J. Physiol. (1981), **311**, *pp.* 147–160
With 8 text-figures
Printed in Great Britain

OPTICAL RECORDING OF DEVELOPMENT OF ELECTRICAL ACTIVITY IN EMBRYONIC CHICK HEART DURING EARLY PHASES OF CARDIOGENESIS

BY SHIROH FUJII, AKIHIKO HIROTA AND KOHTARO KAMINO*

From the Department of Physiology, Tokyo Medical and Dental University School of Medicine, Bunkyo-ku, Tokyo 113, Japan

(*Received 26 March 1980*)

SUMMARY

1. Developmental changes in the spontaneous action potential were measured using optical signals from early embryonic chick heart stained with a potential sensitive merocyanine-rhodanine dye.

2. The wave-length dependence of the dye signal is triphasic in early embryonic chick heart with a decrease in absorption from 525 to 600 nm, an increase from 625 to 720 nm, and a decrease at 750 nm. The signal was largest at 700 nm.

3. The magnitude of the spontaneous absorption signal increased as the development of the embryonic heart proceeded. In addition, the magnitude of the absorption signal differed among the various regions of an early embryonic chick heart and the number of electrically active cells increased dramatically throughout the 7–9 somite developmental stages.

4. The number of the electrically active cells was largest in the right portion of the ventricle at the 7–9 somite developmental stages.

5. The shape of spontaneous absorption signals could be classified into four types in the developmental stages between 7 and 9 somites: Signals resembling the cardiac action potential and the pace-maker potential were not recorded until the 8–9 somite developmental stage.

INTRODUCTION

The early process of 'morphological' cardiogenesis has been investigated in detail (for reviews see DeHaan, 1963; Patten, 1949; Manasek, 1978). However, studies on the origin and organization of the cardiac 'functions' throughout the early phases of heart development made little progress as the small size and fragility of the cells made the application of conventional micro-electrode methods impractical.

In previous papers (Hirota, Fujii & Kamino, 1979; Fujii, Hirota & Kamino, 1980) we described the spontaneous absorption signal detected from early embryonic hearts stained with a potential sensitive merocyanine-oxazolone dye (Salzberg, Grinvald, Cohen, Davila & Ross, 1977). The properties of this signal resembled those of a spontaneous action potential, and it was concluded that electrical excitability occurs

* To whom correspondence and reprint requests should be sent.

0022-3751/81/3410-0885 $07.50

in the 7–8 somite embryonic heart, before the initiation of the heartbeat. Optical monitoring of spontaneous electrical activity in early embryonic heart cells might therefore provide a powerful tool for answering functional questions in cardiogenesis.

A closely related merocyanine-rhodanine dye also exhibits an absorption signal in response to changes in membrane potential in squid giant axon (Ross, Salzberg, Cohen, Grinvald, Davila, Waggoner & Wang, 1977), and has been used as a rapid molecular probe for monitoring action potentials in *Aplysia* central neurones (Woolum & Strumwasser, 1978), barnacle supraesophageal ganglion cells (Cohen, Grinvald & Lesher, 1978), superior cervical ganglion cells from the newborn rat (Ross & Reichardt, 1979), skeletal muscle (Baylor & Chandler, 1978), adult frog heart muscle (Morad & Salama, 1979), and *Limulus* ventral photoreceptors (Brown, Harary & Waggoner, 1979).

In the present paper, we characterize the spectra and multicellular origins of the spontaneous optical signals detected from very early embryonic hearts stained with a merocyanine-rhodanine dye, and second we shall describe the time course of development and regional variation of the optical signal, in connection with development of numbers of the synchronously active cells. Furthermore, we demonstrate development of the characteristic features of the cardiac action potential.

METHODS

Experimental methods were similar to those described in our previous paper (Fujii *et al.* 1980).

Preparations. The embryos were removed from fertilized chicken eggs (white Leghorn) after 25–150 hr of incubation. After the amnion and the splanchnopleure were carefully peeled off, the isolated embryos were placed in a bathing solution containing 0·5 mg/ml. of a merocyanine-rhodanine dye (Dye XVII in Ross *et al.* 1977, and available as NK 2495 from Nippon Kankoh Shikiso Kenkyusho Co., Okayama Japan) for 15 min. The dye solution was then replaced with the normal bathing solution. The dyed embryo was attached to the bottom of a simple chamber; the surface of the solution was covered with a coverglass with the thickness of 0·13–0·17 mm.

Bathing solution. The solution in which the embryos were placed usually contained: 138 mM-NaCl, 5·4 mM-KCl, 1·8 mM-$CaCl_2$, 0·5 mM-$MgCl_2$ and 5 mM-Tris-HCl buffer (pH 7·2).

Optical measurements. The chamber was mounted on the stage of an Olympus Vanox microscope (Type, AHB-LB-I Olympus Optical Co Ltd., Tokyo, Japan). Transmission of light through the area of the embryonic chick heart was measured using an apparatus similar to that used originally by Salzberg *et al.* (1977) with slight modification. Light from a halogen-tungsten-filament lamp (JC 24V-300W, Kondo Sylvania Ltd., Tokyo Japan) was collimated, passed through an interference filter and heat filter, and then focused on the embryonic heart by means of a bright-field condenser. An Olympus 10×, 0·30 N. A. long working-distance objective and 3·3× photographic eyepiece formed a ×33 magnified real image of the embryonic heart in the image plane. The light guide (4 mm in diameter) glued to the active surface of a type 509-10 photo-detector (Bell & Howell, Bridgeport, Conn. U.S.A.) was used. The tip of the light guide was positioned over the image of the area of interest in the heart. The output of the photodiode was amplified and then displayed on an Addscope (ATAC-250, Nihon Kohden, Tokyo, Japan).

Intracellular micro-electrodes. Experiments using micro-electrodes were done in the 50–150 hr old preparations. The electrodes were filled with 3 M-KCl and had resistances between 30 and 50 MΩ.

RESULTS

Absorption signals accompanying action potential

Fig. 1 *A* shows a simultaneous recording of an absorption change (top trace) and the spontaneous action potential (bottom trace) obtained in a single sweep when 5

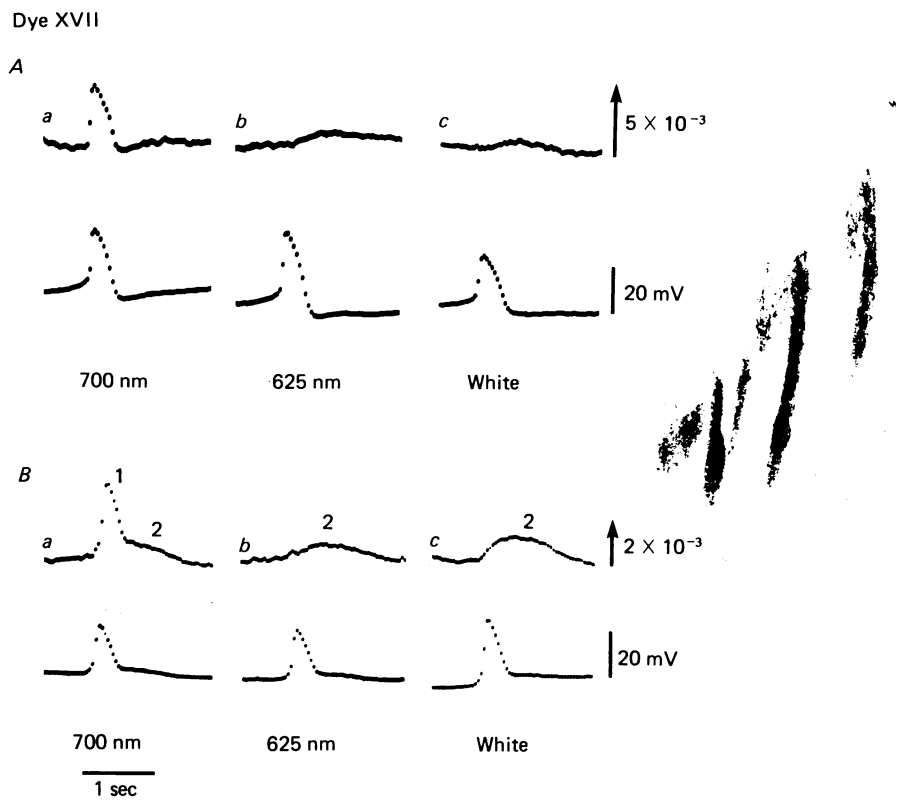

Fig. 1. Optical transmission changes (top traces) with a 700 ± 16 (trace *a*) or a 625 ± 16 nm (*b*) interference filter or white light (*c*) accompanied by an action potential (bottom traces) recorded with a micro-electrode in a 5 day old embryonic heart stained with the merocyanine-rhodanine dye (Dye XVII). The transmission changes were recorded simultaneously with a measurement of the action potential in one of the ventricular cells (epi-myocardium cells) within the area of the light beam. In *A*, the transmission change was eliminated with a 625 ± 16 nm interference filter or white light. In *B*, an example accompanied by small heartbeats is demonstrated. The preparations were bathed in a hypertonic solution made by increasing the NaCl concentration (about 532 mM) to minimize the heartbeats. The measurements were made in a single sweep, and carried out at room temperature. The direction of the arrows to the right of the optical traces indicates a decrease in transmission (increase in absorption) and the length of the arrow represents the stated value of the change in intensity divided by the DC-background intensity.

day old embryonic chick hearts, stained with a merocyanine-rhodanine dye (Dye XVII), were illuminated. During the action potential, the light intensity reaching the photodiode decreased; evidence presented below indicates that the intensity decrease resulted from an absorption increase. The absorption changes depended on the

wave-length of the incident light. The change in the absorption was largest for light of 700 nm. The spectra are summarized in Fig. 3. The absorption changes, especially the absorption increase at 700 nm, had a time course that was very similar to the time course of the action potential recorded with a microelectrode. The absorption signals that accompanied the action potential were minimized with light of 625 ± 16 nm or eliminated when the preparation was illuminated with white light.

In squid giant axon it has been shown that changes in absorption of membrane stained with the merocyanine-rhodanine dye (Dye XVII) are linearly related to membrane potential changes (Ross *et al.* 1977). Quite recently, it was reported that reflectance changes exhibited by the merocyanine-rhodanine dye are also linearly related to membrane potential in adult frog heart (Morad & Salama, 1979). This dye appears to behave similarly in the early embryonic heart. As shown in Fig. 1*A*, the absortion signal of the merocyanine-rhodanine dye faithfully reproduced the time course of the action potential in a manner similar to that of the merocyanine-oxazolone dye (NK 2367) (Hirota *et al.* 1979; Fujii *et al.* 1980).

Fig. 1*B* shows second experiment where the optical signal has two distinguishable components; an early (denoted by 1) phase and a later (denoted by 2) phase. The time course of the early component was similar to that of the action potential. The early component always had the same direction at a fixed wave-length; it had a minimum at 625 ± 16 nm and it was completely eliminated in white light. The sign of the later component, however, was variable; it was sometimes monophasic; sometimes biphasic, and occasionally triphasic; also, the later components remained in white light and at 625 nm, the null wavelength for the potential-dependent signal. In addition, the later component was detected in unstained preparations, and was minimized in a Ca^{2+}-free, strictly speaking nominally free, bathing solution (Fig. 1*A*). These results suggest (Fujii *et al.* 1980) that the early component is due to the membrane potential change and the later component to contraction of the heart.

We also examined pharmacologic and photodynamic effects on the heartbeats and on the action potentials. In 5 day old embryonic hearts stained with the merocyanine-rhodanine dye, neither pharmacologic nor photodynamic damage was serious, and measurements could be made continuously for 30–60 min under the usual conditions.

In the early embryonic chick heart, the merocyanine-rhodanine dye (Dye XVII) exhibited the same signal size, signal-to-noise ratio and photodynamical or pharmacological action as the merocyanine-oxazolone dye (NK 2367). However, we found that embryonic heart stained with the merocyanine-oxazolone dye photobleached rapidly compared to tissue treated with the merocyanine-rhodanine dye; the former photobleached in around 30 min and the latter in 1–2 hr. Thus, the merocyanine-rhodanine dye seems to be a more useful probe for monitoring spontaneous electrical activity in early embryonic chick hearts.

Spectral properties

Fig. 2 shows spontaneous absorption signals from the right portion of the ventricle in an 8 somite embryonic chick heart stained with the merocyanine-rhodanine dye. As shown in this Figure, both the sign and magnitude of the absorption signal are wave-length dependent, and there are absorption maxima at 700 and 650 nm. The spontaneous absorption signals from the right ventricle of the 8 somite embryonic

heart were also eliminated with white light and minimized at 600 ± 14 nm. As expected, there were no signals from unstained preparations; the phase 2 component was not detected.

Plots of spontaneous absorption signal-size *vs.* wave-length for eight different developmental stages are shown in Fig. 3 (*A* and *B*). In all cases, there was a decrease in absorption between 525 and 600 nm, an increase between 625 and 720 nm and a decrease at 750 nm. Resting spectra of dye bound to embryonic heart muscle at different developmental stages are also shown in Fig. 3*C*. These spectra each

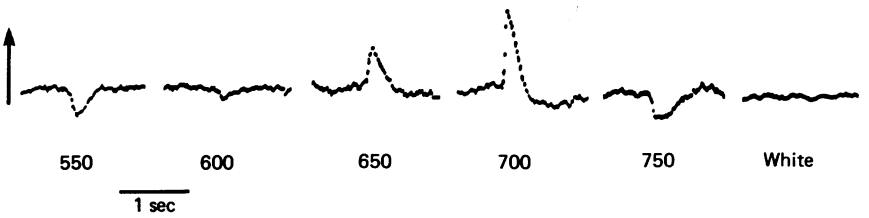

Fig. 2. Spontaneous absorption changes at several wave-lengths recorded from the right portion of the ventricular region in an 8 somite embryonic heart stained with the merocyanine-rhodanine dye (Dye XVII). It must be kept in mind that *a conventional micro-electrode cannot be employed in these early developmental stages because of the small cell size (4–7 μm of the diameter) and the preparation's fragility.* The measurements were carried out with interference filters having the following transmission maxima: 550 ± 14, 600 ± 14, 650 ± 16, 700 ± 16, or 750 ± 16 nm. A recording made with white light is also shown. The traces were detected in a single sweep at 37·3 °C. The direction of the arrow has the same meaning as in Fig. 1. Transmittance calibration bar is $1{\cdot}56 \times 10^{-3}$ for 550 nm; $1{\cdot}45 \times 10^{-3}$ for 600 nm; $0{\cdot}99 \times 10^{-3}$ for 650 nm; 10^{-3} for 700 nm; $1{\cdot}41 \times 10^{-3}$ for 750 nm; and $1{\cdot}65 \times 10^{-3}$ for white light. The shape of the optical change in this Figure exhibits a cardiac type (type III in Fig. 8) signal.

exhibited a shoulder at 650 nm and a maximum at 700 nm where the largest signal was observed. There were no significant differences in the pattern of the action spectra among the different developmental stages; neither the 650 nm- nor the 700 nm-peak shifted. However, the signals at 750 and 625 nm did diminish in the size during the course of development. We feel that this observation provides important limitations on the possible molecular mechanisms responsible for the signal (see Discussion).

Signal-size

In an 8 somite embryonic heart, the fractional change in absorption at 700 nm was on the order of one part of 10^4. The fractional absorption change ($\Delta A/A_r$) is related to the fractional change in transmitted intensity, change in transmission/resting transmission ($= \Delta I/I_r$). Thus, $\Delta A/A_r$ is equal to $-\Delta I/(I_{r,\text{ before}} - I_{r,\text{ after staining}})$ (Ross *et al.* 1977).

The magnitude of the spontaneous absorption signal depends strongly on the developmental stage of the embryonic heart. As shown in Fig. 4, the fractional intensity change in the optical action potential of embryonic ventricles increased from 5×10^{-4} at the 7–8 somite (corresponding to 25–35 hr) developmental stage to 4×10^{-3} at 120 hr.

In a multicellular tissue such as heart, a single photo-detector receives signals from

many active cells. It is reasonable, therefore, to assume that the signal size is a function of the number of active cells which are electrically coupled (synchronized) in the object field of the detector. Fig. 5 shows two kinds of signals obtained in a continuous recording from the ventricle of an 8 somite embryonic heart. In the

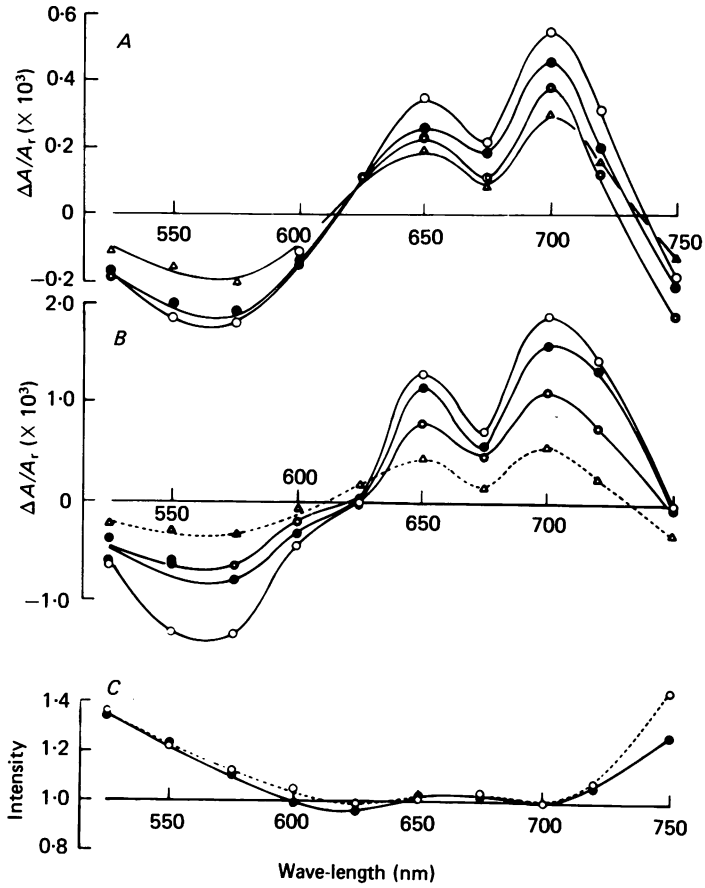

Fig. 3. Spectra of absorption changes of embryonic heart tissue stained with the merocyanine-rhodanine dye (Dye XVII) in early developmental stages. In *A*, the spectra in 7 (△), 8 (◎), 9 (●), and 12 (○) somite embryonic hearts are illustrated, and in *B*, 36 (△), 88 (◎), 103 (●) and 115 (○) hour old embryonic hearts. The scale for the ordinate indicates the fractional absorption change ($\Delta A/A_r$, see text). The data were corrected for apparatus efficiency. The action spectra in this figure were obtained from the right portion of the ventricular region. The measurements were carried out at 38–39 °C. The wave-length dependence of the intensity transmitted by an 8 somite (○) and a 6 day old (●) embryonic heart stained with Dye XVII (*C*). In *C*, the ordinate is the transmitted intensity normalized to that at 700 nm.

beginning, the signal *a* appeared rhythmically; later the signal *a* seemed to split into two signals, *b* and *c*; subsequently the two signals fused to become one signal again. It seems likely that the two signals *b* and *c* are recorded from two different populations of active cells which were each synchronized electrically, and that signal *a* represents the joint population, synchronously active. Indeed, signal *a* can be reconstructed by adding signals *b* and *c* (see Discussion).

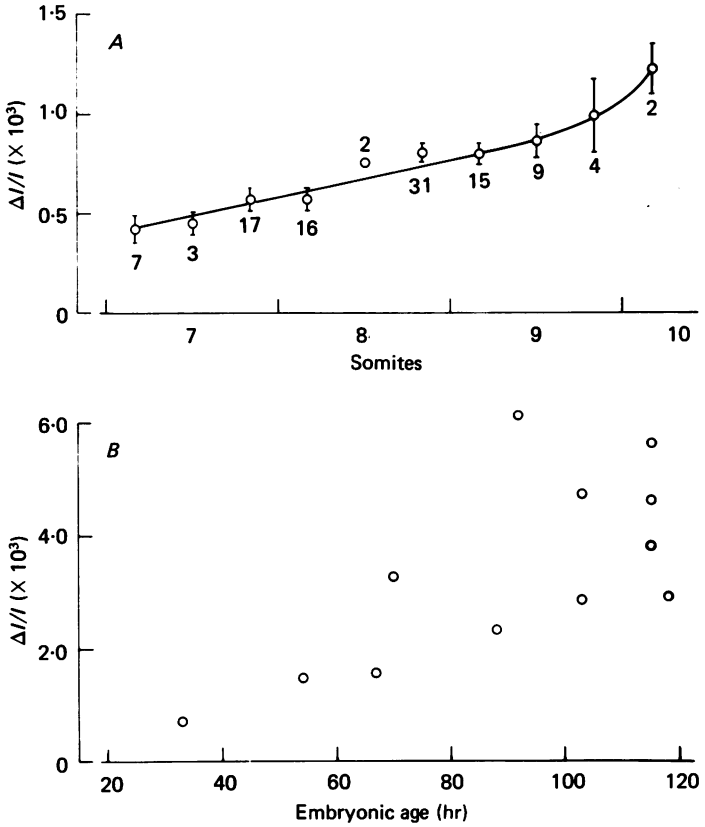

Fig. 4. The magnitude of the spontaneous absorption signals *vs.* the developmental stages. The spontaneous absorption signals at 700 nm wave-length were recorded from the right portion of the ventricular region at 37 ± 1 °C. The signals were detected from area of 0·01 mm² in the heart. Scale for the ordinate indicates the fractional change in the transmission intensity. Number of observations (on each point) (in *A*). The abscissae indicate the developmental stages, somites (in *A*) or hours after incubation (in *B*). Especially, in *A* the abscissae should be read as the early, middle or later period of a stated stage. In *B*, the experimental values are scattered. This suggests that there are individual differences in the developmental process. It should be noted that the time (in a physical sense) does not uniquely specify the ontogenetic period.

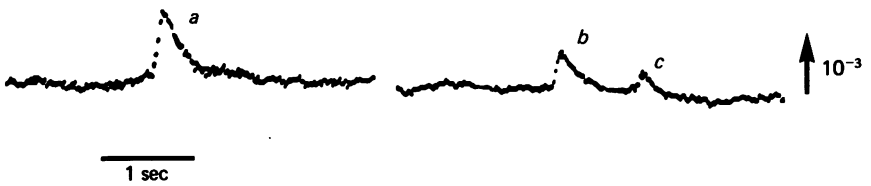

Fig. 5. An example of the spontaneously splitting of an absorption signal. The signals were recorded from the right portion of the ventricular region of an 8 somite embryonic heart stained with the merocyanine-rhodanine dye at 37·6 °C. The signal (*a*) belongs to type III in Fig. 8, respectively. During the course of measurement, the signal suddenly split into two signals (*b* and *c*), and later the two signals were fused into one. This behaviour was occasionally observed in 7–8 somite embryonic hearts. The traces were obtained in a single sweep with a 700 ± 16 nm interference filter.

Regional distribution of electrically active cells

Fig. 6 demonstrates spontaneous absorption signals recorded from various regions in a 7 somite embryonic heart. There are differences in the signal size among the various regions. In this preparation, the signals recorded from the right portion of the ventricle (traces 1 and 2) were the largest in size; somewhat smaller signals were detected from the unfused primordia at the atrium level (traces 3 and 7). In recording 4, there were three different signals, which apparently were not synchronized. On the

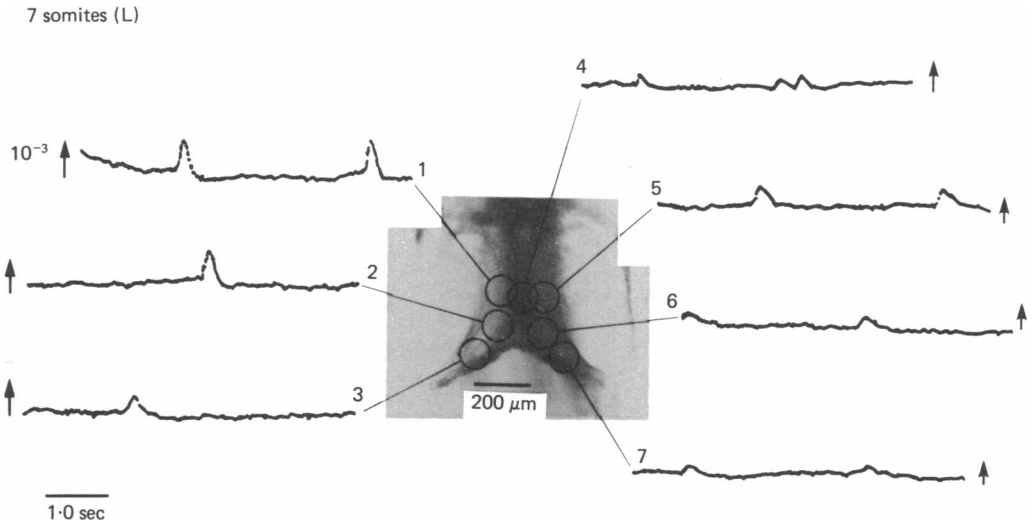

Fig. 6. Spontaneous absorption signals from various regions of a 7 somite embryonic heart stained with the merocyanine-rhodanine dye (Dye XVII), using a 700 ± 16 nm interference filter. A light guide 4 mm in diameter was moved in the 33× image plane so that the absorption signals from a stained embryonic heart could be recorded from the desired regions. The measurements were made in a single sweep, and carried out at 35·3 °C. When the temperature decreased, the frequency of the spontaneous absorption signals decreased, but the size and the shape were not altered. Trace 4 exhibits two kinds of signals. L: later period of a particular somite stage.

assumption that the signal size is a function of the number of cells that are electrically active, the results in Fig. 6 suggest the regional distribution of synchronous electrical activity. Although we cannot yet make quantitative estimates of absolute numbers, it is quite evident that the active cells are already widely dispersed in the 7 somite embryonic heart (see also Discussion).

We also attempted to determine qualitatively the regional distribution of the active cell populations at various developmental stages from 7 to 9 somites by measuring the absorption signal size. The areas circled in Figs. 6 and 7 correspond to the regions monitored by one detector. As may be seen in Fig. 7, there is a maximum in the number of active cells in the right portion of the ventricle. Throughout the very early developmental stages from 7 to 10 somites, the population distribution appeared asymmetrical about the midline of the heart. The areas where the number of active cells are maximal shifted anteriorly with development from 7 to 9 somites. Very small signals were detected near the midline (trace 4 in Fig. 6).

The apparent numbers of active cells in Fig. 7 are relative values obtained by normalizing the signal size to the largest size. It would be necessary to record the absorption signal from a single active cell in order to estimate the absolute number of active cells. However, this is difficult for a number of reasons including photodynamic damage, photobleaching, geometrical complication, and/or, other unknown factors.

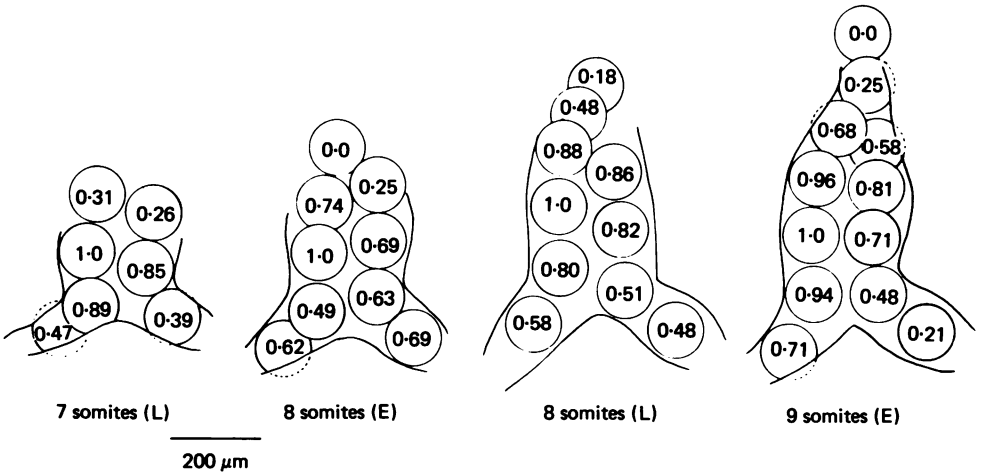

Fig. 7. Regional differences in the magnitude of the spontaneous absorption signals recorded with the merocyanine-rhodanine dye (Dye XVII) from 7–9 somite embryonic chick hearts. A light guide 4 mm in diameter was connected to a photodiode positioned over the 33 × enlarged real image. Thus, the field of the optical recording indicated by circles in this Figure was estimated to be about 0·01 mm². The values of magnitude of the signal recorded in each location was normalized to that from the right portion of the ventricular region, and we think these values are related to the relative number of electrically synchronized active cells in the embryonic hearts (see text). E or L indicates the early or the later period of stated stage. The illuminating power of the incident light was constant in each preparation, and the experimental values were obtained from the areas in which the DC-background intensities were nearly equal, eliminating the possibility that the optical signal is affected by the amount of dye binding. The transmittance intensities from the different regions also were nearly equal in these preparations before staining. Note that the right portion of the ventricular region exhibits the maximum values. The measurements were carried out at $37 \pm 1{\cdot}5$ °C, and preparations having one kind of signal were used. A slight photobleaching effect (about 10%) was compensated for by interpolation.

Shape variation of the optical action potential

Developmental changes in the shape of the spontaneous absorption signal appear to be related to the differentiation of embryonic heart cells. During the early phases of development, there are apparently four types of spontaneous absorption signals. The first type is a symmetrical hanging bell-type. This type can be divided into two patterns, I_a and I_b; I_a is relatively long in duration (500–700 msec) and I_b is spike-like with short duration (100–200 msec). Throughout the 7–8 somite developmental stages, type I_a was most frequently recorded, while I_b was rare. Neither I_a nor I_b could be characterized regionally. Two additional types (II and III) were recorded occasionally during early stages of development, from the last period of 8 somites

to the early period of 9 somites, just before initiation of the heartbeat. In type II, the slow diastolic depolarization or pacemaker potential is clearly recognized. Type III is a cardiac-type optical action potential with a rapidly rising phase and a distinct plateau phase. As seen in Fig. 8, the plateau phase is apparently not yet fully developed. We have not yet been able to predict which location in the embryo will have the type II and III signals.

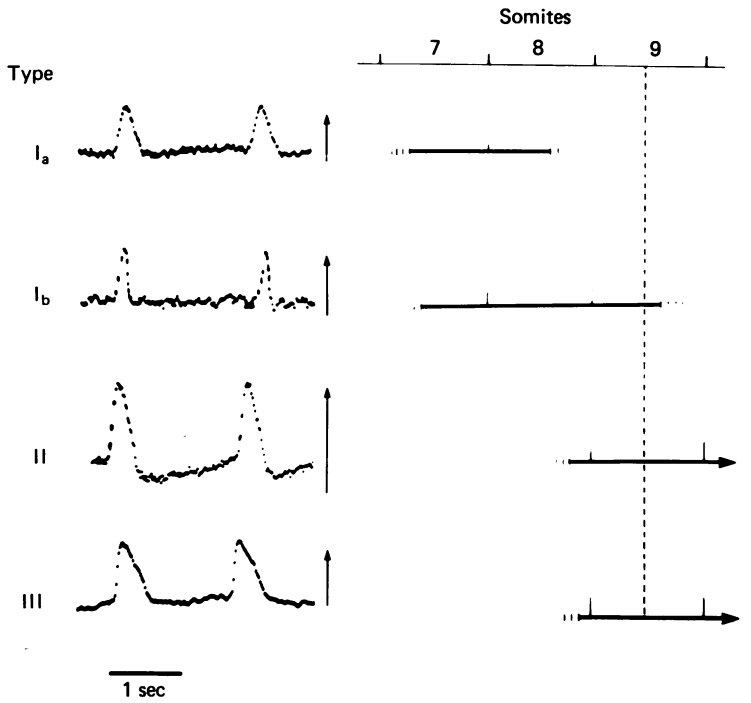

Fig. 8. A possible classification of the shape of the spontaneous absorption signals recorded from 7–9 somite embryonic hearts. The bold horizontal lines on the right represent the developmental stages at which each type of signal was monitored. The signals demonstrated in this Figure were recorded from the right ventricular region in 8 somite embryonic hearts. First contractions are optically detected at the middle period of the 9 somite developmental stage near the period indicated by a vertical dashed line.

DISCUSSION

The main results in this optical study of immature cardiac muscle using the merocyanine-rhodanine dye (Dye XVII) were that the spontaneous absorption signals increase in size during development of the embryonic heart, and that the signal size seems to depend on the number of electrically synchronized cells. In addition, changes in the time course of the cardiac action potential during development were noted.

1. The spectra of the absorption changes with the merocyanine-rhodanine dye were triphasic. Similar spectra with this dye, and the closely related merocyanine-oxazolone, were found in the superior cervical ganglion of the newborn rat (Ross & Reichardt,

1979), in adult frog heart muscle (Morad & Salama, 1979), and in salivary acini of the snail (Senseman & Salzberg, 1980). There are however minor differences among the species which show the triphasic spectra. In squid giant axons (Ross *et al.* 1977), the triphasic signal was seen only with linearly polarized light with the polarization vector perpendicular to the axon's longitudinal axis, while light polarized parallel to the axon axis yielded a monophasic signal. Such effects are probably related to the mechanism(s) responsible for the optical signal, and the shape of the action spectrum is very likely sensitive to changes in the microenvironment that the dyes may encounter in a membrane. A reduction in the intensity changes at 750 and 625 nm, during embryonic development, may be related to developmental changes in the molecular composition of the membrane (Ishima & Waku, 1978). Unfortunately, however, at the present stage, we do not have enough evidence to elucidate the mechanism(s) in detail. Possible mechanism(s) for the absorption signals of fast molecular probes have been discussed by Waggoner & Grinvald (1978), Cohen & Salzberg (1978), and Morad & Salama (1979).

2. As shown in Figs. 3 and 4, the magnitude of the optical action potential increased during development of the embryos. Generally, in a multicellular tissue such as heart, a single detector records the signals from many different cells at once. Therefore, it may be assumed that the observed size of the optical signals is a function of the active membrane area and of the size of the signal generated by a unit area of active membrane undergoing an action potential. Here the active membrane area in the field of optical recording is dependent upon the *number* and or, *size* of active cells.

The actual expression will depend, of course, upon the morphology of the embryonic heart cells and we shall consider the following two cases.

(*a*) *Case where the active cell size and the signal size of a unit active cell membrane area are held constant.* Throughout the early phases of development (less than 45 hr), the diameters of myocardial cells do not markedly increase (Manasek, 1968, 1969). In this case, the fractional change in the optical signal is probably directly proportional to the number of active cells in the object field of the detector. The results shown in Fig. 4*A* are consistent with this idea. According to this interpretation, it seems possible that the increase in size of the spontaneous absorption signal throughout the 7–10 somite developmental stages (Fig. 4*A*) is mainly due to an increase in the number of active cells which are electrically coupled, and that the number of electrically active cells at the middle period of the 9 somite stage, when the spontaneous heartbeat begins, is apparently twice that at the 7 somite stage, when fusion of the paired cardiac primordia begins. It has previously been noted that the magnitude of the optical signal depends upon several factors including membrane area and the amount and site(s) of dye binding (Cohen, Salzberg, Davila, Ross, Landowne, Waggoner, & Wang, 1974). Quite recently, Morad & Salama (1979) noted that the amplitude of the optical signals was dependent on the ratio of excitable membrane to non-excitable endothelial membrane in adult frog heart muscle.

(*b*) *Case where the active cell size and the signal size of a unit active cell membrane area vary.* As shown in Fig. 4*B*, the fractional changes in the signals increased during the development of the chick embryos; the magnitude of the signal in a 120 hr old embryonic heart was approximately 3 times that in the 50 hr old preparation.

Speralakis & Shigenobu (1972) reported that the transmembrane resting potential and the action potential of ventricular cells, recorded with micro-electrodes, increase during development of the chick embryonic heart. In their report, the magnitude of the action potential increases, from a mean value of about 55 mV at 2–3 days to 60 mV at 4–5 days. Shimizu & Tasaki (1966) also reported 65 mV at day 3 and 110 mV at day 16. Thus, it is possible that the signal size of a unit area of active membrane increases during the development of the embryos. In addition, throughout the 50–120 hr developmental stages, the cell size increases rapidly (Manasek, 1978). The rate of increase in the absorption signal size during the 50–120 hr development stages is larger than the rate of increase in the action potential recorded with micro-electrodes. In our preparation also, the rate of the developmental changes in the absorption signal size is large compared to the change in the amplitude of the action potential recorded with micro-electrodes. Thus, it seems likely that the increase in the size of the absorption signal is mostly due to an increase in the number of electrically active cells in the embryonic hearts, and partly to the increased cell size and, or, the magnitude of the action potential during the development of the older embryos (50–120 hr).

3. Although the possibility remains that the optical signal from a unit area of active cell membrane increases slightly throughout the 7–9 somite developmental stages, it is reasonable to assume that the observed fractional change in the signal is approximately proportional to the number of synchronized active cells in the field of optical recording. Fig. 5 strongly supports this assumption. This idea led to the determination of both the regional distribution in the number of synchronously active cells and the developmental changes in these numbers.

The merocyanine-rhodanine dye is membrane impermeant (Salzberg, 1979) and is bound only to the outer surface of the embryonic heart, as is the case with the merocyanine-oxazolone dye (NK 2367) (Fujii *et al.* 1980). The outer surface of the primitive tubular heart is the myocardium (Manasek, 1978). Therefore, the results shown in Fig. 6 and 7 indicate the regional distribution of the number of electrically synchronized cells in the myocardium, and developmental changes in these numbers. A shift in the centre of the most intense activity seems to parallel the morphological organization process of the cardiogenic plate demonstrated by DeHaan (1963) and Stalsberg & DeHaan (1969). In addition, this area coincides with the position at which the first contraction of the heart will occur during the middle period of the 9 somite stage (Patten, 1949).

4. Perhaps the most interesting observation concerning the shape of the optical signals is the detection of absorption changes associated with the generation of the pace-maker potential (type II) and the cardiac action potential (type III) during the early developmental stages that precede the initiation of the heartbeat.

The rate of rise of the signal corresponding to the cardiac action potential in the 8 somite stage is significantly smaller than that observed in the 4 day old embryonic heart shown in Fig. 1. This characteristic is consistent with the finding that the maximum rate of rise in the action potential ($+\dot{V}_{max}$) increases from a mean value of about 19 V/sec at 2–3 days, to about 80 V/sec at days 5–10 (Speralakis & Shigenobu, 1972). Thus, it was suggested that there are only slow Na^+ channels in the early phases of development before the first contraction. This assumption is also strongly supported by our finding that the spontaneous absorption signal occurring

at approximately the 8 somite stage is not sensitive to tetrodotoxin (Fujii *et al.* 1980). On the other hand, it seems likely that the generation of the pace-maker-like potential at the 8 somite stage results in the rhythmicity of the first contraction during the middle period of the 9 somite developmental stage. Type I_a might reflect the action potential in the functionally underdeveloped active cells, and type I_b the primitive pattern of the cardiac type action potential. From the observation that the type I_a signal is usually recorded from the embryonic heart between the 7 and the early period of the 8 somite stage, and that types I_b, II and III are mostly recorded from various regions in the 8–9 somite embryonic hearts, we may speculate that myocytes in the very early stages are rather homogeneous, and that there is not yet a differentiation between pacemaker cells and ordinary myocytes.

We are very grateful to Drs L. B. Cohen, B. M. Salzberg, and H. Irisawa for thoughtful reading of the manuscript and for their many helpful comments and suggestions and to M. Ohara for pertinent advice on the manuscript. We also thank Professor T. Furukawa for the loan of an Addscope and for encouragement. The work was supported by grants nos. 457021, 422007, and 387039 to K.K. from the Ministry of Education, Science and Culture of Japan.

REFERENCES

BAYLOR, S. M. & CHANDLER, W. K. (1978). Optical indications of excitation–contraction coupling in striated muscle. *Proceedings of an International Symposium on Cardiac Muscle* (Shiraz, Iran), ed. MORAD, M., pp. 207–228. New York: Academic Press.

BROWN, J. E., HARARY, H. H. & WAGGONER, A. S. (1979). Isopotentiality and an optical determination of series resistance in *Limulus* ventral photoreceptors. *J. Physiol.* **296**, 357–372.

COHEN, L. B., GRINVALD, A. & LESHER, S. (1978). Simultaneous recording of activity of most (all?) of the large neurons in the barnacle supraesophageal ganglion. *Soc. Neurosci.* **4**, 189.

COHEN, L. B. & SALZBERG, B. M. (1978). Optical measurement of membrane potential. *Rev. Physiol. Biochem. Pharmacol.* **83**, 33–88.

COHEN, L. B., SALZBERG, B. M., DAVILA, H. V., ROSS, W. N., LANDOWNE, D., WAGGONER, A. S. & WANG, C. H. (1974). Changes in axon fluorescence during activity: Molecular probes of membrane potential. *J. membrane Biol.* **19**, 1–36.

DEHAAN, R. L. (1963). Organization of the cardiogenic plate in the early chick embryo. *Acta Embryol. exp. morphol.* **6**, 26–28.

FUJII, S., HIROTA, A.& KAMINO, K. (1980). Optical signals from early embryonic chick heart stained with potential sensitive dyes: evidence for electrical activity. *J. Physiol.* **304**, 503–518.

HIROTA, A., FUJII, S. & KAMINO, K. (1979). Optical monitoring of spontaneous electrical activity of 8-somite embryonic chick heart. *Jap. J. Physiol.* **29**, 635–639.

ISHIMA, Y. & WAKU, K. (1978). Phospholipid analysis of the chick ventricles in the early stages of development when their excitability changes in tetrodotoxin or in sodium substitute media. *Comp. Biochem. Physiol.* **61**, 283–386.

MANASEK, F. J. (1968). Embryonic development of the heart. I. A light and electron microscopic study of early chick embryo. *J. Morph.* **125**, 329–366.

MANASEK, F. J. (1969). Embryonic development of the heart. II. Formation of the epicardium. *J. Embryol. exp. Morphol.* **22**, 333–348.

MANASEK, F. J. (1978). Organization, interactions, and environment of heart cells during myocardial ontogeny. In *Handbook of Physiology*, section 2: The Cardiovascular System, vol. 1, ed. BERNE, R. M., SPERELAKIS, N. & GEIGER, S. R., pp. 29–42. Bethesda: Am. Physiol. Soc.

MORAD, M. & SALAMA, G. (1979). Optical probes of membrane potential in heart muscle. *J. Physiol.* **292**, 267–295.

PATTEN, B. M. (1949). Initiation and early changes in the character of the heartbeat in vertebrate embryos. *Physiol. Rev.* **29**, 31–47.

ROSS, W. N. & REICHARDT, L. F. (1979). Species-specific effects on the optical signals of voltage-sensitive dyes. *J. membrane Biol.* **48**, 343–356.

ROSS, W. N., SALZBERG, B. M., COHEN, L. B., GRINVALD, A., DAVILA, V., WAGGONER, A. S. &

WANG, C. H. (1977). Changes in absorption, fluorescence, dichroism, and birefringence in stained giant axons: Optical measurement of membrane potential. *J. membrane Biol.* **33**, 141–183.

SALZBERG, B. M. (1979). Perfusion and superfusion of giant axons with potentiometric probes – Fast optical signals. *Biophys. J.* **25**, 13*a*.

SALZBERG, B. M., GRINVALD, A., COHEN, L. B., DAVILA, V. & ROSS, W. N. (1977). Optical recording of neuronal activity in an invertebrate central nervous system: Simultaneous monitoring of several neurons. *J. Neurophysiol.* **40**, 1281–1291.

SENSEMAN, D. M. & SALZBERG, B. M. (1980). Electrical activity in an exocrine gland: Optical recording using a potentiometric dye. *Science, N.Y.* **208**, 1269–1271.

SHIMIZU, Y. & TASAKI, K. (1966). Electrical excitability of developing cardiac muscle in chick embryos. *Tohoku J. exp. Med.* **88**, 49–56.

SPERELAKIS, N. & SHIGENOBU, K. (1972). Changes in membrane properties of chick embryonic hearts during development. *J. gen. Physiol.* **60**, 430–453.

STALSBERG, H. & DEHAAN, R. L. (1969). The precardiac areas and formation of the tubular heart in the chick embryo. *Devl Biol.* **19**, 128–159.

WAGGONER, A. S. & GRINVALD, A. (1978). Mechanisms of rapid optical changes of potential sensitive dyes. *Ann N.Y. Acad. Sci.* **303**, 217–241.

WOOLUM, J. C. & STRUMWASSER, F. (1978). Membrane-potential-sensitive dyes for optical monitoring of activity in *Aplysia* neurons. *J. Neurobiol.* **9**, 185–193.