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[Reprinted from The Journal of General Physiology, March 20, 1927, Vol. x, No. 4, pp. 501–518.]

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(Accepted for publication, January 20, 1927.)

I.

The heart of *Limulus* is classic as example of neurogenic cardiac rhythm (Carlson, 1905-06, 1909). The frequency of its pulsations may be controlled by influences affecting the heart ganglion alone. When the temperature of the ganglion alone is varied (Garrey, 1920–21, *a*, *b*, *c;* 1921–22) the frequency of contractions adheres to the Arrhenius formula, μ in the equation

$$Frequency = Ke^{-\frac{\mu}{RT}} + C$$

being = 12,200 (Crozier, 1924–25, *a*); in some preparations there occurs a "break" at 15°, with μ at lower temperatures = approximately 23,500.

In the embryo of *Limulus* there is an early developmental period in which the heart is visible, contracting rhythmically, while the cardiac nervous system is still unformed; during this interval the heart rhythm is "myogenic" (Carlson and Meek, 1908). We have determined the relationship between temperature and frequency of heart beat during this period of "myogenic" rhythm, in order to compare the temperature effect with that in the heart ganglion of the adult. There seem to be clearly defined differences.

There is no reason to suppose that a difference in μ obtained in this way necessarily corresponds to or is diagnostic of myogenic as contrasted with intrinsically neurogenic processes. In so far as values of μ appear to be specific, and thus to correspond to physical realities

which may be utilized for purposes of classification and analysis,[1] it must be held that particular magnitudes of μ may reappear in all sorts of situations and do not pertain individually to particular types of function. But at the same time it is apparent that the occurrence of different systems of temperature characteristics for the two cases, embryonic heart and adult, is fully consistent with the idea that the respective essential controlling processes are unlike.

Such a result is of course not unexpected. The metabolic state of embryonic cells must differ materially from that of relatively greater dynamic stability enjoyed by the protoplasm of fully differentiated tissues. From this standpoint the apparently "irregular" variation of μ for rhythmic contraction in cultures of explanted chick myocardium (Murray, 1925–26) might be understood without reference to obscure regulation by the organism as a whole in order to account for greater uniformity in results when organs of intact animals are observed, even without appeal to structural conditions. When whole organisms are used it is possible to obtain modifications of temperature characteristics (μ), and these modifications appear to be specific (Crozier and Stier, 1924–25, *a;* 1925–26, *b*). It is entirely possible that the regularity of μ for comparable activities is partly determined by structural conditions in normal organs, such as permit of active control by definite "pace makers." Certain effects which seem to necessitate this view are discussed in a later section. It is perhaps of interest for this interpretation that the frequency of pulsation in the hearts of intact embryos may show considerable differences in μ among similar individuals (*cf.* Crozier and Hubbs, 1924, and other cases), or in relation to age and other variables. The present observations show differences between individuals comparable to those experimentally induced in the breathing rhythm of the grasshopper (Crozier and Stier, 1924–25, *a*).

[1] Crozier, 1924–25, *a*, *b;* 1925–26, *b*. Crozier and Stier, 1924–25, *a;* 1925–26, *a*, *b*. Fries, 1926–27. It may be noted that change of μ coincident with the institution of neurogenic control would not necessarily prove diagnostic either, for we should require study of comparable developmental stages in the absence of nervous elements.

II.

The heart of *Limulus* embryos within the egg envelopes becomes visible as a pulsating organ at the stage labelled *H* in accounts of the differentiation of the embryo (Kingsley, 1893; Kishinouye,1893). For a period of about 6 days, or until Stage *K* (before the appearance of the telson), at laboratory temperature (Carlson and Meek, 1908), the activity of the heart continues to be visible, in the absence of nervous control.

The heart is not at any time particularly easy to see. This difficulty, together with expected variation in the effect of temperature, led us to practice special precautions in obtaining a large number of observations. The frequency of the heart beat is made visible by horizontal light of fair intensity, under which the cardiac tube appears as a delicate white ghost against the yellowish background of the substance of the embryo. In order to maintain the animal in a position suitable for observation, the egg membrane was punctured and a segment of it folded outward. This segment was fastened by white vaseline to a small glass block. The collapsed membrane holds the embryo in a relatively fixed position. Such preparations live in an apparently normal way for many days and continue to develop.

A number of glass blocks, carrying labelled embryos, are placed in a thin walled crystallizing dish with sea water. Into this projects a microscope with paraffined objective used as an immersion lens. Dish and microscope are securely fastened to an iron frame supported on the rim of a large water thermostat. The vessel containing the embryos is submerged so that its water level is below that of the water in the thermostat. The temperature is read on a thermometer with enclosed stem, calibrated, and graduated to 0.05°.

In all such experiments difficulty is met in maintaining temperatures below that of the room. The latitude of fluctuation in frequency of contractions, at constant temperature, necessitates a number of observations at uniform temperatures on each embryo. Yet it is required to change the temperature by a known small amount at intervals of about an hour.

To do this we constructed a thermostat from which heat could be abstracted by a SO_2 compression circuit. The coil and brine tank of a refrigerating unit were replaced by a considerable length of half-inch Cu tubing, coiled so as to form a helical shell within the wall of a 10 gallon glass tank. The motor operating the compressor was started through a relay actuated by a large mercury thermoregulator.[2] "Sticking" and sparking were obviated by using a Ni-steel needle to make contact with the fluctuating Hg surface, and by having the relay of high

[2] For aid in this construction we are greatly indebted to Mr. H. V. Rivinius, refrigeration engineer of the Metropolitan Ice Co.

resistance (5000 ohms).[3] With adequate stirring, and felt insulation, this device enabled us to maintain for as long as desired any required temperature between 0° and that of the room. When an electric heater, constant or relay-controlled, is added to this arrangement, temperatures above that of the room are similarly obtainable, and the slight lag in temperature adjustment is still further reduced. The constancy of temperature within the body of the thermostat is then ±0.001°. In the vessel carrying the objects to be studied the constancy is well within 0.01°, and this is improved by a cover.

The temperature is changed quickly by adding hot or cold water from a reservoir, excess in the thermostat being removed by a constant level device. The thermoregulator is readjusted by sucking Hg out of the regulator bulb, or by forcing more Hg into it from an accessory bulb. Finer adjustment is made by the screw-mounted contact needle. This operation required but a minute or so.

The thermostat is mounted upon a box, and a window is left in the insulation of the bottom. Through this window a beam of light is projected vertically upward, and may assist observation through the microscope or be employed in other ways.

The microscope, of ordinary type or a binocular with "Planktonsucher" objectives, is so mounted as to be movable over the observation chamber; but in the present experiments it was found that the glass blocks bearing embryos could easily be manipulated with a needle and in turn brought into position for the readings. These movements were found to be without influence upon the frequency of the heart beat, but some minutes were allowed to elapse before readings were taken. Lateral illumination was supplied by a small submerged electric lamp. It was easily shown that the light was without effect upon the frequency of contractions, but the general illumination was kept reduced as an aid to seeing the heart, thermometers, and thermoregulator being viewed by means of small accessory lamps.

III.

When the frequency of the heart beat or of breathing movements is to be timed with precision in an intact animal it is necessary to avoid carefully the effects of concurrent movements of the body or appendages.[4] In some instances it seems as if the execution of such movements is the cause of accelerations or retardations in the rhythm under observation; in others, it appears more probable that both disturbances have a common and simultaneous origin in the central nervous system. If it is desired to study intrinsic fluctuations of frequencies and to obtain temperature characteristics as precisely

[3] Regulation may also be conveniently made by means of a system such as that described by Beaver and Beaver (1923).

[4] *Cf.* Crozier and Stier, 1924–25, *a*.

as possible, such deviations must be taken account of. In the *Limulus* embryo, as in other embryos, there are evident from time to time "spontaneous" movements of body and legs. But it happens that in *Limulus* these movements, like the photokinetic movements of the legs which appear when the light intensity is suddenly changed, seem to have no influence whatever upon the sequence of the heart beats.

The frequency and the amplitude of the cardiac movements nevertheless go through a rather definite cycle, at constant temperature. This was ascertained by repeated observations on single individuals; since the data add nothing new to the theory of the case, they are not given here. The latitude of variation is slightly greater than 10 per cent of the mean frequency, and, as in some similar cases earlier described, is constant over the workable temperature range but varies from one individual to another. The latitude of variation seems quite unrelated, in general, to the relative rate of the process considered, and since it varies within pretty narrow limits for a variety of activities (5 to 10 per cent of the mean), it must be regarded as chiefly determined through some property of protoplasmic organization rather than by the specific process whose temperature characteristic is being measured. In a small number of known cases (Crozier and Stier, 1926–27, *a*) the latitude changes when μ differs on either side of a critical temperature, and in such cases a specific association must be assumed.

The slow developmental pace of the *Limulus* larva, together with the great resistance to asphyxiation (*cf.* Kingsley, 1893; Newman, 1906; Redfield and Hurd, 1925) contribute to its suitability for our purpose. The majority of the individuals used were kept at 20°, when not subjected to temperature changes experimentally. No differences were seen in other embryos maintained at 4.5° for several weeks. It was possible in this way to have embryos of various stages of development available at one time. Reversing the course of the temperature changes gave observations in good agreement. Within certain limits duplicate "runs" on successive days also agreed well; but as a rule the frequency of heart beat changed after 1 or more days, although—so far as ascertained—without change of temperature characteristic. The total number of observations was 3400.

IV.

Sixteen series of readings, on fourteen different individuals, were well controlled by repeated check observations at the same tempera-

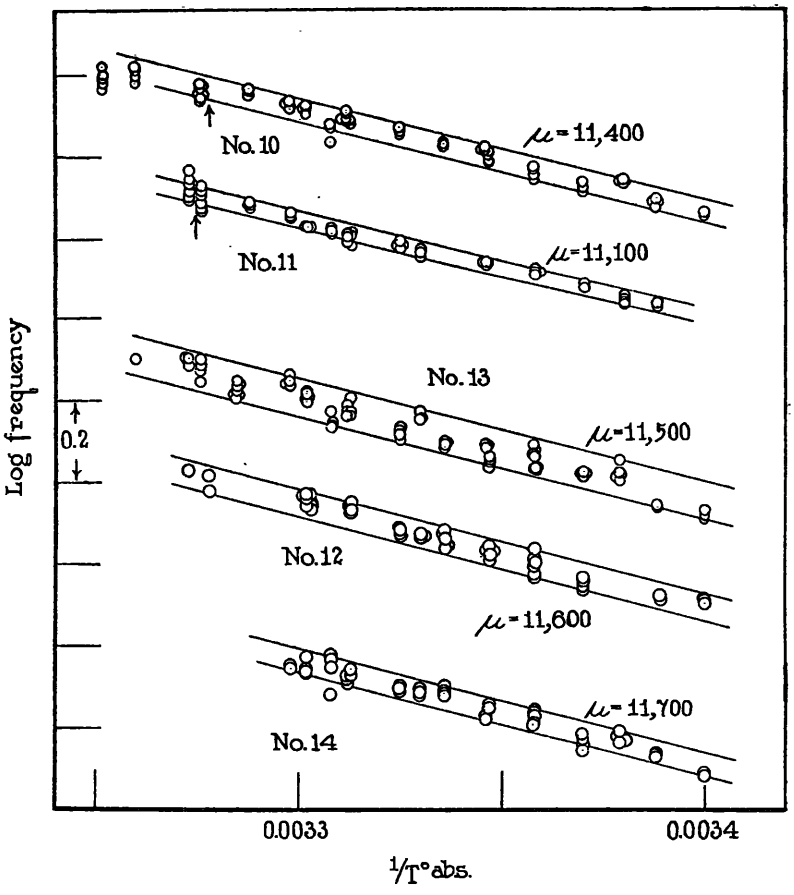

FIG. 1. Observations on the frequency of heart beat in five embryos of *Limulus*, at temperatures between 20° and 35°±, for which μ = 11,400. (The frequencies at constant temperature are very nearly the same; the frequency is taken as 100 ÷ seconds for ten beats.)

tures. Of these individuals seven provide increments ranging from 11,000 to 12,280 (the latter value is probably too high); for these the average μ = 11,520 ± 100. Three series gave μ = 16,430 ± 200.

Two gave μ = 20,000 ± 100, and two μ 25,500 ± 300. When data from any one animal are considered over a range of temperatures the lower value of μ pertains to the higher temperature interval (20°–30° +), but one embryo gave μ = 11,000 over the range 10°–20°, and μ = 16,400 occurs both in the range 10°–20° and in 20°–30° (with different embryos).

Above 30°–34° the rate of increasing frequency of heart beat with elevation of temperature is very slight; most embryos of this

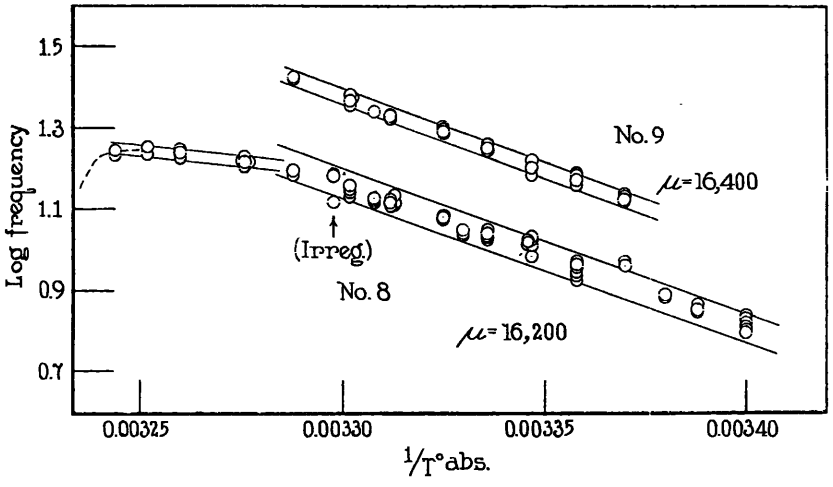

FIG. 2. Data from two *Limulus* embryos for which μ = 16,300 (20°± to 30°±); the rates for No. 9 have been multiplied by 1.59. The latitude of variation, as seen also in Fig. 1, varies with the individual. Above 30°± the frequency changes very little with increasing temperature; the curve, within the region shown (No. 8) is perfectly reversible. This phenomenon has already been referred to as apparent in other cases (*cf.* Crozier, 1925–26, *a*).

age show slight but easily detected decreases in frequency of heart contraction, which are only very slowly reversible on return to temperatures below 30°. Other individuals do not exhibit this hysteresis, but above 30°± the thermal increment is very small (μ = 5,000 ±) between 30° and 40.5°. This effect resembles that already noted in some other instances (Crozier, 1925–26, *b*), and is suggestive of the control of heart beat frequency by some purely physical condition, such as fluidity of substance or the saturation of some reactive sur-

face which gives a mechanical limit to the maximum frequency of pulsation. In one case which is illustrated (Animal 2, Fig. 5) no hysteresis was apparent on return to lower temperatures.

The temperatures for cessation of regular cardiac rhythm were 9° and 45°; above 40.5° the frequency of contraction decreases; at 44.4° the heart beat was still regular, but at 45.4° only an occasional beat was apparent. For the adult *Limulus* (Carlson, 1906) the thermal limits for contraction of the heart muscle are given as 0°± and 32°,

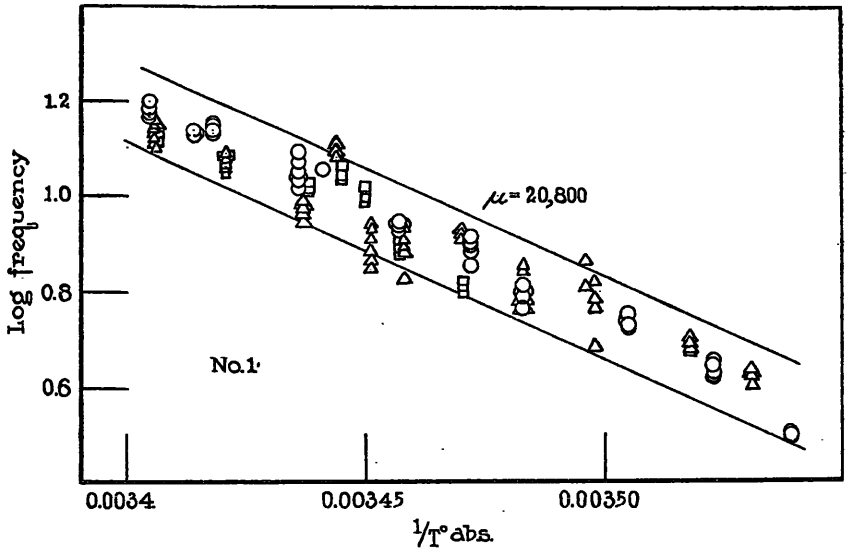

FIG. 3. One individual gave data (three "runs" of observations) yielding μ = 20,800 for frequency of cardiac rhythm, below 20°.

with "heat rigor" appearing at 47°; for the cardiac ganglion and nerves, the limits of activity are 0°– and 42°–43°. At the lower temperatures, beating was observed to become regular at 8.7° to 9.2°; below 8.7°, down to 5.9°, an occasional isolated beat was seen; on warming up to 8.8° to 9.0°, regular contractions were uniformly observed. It is worth noting that these thermal limits, determined from observations upon a large number of embryos, were found to be the same in embryos kept for 15 days at 4.0° as in those maintained at room temperature or used in warming or cooling experi-

ments. This agrees with the essence of Mayer's (1914) findings upon the adult *Limulus* and points to the conclusion that in this instance the thermal effects depend upon the composition of the animal, rather than upon thermal adaptation.

Between the extremes of temperature which limit the exhibition of regular rhythm (9°, 40.5°) the following are found to be critical temperatures, in the sense (Crozier, 1925–26, *a*) that abrupt change or irregularity may there appear in the curve relating frequency to

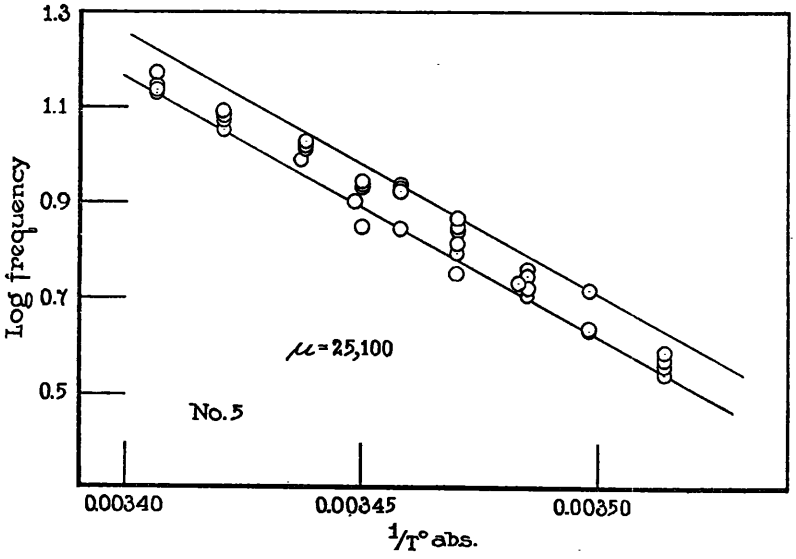

FIG. 4. *Limulus* Embryo 5 gave μ = 25,100, below 20°.

temperature: 20°, 27°, 30° to 34.5°. We purposely avoided the possible influence of the "break" at 20°± by largely working below or above this temperature, with different embryos. One individual (No. 2; Fig. 5) was found by repeated runs of observations to exhibit a sharp "break" in the curve of heart beat frequency at 20°–21° of such a character that a change in frequency accompanied a change of temperature characteristic. This is the first instance of the kind which we have been able to study carefully. The probable existence of such cases has earlier been mentioned (Crozier, 1925–26, *b*); they are of particular interest for the theory of critical temperatures.

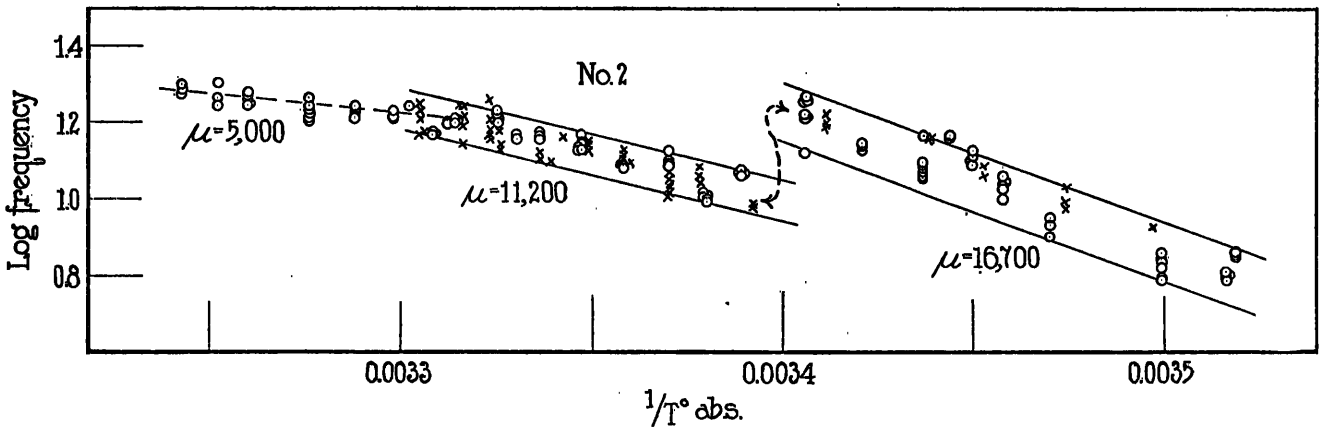

FIG. 5. Embryo 1 gave μ = 16,700 below 20°, μ = 11,200 between 20° and 30°; above 30° the curve flattens out (as with No. 8, Fig. 2, and in other instances not plotted). The particular interest of this graph is in the type of "break" found at 20° (see text). Two series of observations are distinguished by symbols; in the second series (crosses) the latitude of variation is less, below 20°, than in the first series.

V.

The temperature characteristics for frequency of cardiac rhythm in *Limulus* embryos thus appear as definite and recurrent quantities. The fact that two individuals apparently similar may yield quite different magnitudes of μ simply means, we take it, that the two pace maker cells or cell groups in these hearts have slightly different metabolic adjustments, or that the pace maker groups are different. If this were correct we might reasonably expect to alter μ experimentally in a more or less predictable way. This we did not attempt in the present observations although it has been accomplished in other instances (Crozier and Stier, 1925–26, *b*). It would also be predicted that the various values of μ obtained should show certain interrelationships. Thus the common association of the values 11,300 and 16,200 in respiratory and other processes (Crozier, 1924–25, *b*) finds rational application in the present case.

For the heart of adult *Limulus* the characteristic μ, so far as can be ascertained, is about 12,200; in several individuals (data from Garrey, 1920–21, *a*, *b*; *cf.* Crozier, 1924–25, *a*) μ = 23,500 below 15°. With one exception, which is not intrinsically of great weight, this value of μ does not appear in connection with the embryonic heart. The data upon adult heart rates came from experiments in which the temperature of the cardiac ganglion alone was varied, and the μ obtained agrees quantitatively with that for a number of other instances among arthropods in which central nervous control may be assumed (Crozier, 1924–25, *a*; Crozier and Stier, 1925–26, *a;* Fries, 1926–27). The increments apparent in the observations on the embryonic hearts, however, are of frequent occurrence in data on the heart rhythms of molluscs and vertebrates (*cf.* Crozier 1925–26, *b*). This sort of result points definitely to different chemical control of heart pulsation in embryo and in adult *Limulus*, and to the relative diversity of the pace-making control in the developing embryos. (If adult *Limulus* were to be used, the possibility of myogenic effects might have to be reckoned with, in addition to the neurogenic automatism, if the temperature of the whole organism were varied; *cf.* Hoshino, 1925.)

The possibility of diverse pace-making processes in the hearts of different individual embryos, and thus in different but functionally

analogous cells, is important for the understanding of thermal effects in isolated heart preparations and in cultures of developing myocardium. To this there must be added the recognition of sources of confusion which may result from the fluctuation of controlling circumstances within single cells, of which at least two kinds of disturbing effects can reasonably be suggested. The behavior of the "accessory hearts" of *Notonecta* is particularly significant in this connection (Crozier and Stier, 1926–27, *b*). At the moment we wish to deal particularly with the consequences of the occurrence in a single pulsating structural mass of a diversity of possible pace-making elements (*cf.*, for the chick heart, Cohn, 1925). Murray (1925–26) found that the apparent temperature characteristics for frequency of pulsation in cultured explants of chick myocardium failed to show uniformity, and failed to be grouped about detectable modal values. In such preparations there must exist at any moment a number of possible pace makers. The net result of their fluctuating control would be expected to obscure or to blur the influence of any one, since it is fair to assume that their respective inner metabolic states might be differently adjusted. In heart cell cultures the controlling influence of the intrinsically faster beating component of compound masses has been demonstrated experimentally by Olivio (1926), and this appears indeed to be a general condition (*cf.* Mayer, 1911; Crozier, 1916).

The sort of situation, therefore, which we believe to exist in pulsating heart cell cultures is one in which a number of distinct "pace-making" cells or cell groups are present in each pulsating mass. The intrinsic frequency of initiation of rhythmic contraction is supposed to differ among these pace makers. If one pace maker definitely possesses a much faster rhythm than the others, its effect is uniformly apparent. But if two or more pace makers have nearly the same intrinsic frequencies, but are metabolically different, so that each exhibits a characteristic relation to temperature, their several influences upon the gross sequence of pulsations should interpenetrate; at one moment pace maker *A*, at another instant, before *A* starts again, pace maker *B* is in control. One consequence of this kind of effect may be tested immediately. The latitude of variation, expressed as a percentage of the mean pulsation-frequency at each

temperature, should not be constant if pace makers A and B have different temperature characteristics. This is precisely the situation disclosed in several of Murray's (1925–26) figures. Therefore temperature characteristics deduced from such data *en mass* must be regarded as without specific significance.

Three corollaries are at once deducible. (1) The situation here pictured and tested differs from that (Crozier, 1924–25, *a*) in which it is supposed that the slowest process of a catenary series of catalyzed transformations dictates the speed and temperature characteristic for the velocity of formation of the end result. In the present case the swiftest pace maker determines the maximum frequency of rhythmic contraction. Therefore we may expect to find cases in which the "mean" temperature characteristic is increased at higher temperatures. The realization of this possibility is suggested in several figures given by Murray (1925–26; Figs. 1, 2, 3). One effect of this would be to bring about instances in which the log latitude of variation increases with increasing temperature as well as others in which the change is in reverse direction. These differences appear in Murray's figures.

(2) Of greater interest is the corollary that if our assumed pace makers A and B, respectively generating the most frequent and the least frequent contractions among the group capable of acting as pace makers at all, should have the same temperature characteristic, then the logarithmic latitude of variation should be constant; and in such cases the μ deduced should correspond well with a value found in homologous instances. In the figures given by Murray (1925–26; Figs. 1, 2, 3) we find, for cases meeting this requirement, $\mu = 8{,}000$, $\mu = 11{,}000$, and $\mu = 16{,}100$; these compare well with values commonly recognized in heart rate measurements (Crozier, 1925–26, *b*).

(3) For cases in which the latitude of variation is inconstant, the slopes of the lines fitting the extreme variates on the semilog plots should be straight and should provide approximate μ values characteristic of the limiting pace makers. And we should expect these to compare favorably with temperature characteristics encountered elsewhere. We have treated in this way the plots given in Murray's Figs. 1, 2, 3. The result in one case is reproduced in Fig. 6. The

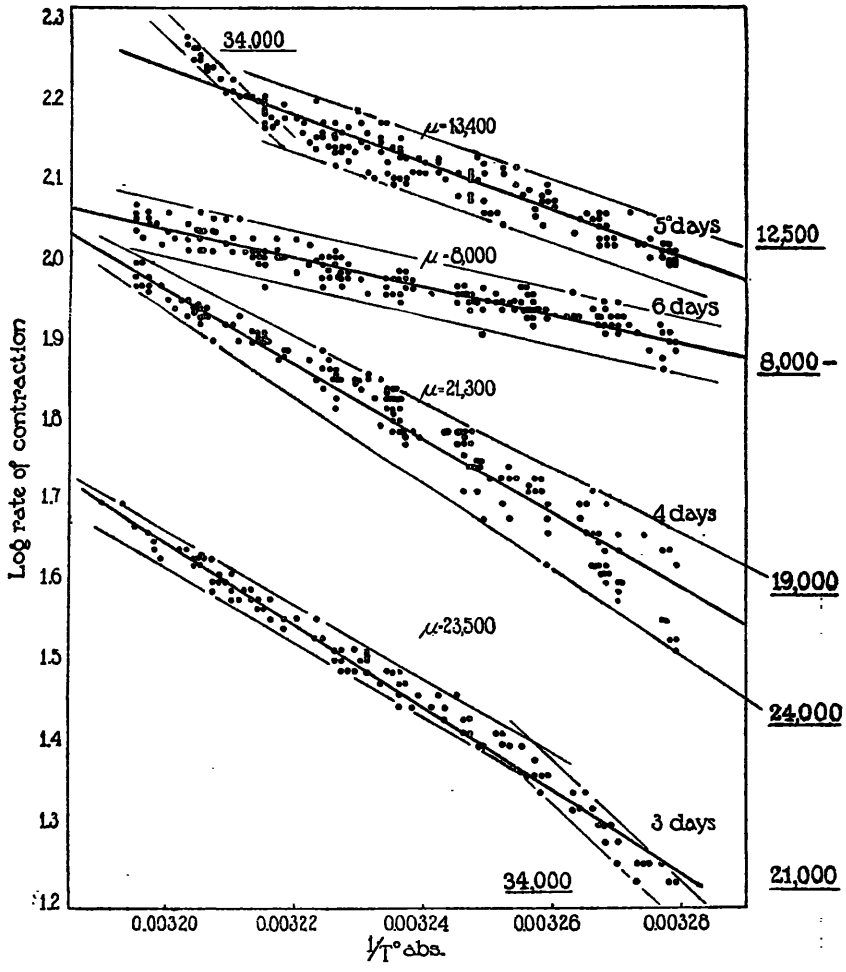

FIG. 6. Reproduced from Murray (1925–26, Fig. 2). The contraction rates of auricular fragments from embryos of the ages indicated. The central line in each plot is that originally given by Murray. To these lines there have been added marginal lines fitting the extreme variates. When attention is given to the latitude of variation it is obviously necessary to deduce values of the temperature characteristics somewhat different from those originally given. Values obtained from the marginal lines are indicated with underscoring. The nature of "breaks" in the uppermost and the lowermost graphs are fairly clear. When the log latitude of variation is constant, values of μ are gotten ("6 days," *e.g.*) which are already well known in other situations. When the latitude of variation changes continuously on the semilog plot the marginal rates are regarded (see text) as due to the operation of diverse pace makers. We consider that the recognition of these sources of confusion in curve fitting is sufficient to remove the force of the contention that temperature characteristics vary at random in this case.

characteristics obtained are noted in the figure, and the result may certainly be taken to agree with the expectation.

The effect of two such limiting pace makers could be imitated by combining the observations from two individuals (*Limulus*) in which μ for frequency of heart beat is different but the rates at given temperature approximately the same. It may be suggested that precisely this condition may appear if one were to measure the frequencies of contraction in the heart of an Ascidian, without reference to the places of origin of the individual beats; or, perhaps better, in a medusa deprived of all but several rhopalia.

These considerations do not completely account for the sources of complexity probably present when such an object as a heart cell culture is studied in this way. The indications already obtained, however, show why in these analyses we have continuously insisted (1) upon the errors which may be involved in the process of averaging rates or frequencies of vital processes in different individuals, or even in the same individual at different times, and (2) upon the ribbon form of significant plottings. There is to be added the further type of difficulty entering when a break occurs in the curve relating frequency or rate to temperature; should this sort of change be present in the activity of one pace maker, absent in others, the logarithmic latitude of variation must again change if the curve for this pace maker falls outside the limits set by the activities of other concurrently effective pace makers. Such a break, furthermore, may or may not be accompanied by an abrupt change of frequency; and changes of frequency may occur without change of temperature characteristic. These are not imaginary situations (*cf.* Crozier, 1925–26, *b;* Crozier and Stier, 1924–25, *b;* 1925–26, *b*). It seems to us inherently probable that disturbances of these types are likely to be encountered with greater frequency in objects such as isolated cell masses in culture than in connection with organs of intact animals, although we may also suggest their probable occurrence in the heart rhythms of embryos. The plottings given by Murray (1925–26) contain features suggestive in this respect, which we venture to predict will find explanation in further studies of embryonic heart rhythm.

There is one general aspect of this whole matter which requires brief additional comment. Murray (1925–26) has suggested that

the essential difference between the gross results of his observations and those in cases where intact organisms have been employed lies in the operation of some regulatory property of the complete organism. Since there is a possibility of vagueness in the understanding of such

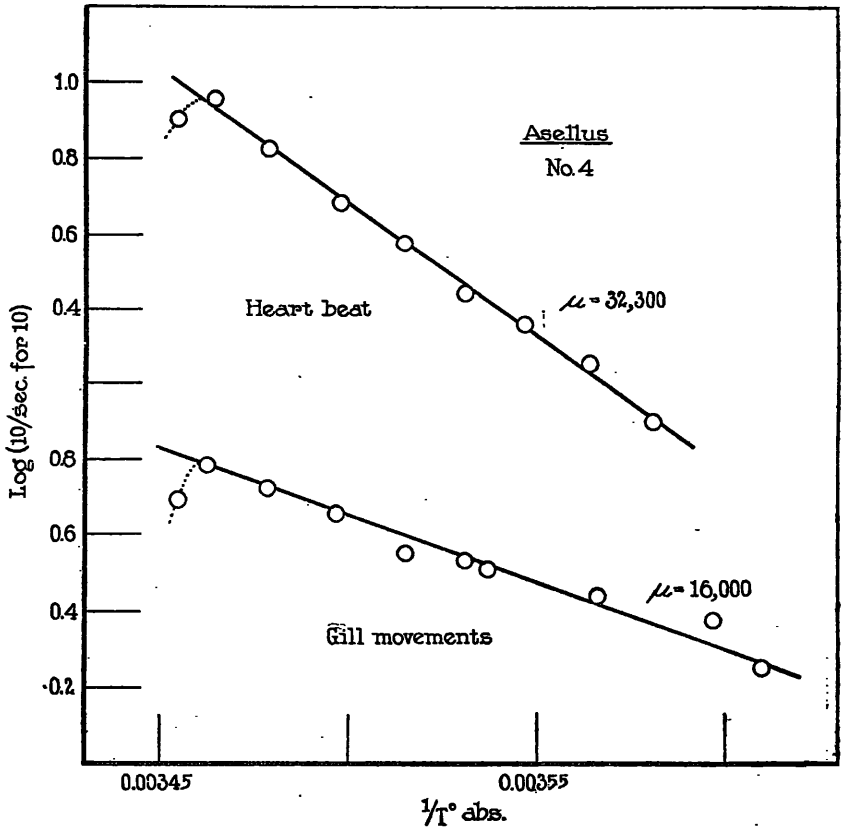

FIG. 7. Simultaneous determinations of frequency of gill movements and of heart beat in *Asellus* show that the temperature characteristic is not the same for the two activities. This disproves determination of μ by the organism as a whole.

a suggestion we may point out that it may be understood in two ways open to test. The "organization factor" might pertain simply to the heart or other structure immediately implicated in the observations; or it might be taken as a feature of the organism as a whole.

The former effect can and must be granted at once as an obvious truism, in the sense that the structure of a heart, for example, permits control by a definite localized pace maker. The latter view can be tested by determining simultaneously the temperature characteristics for two or more different activities in the same individual. We have previously made such experiments. The frequencies of heart beat and of respiratory movements in the same individual arthropod, synchronously determined, do not vary together and do not have the same temperature characteristics. This holds also for embryonic *Limulus*, the frequencies of gill movements providing increments quite different from those here obtained for the hearts (*cf.* Crozier and Stier, in a subsequent paper). For the moment we may illustrate the point by means of data from experiments with *Asellus* (Fig. 7). Therefore a general control by the whole organism is excluded. The results of these experiments will be detailed in another place. They are patently significant for the theory that a specific thermal increment has a particulate locus.

SUMMARY.

Temperature characteristics for frequency of myogenic heart beat in *Limulus* embryos, before the onset of nervous control of the heart, were found to be 11,500; 16,400; 20,000; 25,500. The two first values are the best established. The different values pertain to the hearts of different individuals outwardly similar, and to the hearts of single embryos in different parts of the temperature range. These values differ from that known in connection with the control of the heart beat through the cardiac ganglion. The occurrence of critical temperatures, also, is not the same in all embryos. These facts are employed in a discussion of temperature relations in pulsating explants of chick myocardium.

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WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.