PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

VI. Differentiation in the Chicken Embryo Heart from the Point of View of Stimulus Production.

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This study concerns itself with the attempt to discover throughout the period of embryonic life of the chick, first, the site in the heart at which the pace-making function resides, and, second, to what extent other regions in the heart either share in this function or fail to share in it; to learn at what ages the various portions lose this capability in whole or in part. Studies of the functions of the embryonic chick heart have before now been undertaken, notably with the view to studying such functions as the conduction of stimuli (Külbs (1), Wertheim-Salomonson (2), Johnstone (3)) and to a less extent that of rhythm production and of reflex irritability (Johnstone). The observation that isolated fragments of the heart muscle such as were used in these experiments contract over long periods of time in appropriate culture media has been made by Carrel (4) and by Burrows (5). The object in the observations now reported has been to learn the extent to which a given portion of the living heart possesses this function.

Method.

After the rate of the heart of the intact embryo had been ascertained in the manner described in another paper (6), the embryo was weighed, the weight found being utilized to aid in establishing its age. The heart was then excised and its area measured (6). The heart was next dissected while still suspended and bathed

* We are indebted to Miss Alma Rosenthal for aid in the early part of these experiments.

in chicken serum. The fragments were planted in a large drop of chicken plasma to which enough chicken embryo extract was added to cause coagulation. So far the method of planting and the preparation of the materials resembled closely that for which we are indebted to Carrel. The modifications of his admirable method for our purposes has been described elsewhere (7). For this particular purpose a further modification was adopted in the choice of the embryo extract. Usually the extract had been that of embryos 10 days old. Here we chose instead embryos of the same age as that of the heart to be tested, with the view to exposing the fragments of the heart only to such tissue juices as were at the same stage of development. So far as the source of the plasma was concerned, we had no choice; even if it were obtainable, enough was obviously not available from embryos of the age in which we were most interested. It may be mentioned that the coagulation of the plasma after the fragments had been planted in it varied with the age of the tissue extract that was used. With extracts made from embryos before the age of approximately 5 days, the time of coagulation was delayed; afterward with increasing age of the extract, coagulation was more prompt. The dissections were carried out, as has been said, on the stage of a dissecting microscope, clean incisions being made by carefully sharpened iridectomy knives. After the fragments were planted and the medium coagulated the mica slide on which the plasma had been spread was inverted and placed on a Gabritschewski dish, in the depressed portion about the circumference of which water was placed to prevent drying of the preparation. The average time from the beginning of weighing to the completion of coagulation averaged 22 minutes, increasing with the increasing age of the embryo. At each step of the preparation care was taken to prevent evaporation as a result of the heat of the room and its low percentage of humidity.

We were, it seems, justified in our methods as the differences in behavior exhibited by the various fragments now to be described will show.

EXPERIMENTS.

Experiments were made on the hearts of embryos from 2 days, 18 hours old to 19 days, 18 hours old.

In the 2 to 3 day embryos, when the heart is still in the stage of a simple tube, before the development of the interauricular septum, the whole heart was divided into four pieces (Fig. 1), the atrium, the proximal and distal parts of the ventricle, and bulbus arteriosus. Later at about the 3rd to the 4th day (Fig. 2) the atrium was divided into two pieces, right and left; and the ventricle into five portions, two of the right ventricle, two of the left, and an apical portion. Later still from about the 7th day onward, the auricle was divided into five portions, a mesial piece, two pieces of the right auricle and two of the left (Fig. 3). When the auricles

were large so that the fragments measured more than 1.0 sq. mm., each of the standard five auricular fragments was further subdivided. In this manner, throughout the experiments, the whole auricular tissue was fractionated, planted, and the rate developed by each fragment counted and recorded.

The treatment of the ventricles differed from this. As soon as the heart was large enough, at about the 7th day, these were divided into seven instead of five pieces, —two each from the right and left ventricles, and one each from the apex, conus arteriosus, and the base of the ventricles behind. The pieces from the ventricles

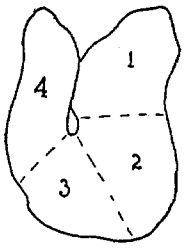

FIG. 1.

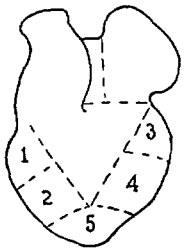

FIG. 2.

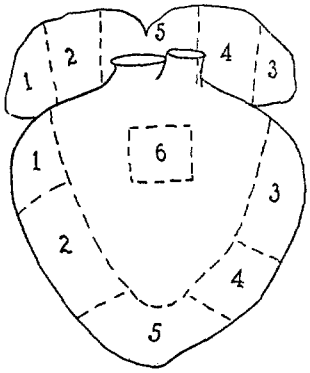

FIG. 3.

FIG. 1. An outline drawing is shown of the heart of a chick embryo 2.7 days old. The heart was divided in a manner indicated by the interrupted line. The portion numbered 1 is the venous end of the tube; that numbered 4, the arterial end.

FIG. 2. In this outline drawing of the heart of a chick embryo 3.9 days old, the manner is shown in which the auricles were divided. The portions numbered 1 and 2 represent the locations of the wall of the right ventricle from which pieces were taken; 3 and 4, those from the left ventricle; 5 that of the apex.

FIG. 3. An outline drawing is shown of the heart of a chick embryo 7.7 days old. The number and location of the fragments of the auricles as well as of the ventricles are shown. Fragment 6 was taken from the conus arteriosus. An additional piece, 7, was taken from the base of the right ventricle posteriorly.

and apex were taken from the margins, right and left, as representative of the convex border of the primitive tube. Except for the conus and dorsal pieces, the V-shaped area between the right and left borders (Fig. 3), including the septum, was discarded. Later when the heart was larger still, seven fragments continued to be taken, but the five pieces from the margins of the heart, instead of being adjoining portions, were specimens only of the five regions. In these large hearts, therefore, only a small portion of the total ventricular tissue was studied.

In the earlier experiments (Nos. 9 to 60) all the fragments were projected and measured according to a technique described elsewhere (7), and the measurements correlated with the rate developed by the fragment. Studies of this sort were made of fragments beginning at 3 days, 18 hours old and continued to 11 days, 18 hours. The measurements ranged from 0.05 to 0.86 sq. mm.; the great majority between 0.1 and 0.25 sq. mm. When the size of the fragments containing the pace maker taken from hearts of various ages is studied in relation to the rate developed by each, the absence of correlation between size and rate quickly appears (Fig. 4). In 4 day embryos the

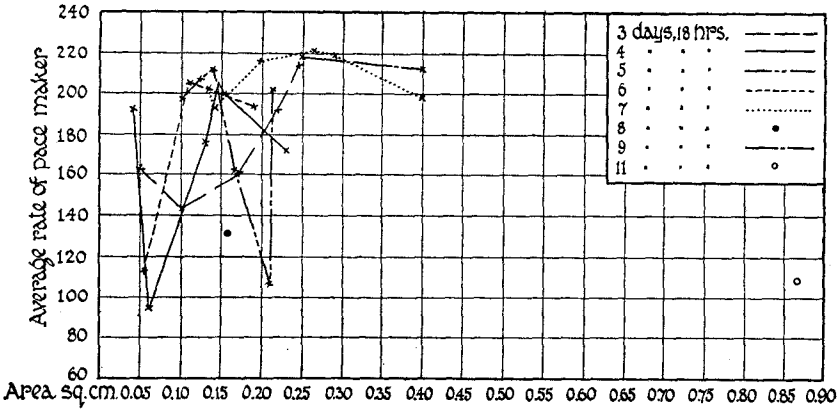

FIG. 4. A series of curves is shown to demonstrate the fact that the size of the fragments of the pace maker makes no difference in its rate-making capability.

smallest pace maker exhibited the highest rate; in 3 day embryos it was the largest piece which did so; in a 7 day embryo pieces ranging from 0.13 to 0.4 sq. mm. all beat at a high rate, from 192 to 220 per minute.

The rate of each fragment was counted as soon after planting as possible. The counting continued for about $1\frac{1}{4}$ hours; in the later ones for 1 hour. In many, indeed in most, experiments the counts after the first 10 minutes of planting were surprisingly constant so that the deviation from the average rate of a given fragment was less than 10 beats in 58.7 per cent of the cases and not more than 30 beats in 94 per cent of the cases (Table I). Obviously therefore the rate of each fragment shortly after planting was approximately uniform.

In another paper (6), the rate of the heart of the intact embryo of various ages is reported and the fact is pointed out that a curve can be drawn which shows the general direction of changes in rate during embryonic life. It is likewise pointed out that, for reasons not yet clear, considerable deviations occur. The specimens included in that study formed the basis also for this one. It is not surprising, therefore, that at a given age the fragments containing the pace maker developed different rates. That the differences are greater than were found in the intact embryos was to be expected, because of the highly artificial environment in which the fragments were placed. In view of this circumstance and of the fact, moreover, that not so much the actual rates developed but the rate of the pace maker relative to that

TABLE I.

Deviation of Each Fragment from Its Average Rate.

No. of fragments.	Deviation from average rate.	Average per cent for average rate.	Per cent of fragments.
Total....577	0–209 beats.		100
46	0	0	8.0
110	1–3	10.7	19.0
183	4–9	18.3	31.7
	Below 10.		58.7
205	10–30	37.3	35.6
33	Above 30.	76.6	5.7

of other fragments can have physiological significance, an analysis of the actual rates has been neglected. It is, however, of interest to notice (Table II) that of 88 instances studied, the difference in rate between the intact heart and that of the fragment containing the pace maker was 3.5 per cent or less in 21 (= 24 per cent) cases; 5.3 per cent or less in 37 (= 42 per cent) cases; and 6.7 per cent or less in 48 (= 55 per cent) cases. The close approximation of the two rates is a striking and surprising circumstance, for we have now a demonstration that the size of the fragment makes no difference to rate production, and that the fragments can actually equal and sometimes surpass the rate of the intact heart. This occurred in point of fact in 28 experiments.

TABLE II.

The Rate of the Pace Maker Compared with That of the Whole Heart.

Age.	No. of cases in which rate of pace maker and of whole heart differ by.																												
	0–10		10–20		20–30		30–40		40–50		50–60		60–70		70–80		80–90		90–100		100–150		150–200		200–250		Total.
	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.	Whole heart higher.	Pace maker higher.			
days *hrs.*																													
2 18*		1		1		1		1			1				1				2		2								10
3 18	2	1				1								1															5
4 18	2			1	1	1		1							1														7
5 18	2		1	1	1	1	1				1		1																9
6 18	2	1	1						1				2		1														8
7 18		1	1	1	1			1													1								6
8 18		2	1				1								2														6
9 18		2	3	1					1												2								9
10 18	1			1		1		1													2								6
11 18																					1								1
12 18	1		1																										2
13 18			1								1	1																	3
14 18	2	1			1		1																						5
15 18													1												1				2
16 18			1								1										1				1				4
17 18					1	1															1								3
18 18																					1								1
19 18															1														1
Total....	12	9	10	6	5	6	3	4	2		4	1	4	1	6				2		11				2				

*18 hours was the average time, varying ± 3 hours.

The Location of the Pace Maker.

The venous end of the cardiac tube was divided in the manner already described, depending on the age of the embryo, the incisions after the 3rd day being made parallel to the long axis of the organ.

TABLE III.

Location of Pace Maker.

Age.	No. of cases.	Right auricle.	Middle piece.	Left auricle.	Ventricle.				Remarks.
					Right base.	Proximal portion.	Distal portion.	Bulbus arteriosus.	
days hrs.		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
2 18*	10	40†				20	20	20	Ventricular portions were equal in rate with the venous end.
3 18	6	50	33			17			
4 18	7	62.5	37.5						
5 18	9		77	22					
6 18	7	13	87						
7 18	6	16⅔	66⅔	16⅔					
8 18	6	83	16						
9 18	8	37.5	37.5		25‡				
10 18	5	80	20						
11 18	1								
12 18	3	100							
13 18	3	66⅔	33⅓						
14 18	5	100							
15 18	2	100							
16 18	4	75		25					
17 18	3	33⅓	33⅓	33⅓					
18 18	2	50	50						
19 18	2	100							

* 18 hours was the average time, varying ± 3 hours.

† At this age there is included the whole venous end of the tube.

‡ To these pieces part of the right auricle was probably attached.

In embryos 2 days, 18 hours old a sharp differentiation of function has apparently not yet taken place. Fragments from the venous end usually (in 40 per cent of cases) set the pace, but this function can at this age be assumed also by the two ventricular and the arterial fragments (Table III). The inference cannot be drawn from these facts

that in the intact heart under natural conditions there is indifference in respect to which portion of the tube initiates the pace. The experiments permit the statement only that at this stage of development a high rate can be developed by fragments from any part of the tube; that the tube in short does not yet show a notable degree of differentiation. At 3 days, 18 hours, a change has already taken place. In the majority of the cases, the rate-making functions appear to lie in the auricle, whereas the ventricles appear to retain the function occasionally. It must be remembered, however, that there is a practical difficulty encountered in dividing the heart. Theoretically if the incision passes proximal to the line of junction between auricle and ventricle, even to a slight extent, a few auricular fibers or cells possessing a high inherent rate if joined to a ventricular fragment may set the pace and give to the ventricles the appearance of possessing a capability which is not inherent in them (Table III). Actually in these experiments at the earlier stages of development the behavior of the fragments anterior to the position of the atrioventricular line shows that they are perhaps the most sensitive.

After the 3 day, 18 hour period, there is no doubt that one or other of the auricular fragments takes on the leadership in rate making. At this time it was usual to divide the auricles approximately into a right portion (usually sub-divided into two fragments), into a left portion (likewise usually divided into two fragments), and into a middle piece at the location of the septum. Obviously it was not possible to separate these structures in such a manner that the middle piece retained in each instance precisely the same structures as the right and left portions. If the pace-making function resided in the mesial portion of the right auricle, it became a matter of chance, depending on the location of the incision, as to whether the pace was to be set by the right or the middle. Occasionally the incision was misplaced sufficiently so that pace making appeared to reside even in the left fragment. In the younger embryos, from the 5th to the 7th day, it was usual to include the pace maker in the middle or right piece. In the larger embryos, where incisions could be placed with greater accuracy, it was the right piece which set the pace. In any case it was not the laterally placed fragments (tip of auricles), but the

mesially placed ones which contained the rate-making structure (Table III).

What the histology of this portion of the auricle is we have not investigated in detail. We know, however, from the study of series of chicken embryos examined at the Harvard Medical School[1] that nerve ganglia are located in the right auricle, in the posterior wall near the interauricular septum. Precisely what function these elements exert we do not know. From the manner, however, in which the incisions were placed, there is no reason to doubt that they were included in the fragment of auricular tissue which usually set the pace. Their presence here throws no light on the question of which tissue, nervous or muscular, is responsible for the cardiac rhythm. A rhythm exists before the inwandering of ganglion cells on the 5th to the 6th days, as is well known since the studies of His (8). What relation to this problem the controversy (Kunz) on the problem whether ganglia wander first into the heart, trailing their nervous connections behind them, or whether the nerve fibrils penetrate the heart tissue first, and the ganglion cells later, is not known. A decision is difficult because there is as yet no adequate method for staining the nerve fibrils and of tracing their course in the heart.

The change in function of rhythm production in fragments taken from typical positions in the heart was studied from the point of view of differentiation. By rhythm production is meant the ability to contract under the experimental conditions, as distinguished from the function of pace making by which is meant the ability to contract at a highest rate in a particular heart. Some fragments contracted; others failed entirely to do so. It is, of course, clear that statements concerning the fragments treated in the manner adopted in the experiments are not generally valid. It is wholly conceivable that the same fragments, had they been suspended in one or another balanced salt solution, might very well have contracted. The problem here was, however, to distinguish diversities if they existed, not uniformity.

[1] It is a great pleasure to be able to acknowledge the courtesy shown to us by Professor F. T. Lewis in placing the beautiful series in his department at our disposal for study, and to thank him for the opportunity he afforded us. To Professor J. L. Bremer and to Dr. E. A. Boyden we are likewise indebted for aid and suggestions.

TABLE IV.

*The Loss of Rhythmic Function by the Fragments According to Age except the Pace Maker.**

Age.		No. of cases.	Auricles.						Ventricles.							
			Right.			Left.			Right.		Left.		Apex.	Arterial end of tube.	Conus arteriosus.	Base of the ventricles posteriorly.
			1	2	3	3	2	1	Base (proximal).	Mid level.	Base (distal).	Mid level.				
days	*hrs.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	18†	9	57					100	88		88			33		
3	18	6	100					100	100		100	100		66		
4	18	7	100	100‡	100	100		100	100	100	100	100	50			
5	18	9	100		100	88	100	80	100	100	100	100	100			
6	18	7	100	100	100	100	100	100	100	100	100	100	100		100 (1)§	
7	18	6	100		100	100		100	100	100	100	100	100		100 (1)§ (root of aorta).	
8	18	6	100		100	100		100	100	80	40	80	80		66 (3)§	100 (2)§
9	18	8	100		100	100		100	100	100	100	100	100			
10	18	5	40		60	80	100	80	60	60	60	20	80		20	0
11	18	1	100		100	100		100	0	0	100	0	0		0	0
12	18	3	33	33	100	66	66	33	66	66	66	66	0		0	0
13	18	3	0	33	66	33	33	0	0	0	33	33	33		0	0
14	18	5	0	40	100	20	0	0	0	0	0	0	0		0	0
15	18	2	0	100	0	0	0	0	0	0	0	0	0		0	0
16	18	4	25	33	50	50	0	0	0	0	0	0	0		0	0
17	18	3	0	33	33	50	0	33	0	0	0	0	0		0	0
18	18	2	0	50	50	0	50	0	0	0	0	0	0		0	0
19	18	2	0	0	100	0	0	0	0	0	0	0	0		0	0

* The pace maker contracted, of course, in 100 per cent of the cases.

† The age of the embryos included varied between 17 and 22 hours or 18 ± 4.

‡ Occasionally the auricles were each divided into three pieces. The mesially placed piece in each case is then numbered 3. This method of division is not shown in Fig. 3.

§ The numbers in parentheses are given only when all of the cases at the given age did not have fragments of the particular location mentioned—and to indicate the number of fragments studied.

The choice of the method we employed proved fortunate in point of fact for it furnished the opportunity of bringing out differences.

In the experiments in the 2 and 3 day embryos the distal, arterial end of the tube has clearly lost the spontaneous contractile function in one- to two-thirds of the cases (Table IV). All the other fragments no matter whence taken retain this function until the 7th day. On the 8th day the ventricles begin to exhibit a loss of the function and by the 13th day this is complete. On the 10th day the auricles, except of course the pace maker, that is to say the lateral fragments especially, also begin to fail. There is, in short, as embryonic life draws to a close apparently a continuous diminution of the capability of independent contraction until at the end it is lost almost entirely, with the exception always of the area to which rhythm production is ultimately confined.

DISCUSSION AND SUMMARY.

In these experiments we have shown that, with the technique adopted, differences in behavior are exhibited by fragments of the heart taken from different localities. The different localities behave in a more or less uniform manner. The pace-making function, for instance, is found at first throughout the cardiac tube but later it is restricted and comes to reside in a special small area at the back of the right auricle near the center. The pace-making system is able to develop a rate comparable to that shown by the whole intact heart, irrespective of the size of the fragment in which it is contained. Later, under the circumstances of the study, the ventricular structures lose the power of spontaneous contraction, and later still, the auricular ones also. It need scarcely be pointed out, however, that this loss refers only to the function of pace making. In its place, the various localities of the heart undoubtedly take on other capabilities. This is what is meant after all by differentiation.

The question whether the pace-making and conduction systems reside in the remains of primitive portions of the cardiac tube in an undifferentiated form, or whether on the other hand these primitive portions develop into differentiated structures which preside over these functions may be reviewed afresh. Obviously the tube in its early state possesses these functions; obviously also the major part of the

heart loses them during the course of development. A knowledge of the changes in form paralleling changes in function would have great interest. On this phase of the problem we hope to report later.

On the basis of these observations, differentiation from the point of view of stimulus production may be viewed perhaps in this manner. Pace making, the conduction of impulses, and contraction are the primitive functions of the tube. As the tube develops into the adult structure, pace making and conduction are supposedly served by tissues resembling in structure the original ones. Whether as a matter of fact a structural change takes place is an interesting and important problem. Those portions of the heart which require to develop greater degrees of energy lose the primitive functions of pace making and conduction, and, in the transformation, take on a differentiated structure. It is, then, not the structures in which the primitive functions of pace making and conduction reside which are differentiated, but the greater mass of ventricular muscle. These reflections have their origin not only from our own work but they grow out of observations to be found in the writings of those (A. Keith and I. McKenzie) who call the nodal and conduction tissues in the heart, embryonic. But whether from the point of view developed here the use of this term is completely descriptive remains an interesting problem.

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