A Comparison of Standard Organ Culture and Standard Transplant Techniques in the Fusion of the Palatal Processes of Rat Embryos

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Numerous investigations have been undertaken to discover the etiology and pathogenesis of cleft palate. Although this anomaly has been produced by various experimental methods in laboratory animals (*5*), morphologic, histologic, and biochemical studies are needed to gain insight into the etiological mechanisms.

In an earlier study, Moriarty, Weinstein, and Gibson (*7*) used organ culture and transplantation techniques to study palatal closure and fusion in normal embryonic rat palates. It was suggested from that investigation that such techniques could be employed to study the morphologic and histologic effects of teratogenic agents on embryonic palatal tissues.

Using modifications of the in vivo and in vitro techniques employed in that earlier study, the current investigation was performed to standardize the methods and provide estimates for the probability of fusion of the palatal processes derived from rat embryos. The standardized methods and probability estimates are needed as guides and controls for future experiments which utilize teratogenic agents.

Materials and Methods

Sprague-Dawley rats were used exclusively as experimental animals in both the organ culture and the transplantation studies. The animals were mated according to the 'weight drop' method described by Hampson (*4*). An age value for the embryos was arbitrarily assigned by designating the day of mating as 'day minus one', the following day, 'day

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zero', etc. All transplanted and explanted tissues were from embryos of a chronologic age of 16 days and six hours. The embryo age was selected on the basis of findings by Zeiler (*13*) which suggested that palatal fusion seldom occurred prior to 16 days and eight hours in the Sprague-Dawley rats.

The operating, transplantation, and cultivation procedures were similar to those described by Moriarty and associates (*7*). A few modifications were employed, however, for improvement in the overall technique. The more significant modifications are discussed in the following paragraphs.

a) The age of the embryos used in the initial study was 15 days plus 14 to 21 hours. In the present investigation the embryos were all 16 days plus six hours of age. This change insured a somewhat closer approximation of the palatal processes at the onset of the experimental procedure. Also, the tongue was not a consideration when using the 16 day six hour embryos since the palatal processes had already assumed a horizontal position cranial to the tongue. In the initial study the tongue was described as being between the processes at the time of the removal of the palatal tissues from the younger embryos and therefore removal of the tongue was necessary prior to cultivation.

b) In some cases in the present study it was noted that the processes were actually contacting at the time they were planted (Figure 1). To be certain that the processes were only in contact and not fused, a fine glass rod was gently teased between the processes during the operation.

c) In the removal of the palatal tissue in the previous study, four excisions were employed. The head was removed first and then a second excision was used to remove the mandible. In this investigation the head was removed above the body of the mandible by an excision which, in essence, combined the two cuts just mentioned. The removal of the excess tissue cranial to the palatal section and the excess distal to the processes were described previously as the third and fourth excisions, respectively, but became the second and third excisions, respectively, for this study.

d) The most significant change in the in vivo study was from a homotransplant method to an isotransplant method. In theory, this change should have minimized the rejection phenomenon that is inherent with the homotransplant (*12*). The fabrication of the components of the transparent chamber in the technique employed by Sabet, Hidvegi, and Ray (*8*) was also modified by the addition of the removable transparent window.

e) Modifications in the organ culture procedure included the use of homologous type medium, that is, the embryonic extract and the plasma were obtained from Sprague-Dawley rats. Also, thrombin was often added to aid in coagulation of the medium. The moist chamber (watch-

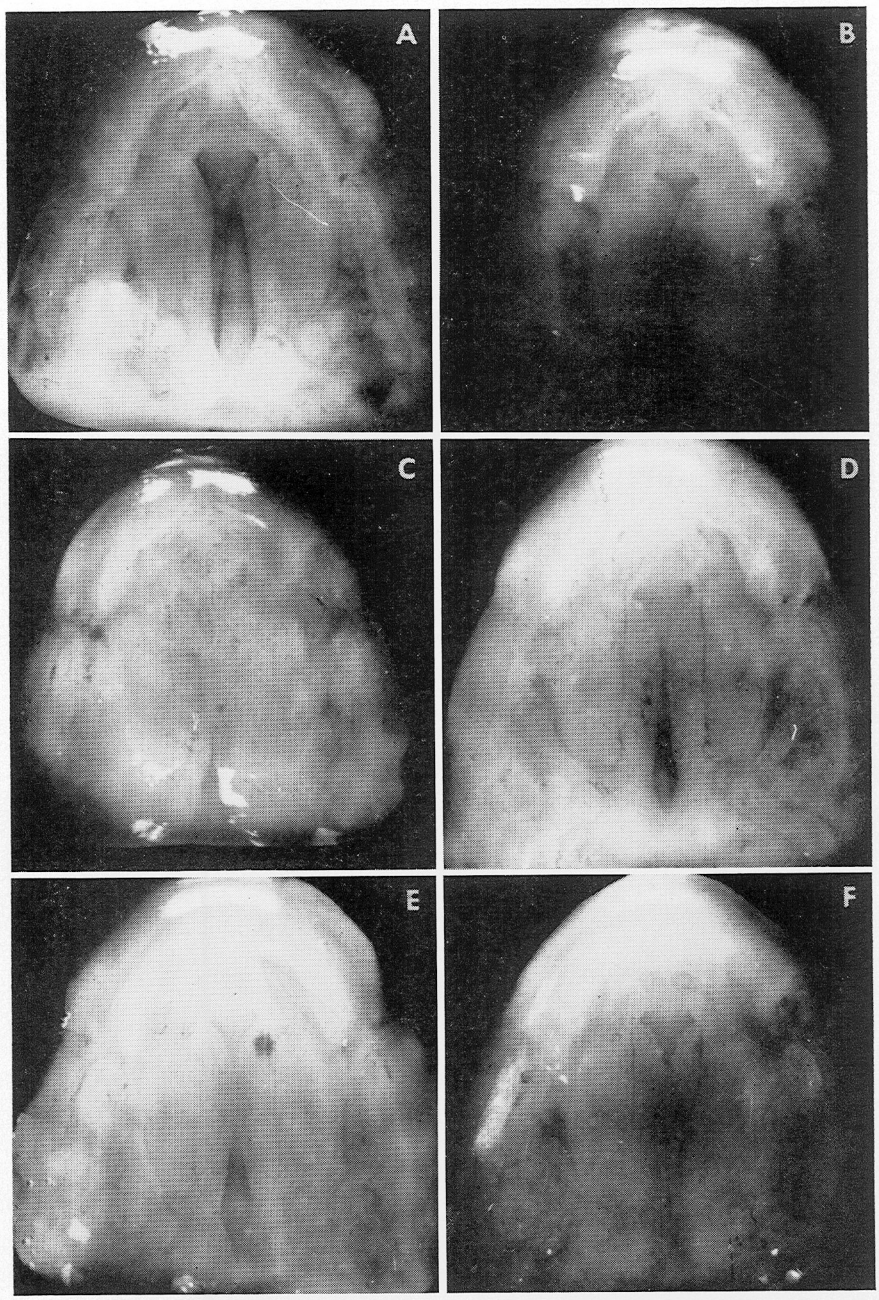

FIGURE 1. Variation in palatal process position of 16 day six hour embryos. A, Tissue R-23 E-2; the medial borders of the processes in a wide open position. B, Tissue R-8 E-6; the medial borders of the processes are overlapped. C, Tissue R-30 E-8; the medial borders of the processes are contacting anteriorly. D, Tissue R-9 E-8; the medial borders of the processes are contacting posteriorly. E, Tissue R-9 E-12; the medial borders of the processes are contacting anteriorly and posteriorly. F, Tissue R-30 E-13; the processes exhibit semi-total contact along their medial borders.

glass) apparatus used in this experiment was similar to that described in the earlier study.

The embryonic extract was prepared in five separate lots. Lot number one was composed of extract from 15 day 12 hour embryos whereas lot numbers two through five were prepared from 16 day six hour embryos. (Clots in the previous work were composed of equal parts of 9½-day-old chick embryonic extract and chicken plasma.)

The plasma was prepared from blood obtained by a cardiac puncture on unanesthetized male rats less than one year of age.

All instruments, equipment, fluids, etc. used in these studies were sterilized by appropriate methods. Operating procedures were performed under sterile conditions.

All experimental tissues were observed, sketched, and photographed initially and approximately every 12 hours during cultivation.

At the termination of the organ culture and transplant period the palatal tissues were prepared for histologic verification of process fusion by using a hematoxylin-eosin stain and standard laboratory procedures.

In accordance with the techniques used in these studies, the data obtained fitted the statistical methods which treat a binomial population. The proportion p of successes in the population is estimated unbiasedly by

$$\text{estimated } p = Y/n$$

where Y is the number of success observed and n is the sample size. A meaningful method of relating the results from these studies to ensuing studies is by applying confidence intervals for p. Confidence intervals were obtained from tables appropriate for binomial populations (*9*).

Results and Discussion

THE TRANSPLANTATION EXPERIMENT. A sample of 60 palatal transplants was studied.

The palatal transplants were maintained on the host animals from 48 to 72 hours. Initially, each transplant was to be maintained on the host of 72 hours. However, it was noted early in the study that there was little change in palatal development after the first 24 hours. If no change was noted in the tissue on two successive observations, these transplants were terminated at 48 to 60 hours.

Investigators have cited the role of the extracellular fluids in the survival of the transplants during the early stages following the transplantation (Mir Y Mir, L., *6*, Taylor and Lehrfeld, *10*, and Conway, Stark, and Joslin, *2*). In the present study the transplantation period was short and was probably terminated before vascularization between the host and the transplant could be established. The survival of the palatal transplants, then, was dependent upon a metabolic exchange occurring between the transplant and extracellular fluid rather than through a direct

vascularization. The value of the extracellular fluid resulted in at least two noteworthy disadvantages in the transplant chamber method. First, the amount of time necessary to prepare the host animals required that the transplantation procedure be performed in two phases. The loss of extracellular fluid that had accumulated between the first and second phases proved to be a disadvantage of a two phase procedure and may have influenced the results. Secondly, the size of the transplant chamber was limited in order to confine the extracellular fluids to the immediate vicinity of the transplant. Limiting the size of the chamber resulted in the tissues impinging upon the chamber walls because of a swelling of the tissues after grafting. How much the impingement affected the growth of the tissues was not discernible.

THE ORGAN-CULTURE EXPERIMENT. Sixty-five embryonic palatal tissues were explanted for periods up to 73 hours. Sixty-one of these tissues were included in the statistical evaluation whereas four tissues were studied separately due to a possible antigen-antibody reaction.

The dynamic age (16 days plus six hours) of each of the cultivated palatal tissues in this investigation eventually exceeded the relatively more static age (15 days 12 hours and 16 days six hours) of the embryonic extract present in the culture medium. Under these circumstances, according to the 'ascending range' theory of Gaillard (*3*), growth (development) of the explant should have been greatly curtailed. This did not occur in this investigation. Perhaps the occurrence of growth (development) could be explained as Walton (*11*) suggests by the presence of the embryonic tissue in a relatively older plasma.

One batch of plasma was obtained from the blood of young male rats which had been used earlier as host animals in the transplantation study. Four tissues were cultivated in a medium containing this plasma. Due to the possibility of an antigen-antibody reaction within the host animal as a result of the original transplantation procedure these four tissues were not evaluated statistically with the other 61 tissues. However, this experiment revealed no observable evidence of a specific incompatability and, in fact, all four tissues exhibited closure and fusion of the lateral palatal processes.

Also, no difference was observed in either the growth or fusion of the tissues cultivated in a medium to which a clot promoting substance (thrombin) had been added.

The palatal tissues were cultured for periods ranging from 48 to 73 hours. The specific time for the removal of the explant from the medium was empirically determined by the investigator in relation to a) the general appearance of the tissue, i.e., distortion, b) the amount of tissue or clot deterioration, and c) apparent fusion of the palatal processes.

HISTOLOGY OF THE EXPERIMENTAL TISSUES. Each of the palatal tissues was studied and evaluated with reference to the following: a) tissue morphology; b) cellular morphology; c) histological determination of

fusion for the transplanted tissue only; d) the amount of vascular infiltration.

The general tissue morphology of the transplanted and cultured tissues was good in all cases and the slight general distortion was considered within normal limits. Disruption of the normal relationship of the nasal cavity, nasal septum, and palatal processes, as well as a lack of development of the nasal septum, were noted. The disruption of the relationship of these structures was more severe in the transplant sample when com-

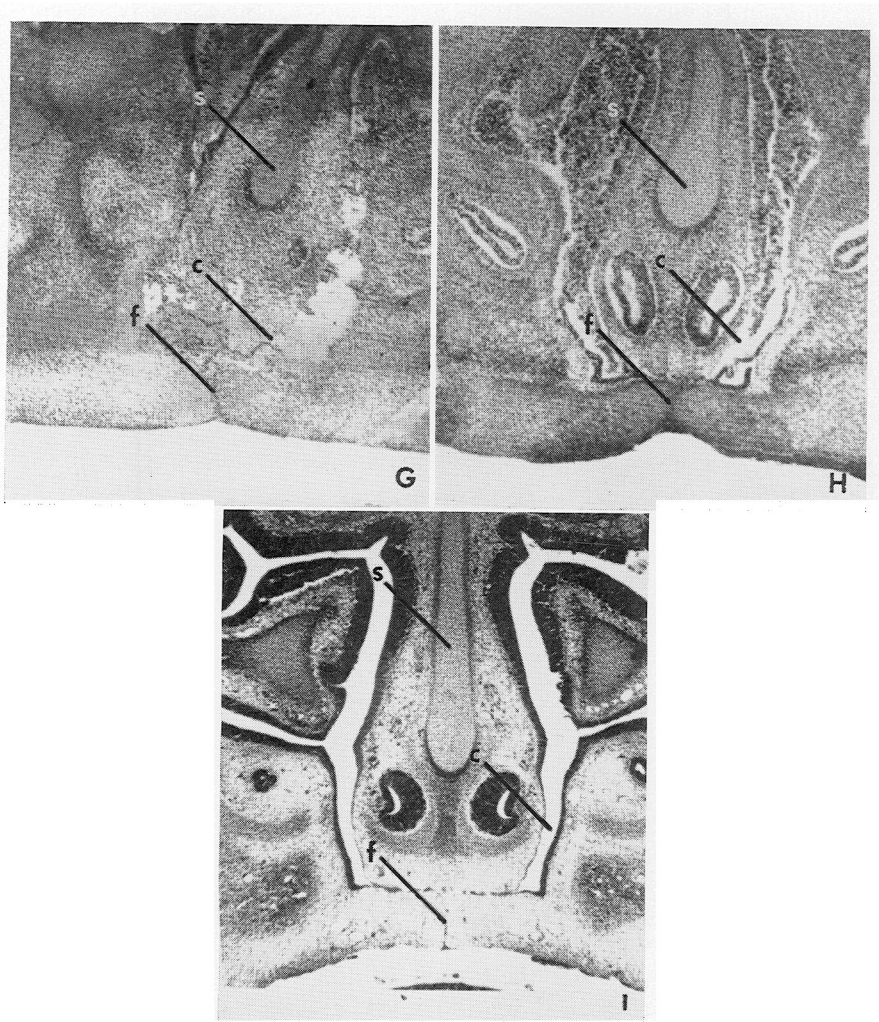

FIGURE 2. Comparative histological morphology of transplanted, explanted, and normally developed tissue. G, Frontal plane section of a 16 day six hour tissue after 50 hours *in vivo*. H, Frontal plane section of a 16 day six hour tissue after 48 hours *in vitro*. I, Frontal plane section of a 16 day 16 hour normally developed tissue. Line 's' denotes the cartilaginous nasal septum. Line 'c' denotes the nasal cavity. Line 'f' denotes the midline fusion area between the palatal processes.

pared with the normal tissue and with the organ culture sample (Figure 2).

When the experimental tissues were examined histologically, they exhibited various degrees of histological 'depression' such as pyknosis, shortening of the stellate processes of the mesenchymal cells, cornification of the epithelial cells, and presence of chromatin debris in the nucleus This 'depression' as described by Moriarty and associates (7) may have been the result of such factors as a lack of adequate nutrition, trauma, rejection reaction of the host animals in the transplant study, etc. The most logical of these explanations seems to be the lack of adequate nutri-

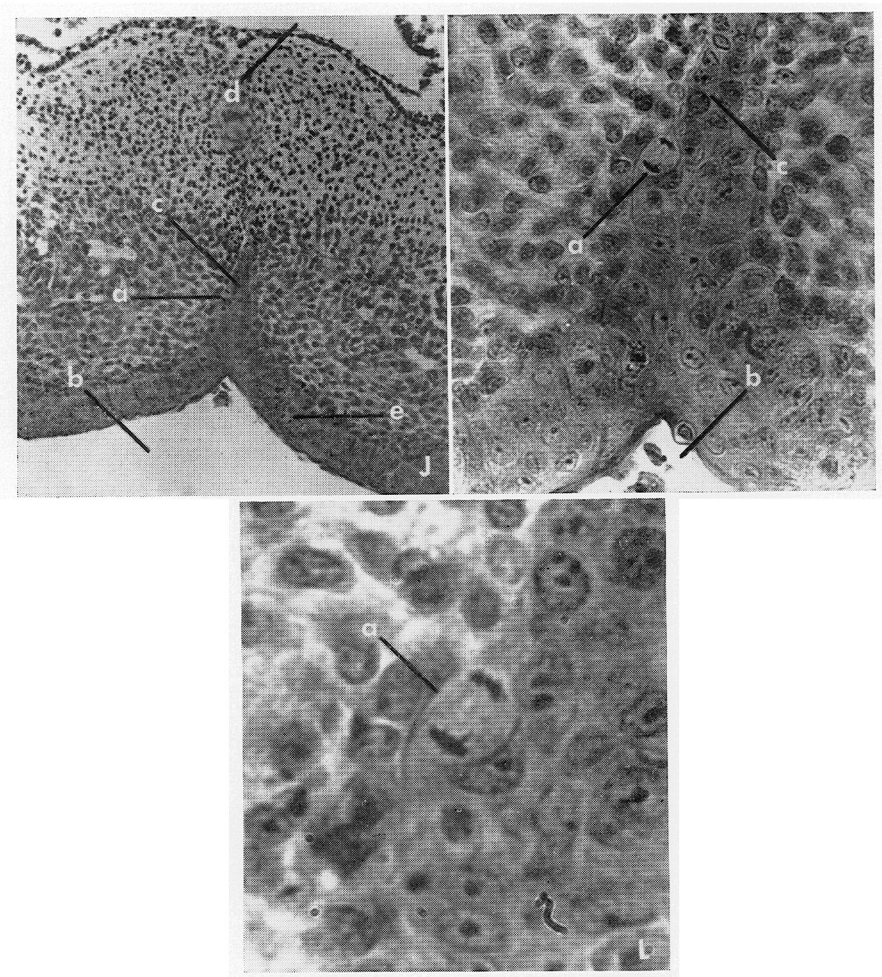

FIGURE 3. J, K, and L are photomicrographs of a frontal plane section of the midline area of fusion of a tissue which developed fusion *in vitro*. The lines represent a) the mitotic figure in the united epithelium along the midline; b) the oral cavity; c) the fusion area of the medial borders of the palatine processes; d) the nasal cavity; e) the mitotic figure in the oral epithelium.

tion due to the size of the experimental tissue and the duration of the experimental procedure.

Numerous mitotic figures were observed in the midline epithelium (between the palatal processes) of the explanted tissues. A few mitotic figures were also noted in the oral epithelium (Figure 3). An actively high rate of proliferation of epithelial cells in the midline has been described by Barry (*1*) as a possible etiologic factor in the production of a cleft palate. There is little doubt that continued epithelial proliferation in this area could result in the presence of epithelial pearls after fusion has occurred.

Infiltration of red blood cells and white blood cells (primarily leucocytes) from the host was evident in all the transplanted tissues.

The determination of fusion of the palatal process of the secondary palate depended upon positive findings from the histological examination of the experimental tissues. In the sample of 60 transplants that was examined, 44 exhibited fusion somewhere along the midline of the secondary palate. Of the 61 explanted tissues, 54 were found to have fused processes somewhere in the secondary palate. Seven explants did not exhibit fusion within the secondary palate but did show fusion of the secondary palate to the primary palate. One explant was destroyed during histologic preparation and could not be examined.

As in the earlier study (*7*), the degrees of fusion ranged from early fusion by epithelial elements (double cell strands of epithelial elements remaining in the line of fusion) to loss of all epithelial elements and complete mesenchymal penetration.

Table 1 shows histological data from the transplanted tissues grouped according to the highest degree of fusion observed for each tissue. A similar grouping is noted in Table 2 for the explanted tissues.

TABLE 1. Data for transplantation technique.

Development During Experiment	*Number of Tissues*	*Duration in Hours (Range)*	*Host Sex*	
			Females	*Males*
Fusion with complete mesenchymal penetration	23	48–72	9	14
Fusion with single cell strand epithelial remnants	10	48–72	3	7
Fusion with double cell strand epithelial remnants	11	48–72	6	5
Process contact without epithelial union	1	58	1	0
Processes did not meet	14	50–72	8	6
Tissue destroyed during histologic preparation	1	48	0	1
Totals	60	48–72	27	33

TABLE 2. Data for organ culture technique.

Development During Experiment	*Number of Tissues*	*Duration in Hours (Range)*
Fusion with complete mesenchymal penetration	35	48–73
Fusion with single cell strand epithelial remnants	5	48–59
Fusion with double cell strand epithelial remnants	14	48–73
Process contact without epithelial union	5	59–73
Processes did not meet	2	48–59
Totals	61	48–73

STATISTICAL EVALUATION. The data obtained from the histological determination of fusion of the palatal processes were evaluated statistically. An estimate for p was calculated for the transplanted tissues as follows:

$$\text{estimated } p = Y/n = 44/66 = 0.73$$

A 95% two-sided confidence interval for the estimated p was determined to be:

$$0.61 < p < 0.84$$

The estimated p for the explanted tissues was calculated as:

$$\text{estimated } p = Y/n = 54/61 = 0.89$$

and the one-side 95% confidence interval is:

$$0.80 < p < 1.0$$

Summary

a) A sample of 60 palatal transplants were evaluated histologically to determine fusion of the processes of the secondary palate. On the basis of the number of successes observed in the experimental sample, an estimated p of 0.73 and a 95% confidence interval for p of $0.61 < p < 0.84$ was obtained. b) The sample of 61 explanted tissues was evaluated histologically for the occurrence of fusion. The probability p of fusion of the palatal processes, using the standard organ culture technique, is 0.89 with a one sided 95% confidence interval of 0.80 to 1.0. c) Some histological aspects of the tissues were described and it was noted that the transplanted tissues exhibited greater disruption of the various tissue structures when compared with the corresponding explanted tissues and with normally developed tissue. d) There were no apparent effects on the growth and/or development of embryonic rat palates cultivated in vitro for periods of time up to 73 hours in a medium: (1) in which the embryonic extract constituent was of a relatively younger age than that of the explant and/or (2) in which the plasma constituent has been derived

from an isografted animal and/or (3) to which the clot-promoting substance, thrombin, has been added. e) Active cell proliferation was observed in the epithelial remnants of the midline fusion area. This phenomena may explain the presence of epithelial pearls after complete mesenchymal penetration has occurred.

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References

1. Barry, A., Development of the branchial region of human embryos with special reference to the fate of epithelial. In S. Pruzansky (Ed.), p. 46–62, *Congenital Anomalies of the Face and Associated Structures.* Springfield: Charles C Thomas, 1961.
2. Conway, H., Stark, R. B., and Joslin, D., Observation on the development of circulation in autografts. *Plastic reconstr. Surg., 8,* 312–319, 1951.
3. Gaillard, P. J., Developmental changes in the composition of the body fluids in relation to growth and differentiation of tissue cultures. *Protoplasma, 23,* 145–174, 1935.
4. Hampson, F. F., A morphological study of palates from rat embryos subjected to a transitory maternal pteroyglutamic acid deficiency. Unpublished M.S. thesis, Univ. of Nebraska, 1962.
5. Kalter H., and Warkany, J., Experimental production of congenital malformation in mammals by metabolic procedure. *Physiol. Review, 39,* 69–115, 1959.
6. Mir Y Mir, L., Biology of the skin graft. *Plastic reconstr. Surg., 8,* 378–389, 1951.
7. Moriarty, T. M., Weinstein, S., and Gibson, R. D., The development *in vitro* and *in vivo* of fusion of the palatal processes of rat embryos. *J. embryol. exp. Morph., 11,* 605–619, 1963.
8. Sabet, T. Y., Hidvegi, E. B., and Ray, R. D., A chamber for *in vivo* observations of living organs. *Plastic reconstr. Surg., 27,* 105–108, 1961.
9. *Tables of the Cumulative Binomial Probability Distribution.* Cambridge: Harvard Univ. Press, 1955.
10. Taylor, A. C., and Kehrfeld, J. W., Determination of survival time of skin homografts in the art by observation of vascular changes in the graft. *Plastic reconstr. Surg., 12,* 423–431, 1953.
11. Walton, A. J., On the variation in the growth of mammalian tissue *in vitro* according to the age of the animal. *Proc. Roy. Soc. Lond. Bull., 88,* 476–482, 1914.
12. Woodruff, M. F. A., *The Transplantation of Tissues and Organs.* p. 59–64, Springfield: Charles C Thomas, 1960.
13. Zeiler, K. B., A study of the morphology and the time of closure of the palate in the albino rat. *Arch. oral Biol., 9,* 545–554, 1964.