 % alcohol with 1 % hydrochloric acid, dehydrated and cleared in methyl salicylate. Operated and control limbs were examined and photographed using a Zeiss Stereo IV stereoscopic microscope and the lengths of humerus, ulna, radius and the elements of digit 3 (the middle finger) measured wherever they were present.

The limb-bud measurements were used to provide an estimate of proportion of the wing-bud remaining after the operation. This was taken as the length of proximal stump plus distal tip divided by the original length.

$$l_{\text{expected}} = \frac{\text{stump} + l_{\text{tip}}}{l_{\text{whole bud}}}$$

The expected result assuming zero regulation was presumed to be a function of this single variable. The 10-day wing measurements were used to provide an estimate of the final proportion of the wing present:

$$\text{wing} = \text{humerus} + \tfrac{1}{2}\,(\text{ulna} + \text{radius}) + \text{hand}$$

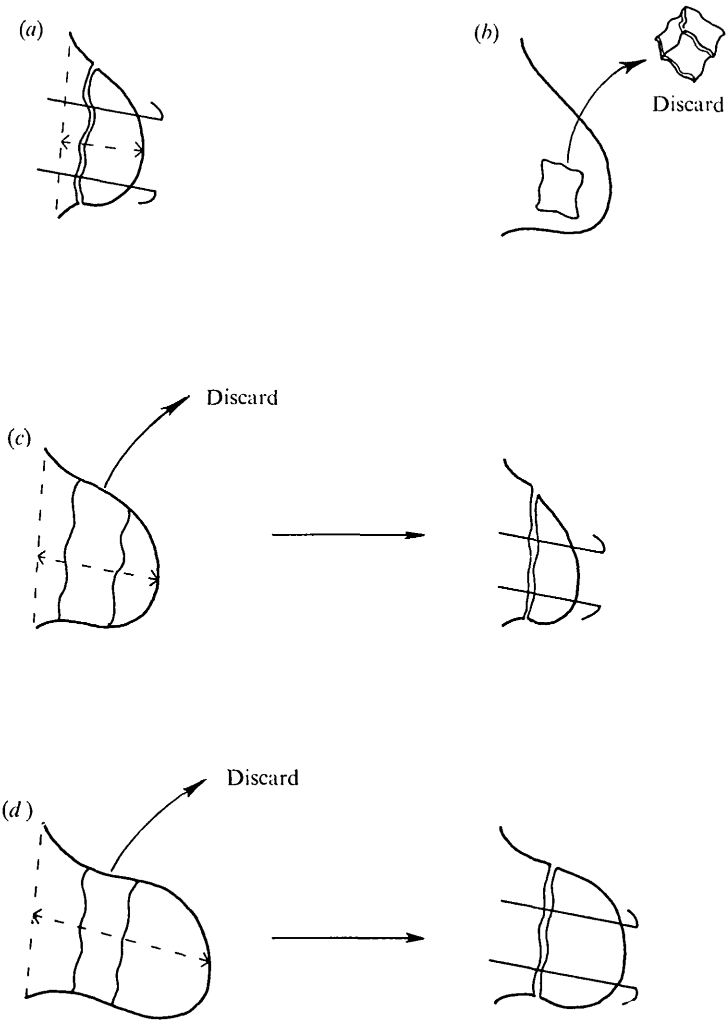

Fig. 1. Slices and holes, schematized. The dashed lines indicate the method of measuring the length of the proximo-distal axis (*a*) Sham control at stage 21, remove tip and replace. (*b*) Demonstration of holes for comparison. (*c*) Removal of 50% of bud at stage 22. (*d*) Removal of 25 % of bud at stage 24.

and then the length of the operated wing divided by the length of the control wing to give:

$$l_{\text{observed}} = \frac{\text{operated}}{\text{control}}$$

This single variable (l_{observed}) was taken as representing the observed result. The control side (left) wing can be used as an accurate estimate of the normal length of the other wing (right) as Summerbell & Wolpert (1972) have shown that the difference between these two measurements is negligible.

RESULTS

Out of 227 embryos successfully incubated to 10 days a comparatively small number (< 5 %) produced limbs lacking recognizable distal parts. I have assumed that in these the tip graft had failed to take and so they are not included in the following results and discussion.

I have studied the remaining limbs (217 embryos) in two different ways. In the first (Table 1) I examine the operated side limb looking for abnormalities of morphology and obvious misproportion. These results are considered in the qualitative estimate of regulation. In the second I measure the lengths of humerus, ulna/radius, wrist and hand and compare them with the lengths of the control side skeleton (see Summerbell & Wolpert (1972), Summerbell (1976)), to acquire an accurate estimate of the difference between operated and control limbs. In both cases the deficiencies tend to lie at about the level of the elbow affecting either the humerus, the ulna and radius or both. Only when a very large slice is removed does the deficiency include the whole zeugopod and so extend into the hand or wrist. In a few of these latter cases there was a strange abnormality. The operated limb possessed two of digit 2. I ignore this result for the purposes of this paper but the results were always scored as abnormal because there were always extensive deficiencies at the level of the zeugopod.

Qualitative data

The ability of the limb-buds to regulate the deficiency as judged by the morphology varied with age and with the size of the deleted slice (Table 1). At stage 19 it was necessary to remove over 60 % of the limb-bud before the resulting wings had a grossly abnormal skeleton and on occasions limb-buds which had had 80 % of the proximal–distal axis removed still produced normal looking wings. These limits gradually became lower with increasing age so that by stage 24 practically any loss of tissue showed itself in some noticeable abnormality of the skeleton.

The types of abnormality produced were the same at all stages, but varied a little depending on the size of the slice removed. There were three common types: loss of one or more epiphyses (Figs. 2*e* and *f*), loss of a whole skeletal element (Fig. 2*f*), fusion of humerus, ulna and radius into a single 'Y' shaped element (Fig. 2*d*). As might be expected loss of whole elements occurred only when a large slice had been removed, but the other two were found more often associated with the loss of smaller slices.

Quantitative data

If one compares the lengths of left and right skeletal elements the difference between them is normally insignificant (less than 4 % in 99 % of cases, Summerbell & Wolpert, 1972). This provides an excellent accurate method of assaying

Table 1. *Number of specimens showing normal or abnormal morphology at day 10*

% bud present	Normal	Abnormal	Total	% bud present	Normal	Abnormal	Total
Stage 19				*Stage 20*			
100	2	0	2	100	5	0	5
80–99	—	—	—	80–99	5	0	5
60–79	2	1	3	60–79	3	1	4
40–59	4	1	5	40–59	2	4	6
20–39	3	2	5	20–39	0	5	5
0–19	—	—	—	0–19	—	—	—
Total	11	4	15	Total	15	10	25
Stage 21				*Stage 22*			
100	4	0	4	100	6	0	6
80–99	4	2	6	80–99	3	1	4
60–79	3	2	5	60–79	4	6	10
40–59	2	10	12	40–59	0	8	8
20–39	0	6	6	20–39	0	6	6
0–19	0	6	5	0–19	0	3	3
Total	13	25	38	Total	13	24	37
Stage 23				*Stage 24*			
100	7	0	7	100	6	0	6
80–99	6	2	8	80–99	1	4	5
60–79	0	12	12	60–79	0	11	11
40–59	0	8	8	40–59	0	9	9
20–39	0	11	11	20–39	0	4	4
0–19	—	—	—	0–19	—	—	—
Total	13	33	46	7	7	28	35
Stage 25							
100	3	0	3				
80–99	1	3	4				
60–79	0	5	5				
40–59	0	3	3				
20–39	0	6	6				
0–19	—	—	—				
Total	4	17	21				

The criterion of abnormality was a clear physical deformity of the skeleton detectable without measuring. The '% bud present' has been divided arbitrarily into six categories, the experiments were actually performed in a more continuous distribution.

whether or not a limb has fully regulated a deficiency. For perfect size regulation the length of the operated side skeletal elements must lie within 4% of the unoperated or control side. If the operated limb varies more than this then it has not fully regulated the deficiency. Using this simple criterion of regulation, it was clear that perfect size regulation was either rare or completely absent. Out of 193 limbs at all stages which had a slice removed, only three managed

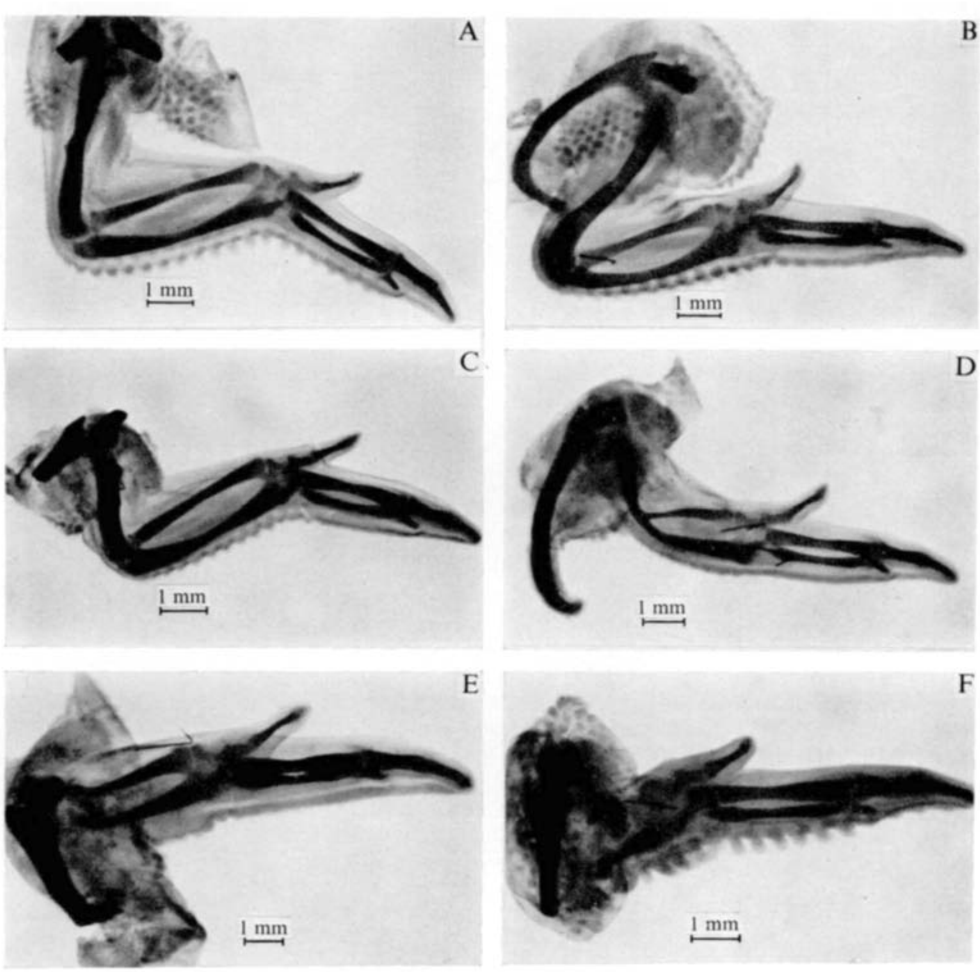

Fig. 2. Typical results at 10 days, A–F represent removal of successively larger slices.

to produce limbs in which the skeletal elements were within 95 % of their normal length, one at stage 19 and two at stage 20. All of the remainder had at least one element significantly shorter than that on the control side. It is also important than none of the limbs showed morphological size invariance, that is reductions in the length of all skeletal elements to produce a small but normally proportioned limb. The deficiency was always concentrated at a single level along the proximal distal axis. To summarize, removal of a slice of tissue resulted almost invariably in a level discrete deficiency in size.

However significant regulation is not necessarily perfect regulation. In Figs. 3–10 I compare the percentage of the limb-bud present after operating with the percentage of the wing present at 10 days. The resulting scatter plots show the relationship between these two variables. Without formal analysis it is clear that at stage 19 and 20, removal of a large part of the bud causes comparatively small defects in the 10-day wing. This may be due to regulation. At later stages large deficiencies in the bud result in equally large deficiencies in the 10-day wing. One can estimate the relationship between the proportion of the bud present and the proportion of the wing obtained by means of regression analysis. One line in each figure (see legends) represents the fitted least squares linear regression line where the length of the wing is the dependent variable so that:

$$\text{proportion of wing} = a+b\ (\text{proportion of bud})$$

where a is the intercept of the y axis and b the slope of the line.

Table 2. *Regression analysis of observed data*

Stage	n	a_0	b_0	r
19	15	$0{\cdot}78 \pm 0{\cdot}04$	$0{\cdot}18 \pm 0{\cdot}07$	$0{\cdot}58$ ($0{\cdot}2 < P < 0{\cdot}5$)
20	25	$0{\cdot}45 \pm 0{\cdot}06$	$0{\cdot}53 \pm 0{\cdot}08$	$0{\cdot}79$ ($P < 0{\cdot}001$)
21	38	$0{\cdot}31 \pm 0{\cdot}04$	$0{\cdot}70 \pm 0{\cdot}06$	$0{\cdot}88$ ($P < 0{\cdot}001$)
22	37	$0{\cdot}10 \pm 0{\cdot}04$	$0{\cdot}92 \pm 0{\cdot}05$	$0{\cdot}96$ ($P < 0{\cdot}001$)
23	46	$0{\cdot}10 \pm 0{\cdot}04$	$0{\cdot}87 \pm 0{\cdot}05$	$0{\cdot}94$ ($P < 0{\cdot}001$)
24	35	$0{\cdot}07 \pm 0{\cdot}03$	$0{\cdot}88 \pm 0{\cdot}05$	$0{\cdot}95$ ($P < 0{\cdot}001$)
25	21	$0{\cdot}06 \pm 0{\cdot}03$	$0{\cdot}87 \pm 0{\cdot}05$	$0{\cdot}97$ ($P < 0{\cdot}001$)

Where n is the number of cases, a_0 the intercept with its standard error, b_0 the slope with its standard error, in $y = a_0 + b_0 x$; and r is the correlation coefficient, with the probability of obtaining as high a value by chance.

These two parameters (a) and (b) are listed in Table 2, together with their standard error, and the correlation coefficient (r) with a test of significant difference of r from 0. In Table 3 the parameters a and b are compared between each stage and every other stage. The groups in bold type indicate the results which were not significantly different from each other. One may assume from this that by stage 22 the parameters have reached a stable value.

Null hypothesis: zero regulation

One can test whether or not these data fit the hypothesis of zero regulation by first constructing a theoretical regression line assuming zero regulation then using this expected line as the null hypothesis in a standard significance test. I have in fact tested the observed regression line against two such estimates.

In the first I make two additional assumptions; (1) that the wing-bud grows uniformly so that 10 % of the proximal–distal axis of the bud at the region and time of operating produces 10 % of the wing skeleton; (2) that the wing skeleton is derived only from tissue lying between the base and the tip of the bud as used in the measurements described in the methods.

If these assumptions are true then the regression line should: (a) pass through the origin (removal of the whole bud results in loss of the whole wing), (b) pass

Table 3. *Comparison of regression lines between stages*

t_b: significant difference between intercepts (rows); t_a: significant difference between slopes (columns)

Stage	19	20	21	22	23	24	25
19		3·29 (0·001 < *P* < 0·002)	5·64 (*P* < 0·001)	8·60 (*P* < 0·001)	8·02 (*P* < 0·001)	8·14 (*P* < 0·001)	8·02 (*P* < 0·001)
20	4·58 (*P* < 0·001)		1·70 (*P* < 0·001)	4·13 (*P* < 0·001)	3·60 (*P* < 0·001)	3·71 (*P* < 0·001)	3·60 (0·001 < *P* < 0·002)
21	8·31 (*P* < 0·001)	1·94 (0·05 < *P* < 0·01)		2·82 (0·005 < *P* < 0·01)	2·18 (0·02 < *P* < 0·05)	2·30 (0·02 < *P* < 0·05)	2·18 (0·02 < *P* < 0·05)
22	12·02 (*P* < 0·001)	4·85 (*P* < 0·001)	3·71 (*P* < 0·001)		**0·71 (0·4 < *P* < 0·5)**	**0·57 (0·5 < *P*)**	**0·71 (0·4 < *P* < 0·5)**
23	12·02 (*P* < 0·001)	4·85 (*P* < 0·001)	3·71 (*P* < 0·001)	**0·0** —		**0·14 (0·5 < *P*)**	**0·0** —
24	14·20 (*P* < 0·001)	5·66 (*P* < 0·001)	4·80 (*P* < 0·001)	**0·60 (0·5 < *P*)**	**0·60 (0·5 < *P*)**		**0·14 (0·5 < *P*)**
25	14·40 (*P* < 0·001)	5·81 (*P* < 0·001)	5·00 (*P* < 0·001)	**0·80 (0·4 < *P* < 0·5)**	**0·80 (0·4 > *P* < 0·5)**	**0·24 (0·5 < *P*)**	

To compare two regression lines at different stages read intercept of horizontal and vertical axes to give t_a and t_b. 'Student's' t-test for significant difference between *a-a′* and *b-b′*. The two groups in bold type show regions where the difference was clearly not significant, cf. the others, i.e. stages 22–25. The brackets show the probability of obtaining as high a value by chance.

through the 1·0:1·0 point (removal of none of the bud results in a normal wing), (*c*) and should be linear with a slope of 1.

This line has not been included in Figs. 3–9 (it made the graph look cluttered) but can be readily visualized. The intercept (a_1) and a test for significant difference (t_a) between a_0 and a_1, the slope (b_1) and a test for significant difference (t_b) between b_0 and b_1 are shown in Table 4 (where t is 'Student's t-test').

In the second hypothesis I examine one of the two additional assumptions in more detail. It is probably unreasonable to assume that the limb-bud, as measured, is exactly equivalent to the 10-day wing (see discussion). It is likely that some of the presumptive skeleton lies in the flank so that removing all of the bud leaves behind a little bit of the wing. To estimate the size of the flank component I examined all the cases in which the proximal cut was at the proximal boundary of the bud and in which there was a clear level specific defect in the wing skeleton. I then measured the proportion (u) of presumptive skeleton lying in the flank. The mean ($\bar{u}$) and standard error of the mean together with a test (t_0) for significant difference between $\bar{u}$ and zero (Student's t) are shown in Table 5. All the means were significantly different from zero except at stage 25 ($0{\cdot}3 > P > 0{\cdot}2$) suggesting that a significant proportion of the limb may remain in the flank. When the values were compared between stages then stages 21 to 25 proved to be not significantly different from each other (Table 5). Stages 21 and 25 (the most disparate values) $t = 1{\cdot}66$ ($0{\cdot}2 > P > 0{\cdot}1$), while for stage 20 to 21 $t = 1{\cdot}79$ ($0{\cdot}1 > P > 0{\cdot}05$). It therefore seemed more reasonable to use a mean value $\bar{u}'$ for $\bar{u}$ taken from stages 21 to 25 and this value ($\bar{u}'$) is inclined in Table 5. All stages have been tested against this mean value ($\bar{u}'$) for a significant difference (Student's t) and the results are shown in the table under $t_{\bar{u}}'$.

These values $\bar{u}19$, $\bar{u}20$ and $\bar{u}'$ can now be used in a second estimate of zero regulation. The null hypothesis being that the regression line should intercept the y axis at $\bar{u}$, pass through the 1·0:1·0 point and be linear with a slope of $(100\text{-}\bar{u})/100$. The only remaining assumption is that the affected part of the proximal–distal axis expands uniformly during growth.

This hypothetical line has been included in Figs. 3–9 using $\bar{u}19$, $\bar{u}20$, and $\bar{u}'$ for the remaining stages. Table 6 consists of the observed intercept (a_0) and its standard error, the hypothetical intercept ($a_2 = \bar{u}$) and its standard error, a test for significance (t_a) the observed slope (b_0) the hypothetical slope ($b_2 = (100\text{-}\bar{u})/100$) and a test for significance (t_b) where t is 'Student's t-test'.

Linearity of curve

It is more difficult to justify the remaining assumption of uniform expansion of the proximo–distal axis. If the rate of expansion varied with the position then the regression line between wing length and bud length would be non-linear. In fact judging by eye, one might guess that stages 20 or 23 scatter plots might be better fitted by a curve drawn through the points. One can test this hypothesis

Table 4. *Comparison observed and expected (bud = wing) regression lines*

	Intercept			Slope		
Stage	a_0	a_1	t_a	b_0	b_1	t_b
19	0·78 ± 0·04	0·0	18·93 (P < 0·001)	0·18 ± 0·07	1·0	11·7 (P < 0·001)
20	0·45 ± 0·06	0·0	7·59 (P < 0·001)	0·53 ± 0·08	1·0	5·80 (P < 0·001)
21	0·31 ± 0·04	0·0	8·16 (P < 0·001)	0·70 ± 0·06	1·0	5·00 (P < 0·001)
22	0·10 ± 0·04	0·0	2·30 (0·02 < P < 0·05)	0·92 ± 0·05	1·0	1·57 (0·1 < P < 0·02)
23	0·10 ± 0·04	0·0	3·16 (0·001 < P < 0·005)	0·87 ± 0·05	1·0	2·45 (0·01 < P < 0·02)
24	0·07 ± 0·03	0·0	2·12 (0·02 < P < 0·05)	0·88 ± 0·05	1·0	2·32 (0·02 < P < 0·05)
25	0·06 ± 0·03	0·0	1·68 (0·1 < P < 0·2)	0·87 ± 0·05	1·0	2·41 (0·02 < P < 0·05)

Where a_0 is the observed intercept with its standard error, a_1 the expected intercept, t_a 'Student's' t-test for significant difference between a_0 and a_1; b_0 is the observed slope with its standard error, b_1 the expected slope and t_b 'Student's' t-test for significant difference between b_0 and b_1. All rounded to two significant places.

Table 5. *Estimate of the proportion (%) of presumptive wing in the flank*

Stage	n	$\bar{u}$ (%)	t_0	$t_{\bar{u}}'$	f (%)
19	6	32·7 ± 0·9	36·3 (P < 0·001)	19·6 (P < 0·001)	32·7 ± 0·9
20	11	17·3 ± 3·4	5·09 (P < 0·001)	3·22 (0·005 < P < 0·01)	17·3 ± 3·4
21	10	8·8 ± 3·3	2·67 (0·02 < P < 0·05)	0·84 (0·4 < P < 0·85)	5·9 ± 1·0
22	4	5·0 ± 3·1	1·61 (0·2 < P < 0·3)	0·28 (0·5 < P)	5·9 ± 1·0
23	17	3·7 ± 1·4	2·64 (0·01 < P < 0·02)	1·28 (0·2 < P < 0·3)	5·9 ± 1·0
24	14	5·7 ± 1·5	3·80 (0·001 < P < 0·005)	0·11 (0·5 < P)	5·9 ± 1·0
25	8	3·8 ± 2·1	1·81 (0·2 < P < 0·3)	0·90 (0·3 < P < 0·4)	5·9 ± 1·0
$\bar{u}'$	53	5·9 ± 1·0	5·73 (P < 0·001)	—	—

Where n is the number of cases (see text), $\bar{u}$ is the estimate of presumptive wing in flank with the standard error of the mean, t_0 'Student's' t-test for significant difference between $\bar{u}$ and 0, $\bar{u}'$ is the mean value of $\bar{u}$ for stages 21 to 25, $t_{\bar{u}}$ is 'Student's' t-test for significant difference between $\bar{u}$ and $\bar{u}'$, f is the estimate of presumptive wing in the flank used to calculate the regression line $y = a_2 + b_2x$.

directly by a test of linearity in which one compares the variance within y for a given x against the variance of mean y about the regression line. In all cases the regression function was not significantly different from linear at $P < 0{\cdot}05$ or less. It is also possible to test indirectly by trying to fit hypothetical curves to the data to see if a better fit can be obtained. This procedure was attempted using $y = ae^{bx}$, $y = a + b\ (\ln x)$ and $y = ax^b$. In most cases the correlation coefficient obtained using these curves was lower than for the linear equation, the exceptions were for the logarithmic and power curves at stages 20 and 22 but here the correlation was only marginally better ($P > 0{\cdot}05$).

Because of these small differences and because I had no theoretical grounds for preferring any of these curves to the linear equation, I have made no attempt to further justify my use of the linear case.

Table 6. *Comparison observed and expected (some wing in flank) regression lines*

	Intercept			Slope		
Stage	a_0	$a_2\ (=f)$	t_a	b_0	b_2	t_b
19	0·78 ± 0·04	0·33 ± 0·01	10·70 ($P < 0{\cdot}001$)	0·18 ± 0·07	0·67 ± 0·01	4·07 ($0{\cdot}005 < P < 0{\cdot}801$)
20	0·45 ± 0·06	0·17 ± 0·03	4·04 ($P < 0{\cdot}001$)	0·53 ± 0·08	0·83 ± 0·03	3·47 ($0{\cdot}005 < P < 0{\cdot}801$)
21	0·31 ± 0·04	0·06 ± 0·01	6·38 ($P < 0{\cdot}001$)	0·70 ± 0·06	0·94 ± 0·01	3·79 ($P < 0{\cdot}001$)
22	0·10 ± 0·04	0·06 ± 0·01	0·90 ($0{\cdot}3 < P < 0{\cdot}4$)	0·92 ± 0·05	0·94 ± 0·01	0·41 ($0{\cdot}6 < P < 0{\cdot}7$)
23	0·10 ± 0·04	0·06 ± 0·01	1·19 ($0{\cdot}2 < P < 0{\cdot}3$)	0·87 ± 0·05	0·94 ± 0·01	1·29 ($0{\cdot}2 < P < 0{\cdot}3$)
24	0·07 ± 0·03	0·06 ± 0·01	0·32 ($0{\cdot}7 < P < 0{\cdot}8$)	0·88 ± 0·05	0·94 ± 0·01	1·18 ($0{\cdot}2 < P < 0{\cdot}3$)
25	0·06 ± 0·03	0·06 ± 0·01	0·16 ($0{\cdot}8 < P < 0{\cdot}9$)	0·87 ± 0·05	0·94 ± 0·01	1·37 ($0{\cdot}1 < P < 0{\cdot}2$)

Where a_0 is the observed intercept with its standard error, a^2 the expected intercept with its standard error, t_a 'Student's' t-test for significant difference between a_0 and a_2; b_0 in the observed slope with its standard error, b_2 the expected slope with its standard error, and t_b 'Student's' t-test for significant difference between b_0 and b_2. All rounded to two significant places. a_2 and b_2 are taken from Table 4.

Fit between observed and hypothetical regression lines

At stage 19 (Fig. 3) all of the points except for the sham controls (bud = 1·0) lie well above both hypothetical zero regulation lines. The fitted regression line passes within 5 % of the 1·0:1·0 point and intercepts the y axis at a point suggesting that removal of all of the bud would still give almost 80 % of the wing. Even when the wing tissue lying in the flank is taken into consideration there is still a wide divergence between observed and expected regression lines. The correlation coefficient for the data, $r = 0{\cdot}58$ ($0{\cdot}02 > P > 0{\cdot}01$) suggested some dependence of the deficiency on the size of the slice removed.

At stage 20 (Fig. 4) almost all of the points lie well above either hypothetical regression line. The fitted regression line passes within 2 % of the 1·0:1·0 point but intercepts the y axis at a level suggesting that removal of all the bud would still give almost half of the wing. Again, even when the tissue lying in the flank is taken into consideration the observed and expected regression lines are still widely different. The correlation coefficient, $r = 0{\cdot}79$ ($P < 0{\cdot}001$) suggested a very strong dependence of the deficiency on the size of the slice removed.

At stage 21 (Fig. 5) the points in general still lie well above either hypothetical regression line. Although the fitted regression line lies much closer the slope is still significantly different ($P < 0{\cdot}001$). The fitted line passes within 1 % of the 1·0:1·0 point and intercepts the y axis at a level suggesting that removal of all the bud still gives 30 % of the wing. Possibly as much as one third of this excess tissue may be explained by the presumptive wing tissue lying in the flank.

The correlation coefficient, $r = 0{\cdot}88$, is very high ($P < 0{\cdot}001$) suggesting a very strong dependence of the deficiency on the size of the slice removed.

At stage 22–25 (Figs. 6–9) the bud seems to have reached a steady state. The

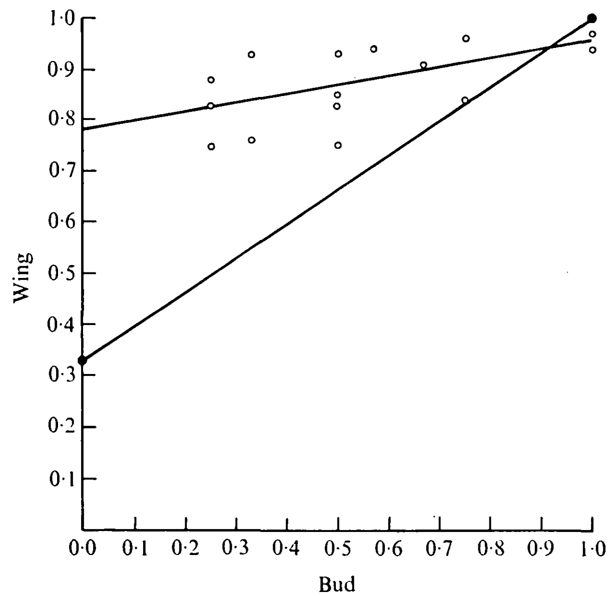

Fig. 3. Scatter plot of stage-19 results. Proportion of bud left after operating against proportion of wing present at 10 days. The upper line is the fitted regression line (—) and the lower is the expected zero regulation line (●—●).

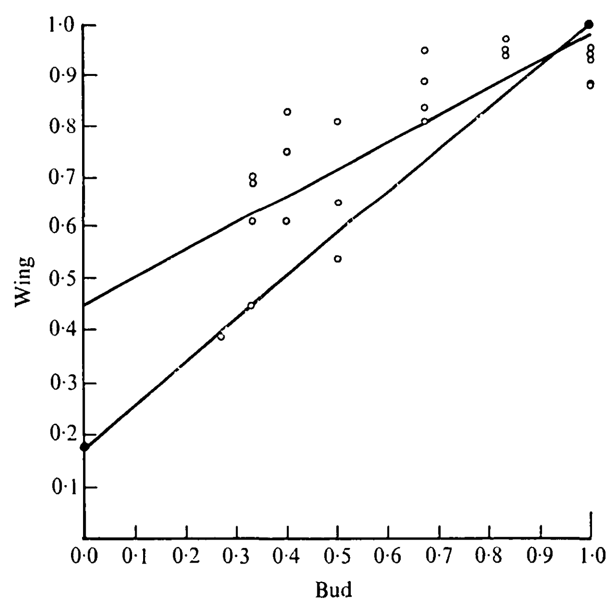

Fig. 4. Scatter plot of stage-20 results. Proportion of bud left after operating against proportion of wing present at 10 days. The upper line is the fitted regression line (—) and the lower is the expected zero regulation line (●—●).

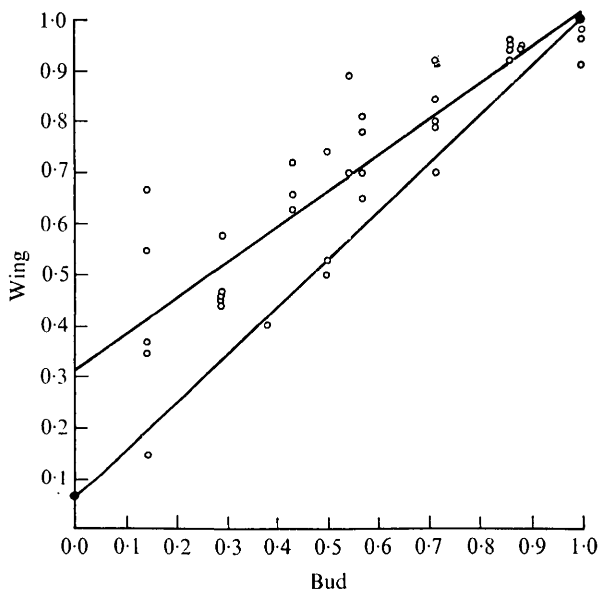

Fig. 5. Scatter plots of stage-21 results. Proportion of bud left after operating against proportion of wing present at 10 days. The upper line is the fitted regression line (—) and the lower is the expected zero regulation line (●—●).

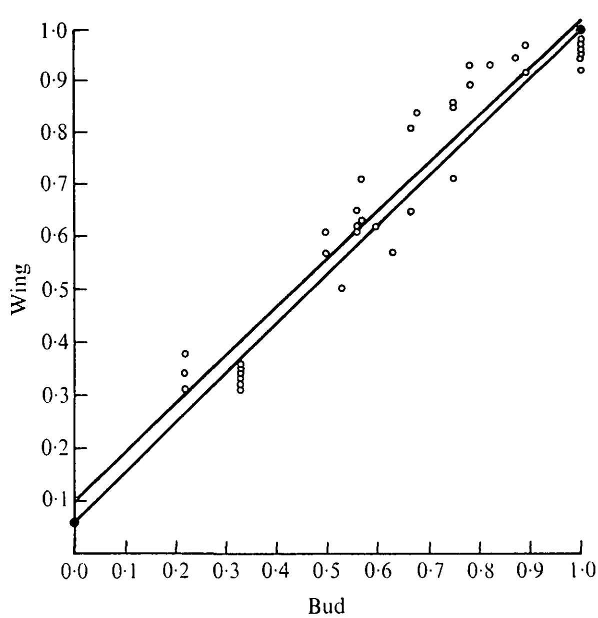

Fig. 6. Scatter plot of stage 22 results. Proportion of bud left after operating against proportion of wing present at 10 days. The upper line is the fitted regression line (—) and the lower is the expected zero regulation line (●—●).

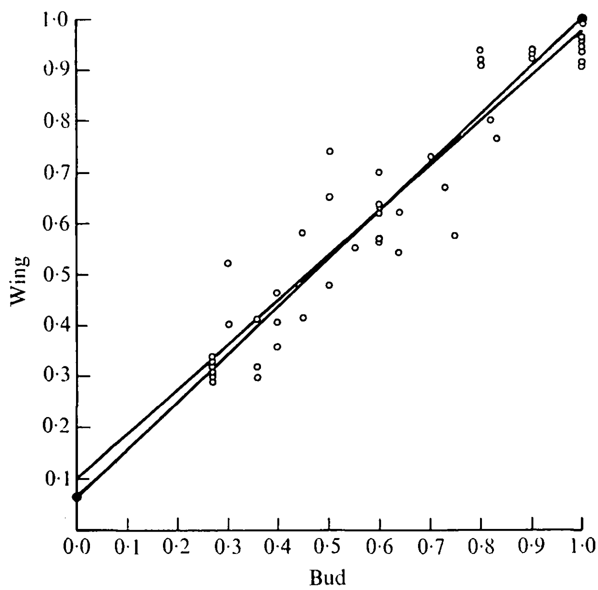

Fig. 7. Scatter plot of stage-23 results. Proportion of bud left after operating against proportion of wing present at 10 days. The fitted regression line (—) intercepts the Y axis at 0·1 then crosses the expected zero regulation line (●—●).

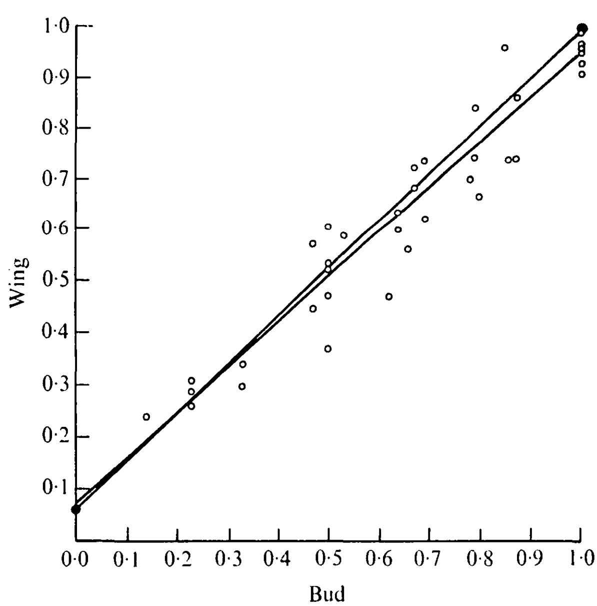

Fig. 8. Scatter plot of stage-24 results. Proportion of bud left after operating against proportion of wing present at 10 days. The fitted regression line (—) intercepts bud = 0·1 below the expected zero regulation line (●—●).

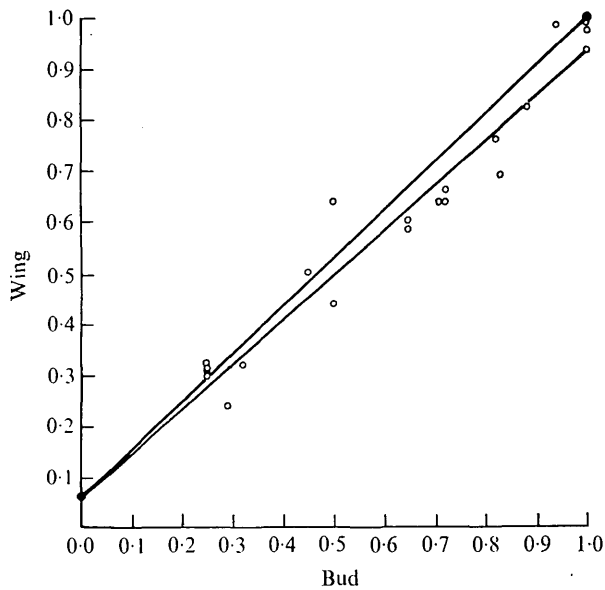

Fig. 9. Scatter plot of stage-25 results. Proportion of bud left after operating against proportion of wing present at 10 days. The fitted regression line (—) lies below the expected zero regulation line (●—●).

points are now scattered about the hypothetical regression lines and the slope of the second estimate (some wing in flank) is not significantly different from the fitted line (see Table 5). The fitted regression line in all four cases passes within 6 % of the 1·0:1·0 point and intercepts the y axis at a level suggesting that removal of all the bud would give less than 10 % of the wing. Most or all of this excess can be explained by presumptive wing tissue lying in the flank. The correlation coefficient varied between $r = 0{\cdot}94$–$0{\cdot}97$, an exceptionally high value ($P < 0{\cdot}001$) suggesting that the length of the wing obtained was very highly dependent on the size of the slice removed.

DISCUSSION

Francis Crick, speaking about developmental biology, observed that it was the mark of a young science that so few of the critical experiments had been repeated. It is therefore disconcerting to discover that when a simple and basic experiment is repeated that different researchers reach opposite conclusions.

The problem is that there are different possible criteria of regulation. Julian Lewis and I (Summerbell & Lewis, 1975) have discussed elsewhere the regulation obtained by Kieny and Hampé and came to the conclusion that using their criterion of the number of segments that develop, the skeletal regulation observed was far from complete. One can apply similar standards to the current work, then ask if the apparent regulation is any better or worse.

If one examines only the morphology of experimental limbs one might think that the limb achieved rather good regulation. For example at stage 22 several buds which had lost 20–40 % of their proximal–distal axis appeared quite normal. Why does this not represent perfect regulation? The answer must lie in the position of the deficiency and in the way in which the cut surfaces heal. If one looks at a typical result as illustrated in Fig. 2*d*, one might interpret this as showing that a slice of tissue corresponding to the elbow joint was removed, then the tip grafted back on to the stump so that the cut edges of presumptive ulna and radius were placed adjacent to the cut edges of the humerus. Not surprisingly the three bones have healed together to produce a single composite 'Y'-shaped bone with no sign of a joint. This produces an obvious abnormality, a piece of tissue is clearly missing, and there is little or no regulation. Now consider an example in which the deficiency lies a little more proximally. The tip is grafted back on to the stump so that the cut edge of the proximal end of the humerus is juxtaposed to the cut edges of the distal end of the humerus. The presumptive bones heal together to produce a skeletal element which has lost a portion of its diaphysis but which look absolutely normal (Fig. 2*c*). Unless a very large part of the diaphysis has been lost one would score this limb as showing good regulation, but in fact it may have behaved in exemplary mosaic fashion.

Deficiencies of this type can only be discovered by some quantitative estimate of the proportion of the wing present. Comparisons of the lengths of skeletal elements in operated (right) and control (left) wings as described in the results demonstrate that in fact there were only three cases in which there was not a significant measurable deficit in limbs from which tissue had been removed. One case at stage 19 with a 100 μm slice removed from a 400 μm limb and two cases at stage 20 with 100 μm slices removed from 600 μm limbs.

So perfect regulation of size and morphology is very rare, even at early stages, removal of a small fraction of the entire proximal–distal axis produces detectable abnormalities.

Theoretical estimates of zero regulation

Before one can assess the extent of regulation in the bud it is necessary to know how the bud would behave if there were no regulation. As I explained in the results my estimates of zero regulation depend on a number of assumptions. Although, as I shall show, these assumptions are compatible with the data contained in this paper, it is fortunate that most of them are either intuitively obvious, or else there is good evidence in the literature to support them (see below).

(1) The first assumption is that if I remove none of the bud I should obtain a normal wing. This assumption clearly should be true but in practice my data show a slight deviation from the expected result. When the sham controls from all stages ($n = 31$) are grouped, the mean wing length was $94 \cdot 3 \pm 0 \cdot 57$ % (standard deviation 3·2 % significantly different from 100 %, $p < 0 \cdot 001$).

Alternatively this figure can be compared with an estimate of the normal variation in the length of unperturbed wing skeletons (Summerbell & Wolpert, 1973) with a mean of 100 % and a population standard deviation of 2·5 % (still significantly different, $p < 0.001$). So although the mean length is significantly lower most cases do come close to the normal range.

One can also compare the length of the control wing predicted from the observed regression line, with the hypothetical value (100). The largest deviations were at stages 24 and 25 (about 7 % short), and a mean value for all seven stages was 97.4 ± 1.18 (not significantly different from 100 %, $0.10 > P > 0.05$). On balance it seems that although the sham controls did not give the expected result, the observed regression lines supported the notion than an entire bud would give an entire wing.

(2) The second assumption is that if all the bud is removed then one will obtain none of the wing. Independent of this paper there are good reasons for supposing that this is not true. Everyone's fate maps (Saunders, 1948; Amprino, 1965; Hampé, 1959; Kieny, 1964*b*; Stark & Searles, 1973; Lewis, 1975; Summerbell, 1976) show that the humerus extends a short distance into the flank so that if the whole bud were removed there would still be a little bit of proximal humerus left. This means that removal of the whole bud ought to leave a little bit of skeleton. In my estimate of the slope for the null hypothesis I consider first the simple case in which I give this remnant the value zero (Table 4) and second the case in which I have estimated its value (Table 5) before calculating a new expected regression line (Table 6).

The difference between the observed intercept and zero is therefore a composite value which includes excess wing tissue due to regulation, and to the presumptive wing tissue in the flank. Much of the apparent regulation suggested by the first hypothesis can be accounted for by the latter factor.

(3) The final assumption is that from the very earliest stage, equal proportions of the proximal–distal axis of the wing bud produce equal proportions of the long axis of the main skeletal elements. With one proviso this assumption is supported by the work of Lewis (1975) and Summerbell (1976), who show that proximal to the wrist the proximo–distal axis of the wing expands uniformly. Fortunately in these experiments it seems probable that the slice removed rarely encroaches on this difficult level as the deletion is always aimed at the elbow. Lewis (1976) has recently shown that a discrete region at the elbow also grows rather slowly, and this region makes up perhaps 10 % of the proximal–distal axis. One would therefore expect the data to overestimate the regulation achieved by up to 10 %. However as this region is normally the first to be removed the overall effect on the regression line will be slight.

The results strongly support the assumption of linear growth for there appears to be an extremely high correlation between the size of the slice removed from the bud and the proportion of the wing that was missing (Table 2, Fig. 10). One problem remains: If one examines carefully the fate maps of say Stark & Searles

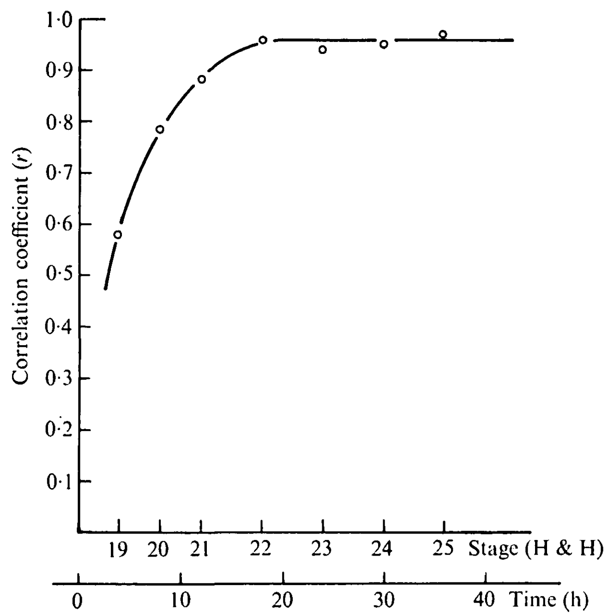

Fig. 10. The change in correlation coefficient with time. Stage 18 set arbitrarily at 0 h, the time when the limb-bud appears as definite outgrowth.

(1973) one sees that particularly at early stages a lot of the wing – the entire hand, is packed into a very small bit of the bud – the distal tip. The experiments in this paper still show a linear relationship because the deficiency does not include hand tissue. This is a good example of the dangers of extrapolating a regression line past the data base. If indeed more wing is packed into the tip, then as slices are removed which encroach on this region the least squares regression should no longer be linear but should dip more steeply. It is a matter of conjecture how much of the difference between observed and expected regression lines this explains. Suffice it to say that the earlier the stage the greater the importance of this factor and that by stage 24 and 25 the impact should be insignificant.

Comparison of observed and expected regression lines

Allowing the above assumptions and neglecting the contribution of the autopod then the slope of the least squares regression line is one possible measure of the extent of regulation. The nearer the slope to 0, the nearer all bud deficiencies are to producing normal length limbs and therefore perfect regulation. The nearer the slope to the hypothetical expected value then the nearer the bud is to perfect mosaic behaviour. One can therefore estimate a maximum value for the percentage of the deficit regulated by the expression:

$$\text{Regulation} = 1 - b_0/b_e$$

where b_0 is the observed slope and b_e the expected slope (some wing in flank).

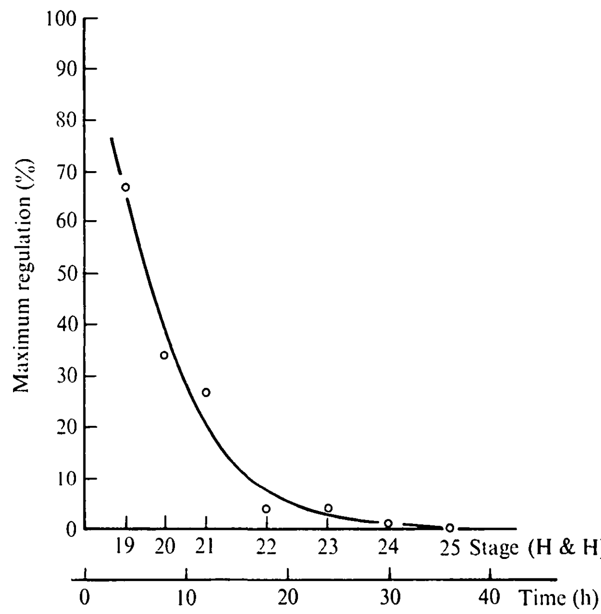

Fig. 11. An estimate of the outside limit of regulation possible, with increasing age, based on the slope of the fitted regression line. Factors other than regulation may explain some of the deviation from zero. Stage 18 set arbitrarily at 0 h, the time when the limb-bud appears as a definite outgrowth.

A graph of this value against time is shown in Fig. 11 where 1 represents perfect regulation and 0 represents perfect mosaicism. At stage 19 the expression approaches 1 but there is a rapid fall off towards 0 with increasing time. One could say that the outside limits for regulation are about 70 % of the deficiency at stage 19, 35 % at stage 20, 25 % at stage 21, and by stage 22 has reached a plateau with a maximum of about 5 % regulation (which is not significantly different to zero ($P > 0{\cdot}20$ or more).

An alternative estimate of regulation is given by the intercept of the regression line on the y axis. This is a measure of the theoretical absolute amount that the bud can regulate. One can again estimate a maximum value for the percentage of the deficit regulated by the expression.

$$\text{regulation} = (a_0 - a_e)/(1 - a_e)$$

where a_0 is the observed intercept and a_e the expected intercept (some wing in flank). A graph of this value against time is shown in Fig. 12 where 100 represents perfect regulation and 0 represents perfect mosaicism. Again at stage 19 the outside limit for regulation is at a maximum at stage 19, about 70 %; and falls off through stage 20, about 35 %; stage 21, about 25 % to a plateau at stages 22 to 25 where the possible maximum regulation is not significantly different to zero ($P > 0{\cdot}2$ or more).

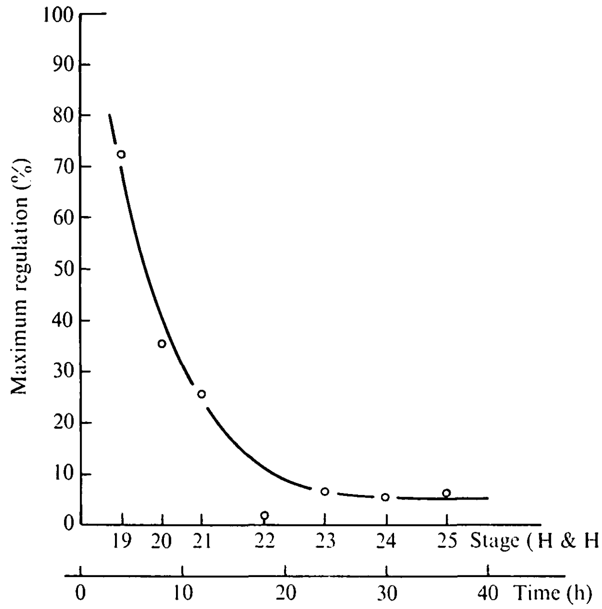

Fig. 12. An estimate of the outside limit of regulation possible, with increasing age, based on the intercept of the fitted regression line. Factors other than regulation may explain some of the apparent deviation from zero. Stage 18 set arbitrarily at 0 h, the time when the limb-bud appears as a definite outgrowth.

GENERAL CONCLUSIONS

(1) The wing-bud behaves as a near perfect mosaic following removal of a whole slice of the proximo–distal axis from stage 22 or later. Prior to this it is possible that it may regulate a maximum of about 25 % of the deficiency at stage 21, about 35 % at stage 20 and about 70 % at stage 19.

(2) Some of this apparent regulation will be due to the fact that at early stages relatively more of the presumptive wing is packed into the distal tip of the bud in a region which is not affected by slices. This experimental artefact tends to cause an overestimate of the regulation at early stages.

(3) Following slices, the morphology of the operated wings often appears relatively normal but in fact the skeleton is almost always (98·6 % of the time) significantly misproportioned.

(4) The limb never demonstrates size independent regulation of form (morphallaxis). None of these experiments gave limbs in which removal of a slice gave a small limb with all skeletal levels proportionately reduced.

(5) If the operation is restricted to the area from the shoulder to the proximal wrist then the deficiency is proportional to the size of the slice removed. This part of the proximal–distal axis must therefore grow relatively uniformly, at least from stage 20 to 25 inclusive. Equal lengths of the bud give equal lengths of the wing skeleton.

No attempt is made to relate this data to current theoretical models. It would seem however to be consistent with the progress zone model (Summerbell, Lewis & Wolpert, 1973). This question will be discussed at a later date along with evidence that regulation can occur when the deficiency is near an apical ectodermal ridge.

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