ENERGY METABOLISM IN ISOLATED EMBRYONIC

RAT HEART

by

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A THESIS

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(Chairman, Graduate Council)

ACKNOWLEDGEMENT

Praise be to God for all things.

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INTRODUCTION

Most of the studies concerning functional development in the embryonic heart have utilized histochemical, biochemical or tissue culture techniques. The histochemical-cytochemical techniques have had their greatest use in localization of storage products or in identification of the sites of action of certain enzymes. Biochemical techniques applied to cellular homogenates have also been useful in the study of developing enzyme patterns, etc. However, precautions must be taken in interpreting the results reported by investigations using these two approaches. For example, it is not axiomatic that the presence of high levels of enzyme activity, as measured by these methods, automatically confers a high level of functional activity in the intact cell. This statement is supported by the results of investigations of Mg^{++}-activated ATP-ase in the chick embryonic heart (Moog, 1965). These investigations have shown that there is no measurable increase in Mg^{++}-activated ATP-ase levels during the time when an active efflux of Na^+ is taking place (between days seven and fourteen of development). Yet the Na^+ transport process is known to be intimately associated with this enzyme. The converse situation also exists in which there are high levels of particular enzymes with no evidence that they are functional within the intact cell (Moog, 1965). Therefore, it appears that the functional contribution of an enzyme in vivo does not necessarily follow the activity as revealed by biochemical or histochemical reactions, although function and measured level of activity often do parallel one another.

The third avenue of investigation, that of culture techniques, allows for a more direct functional analysis of certain metabolic systems, particularly as concerns substrate utilization and energy metabolism in embryonic tissue.

Spratt (1949, 1950a, 1950b) used this technique to great advantage in his classic studies of nutritional requirements in the explanted chick embryo. This investigator cultured embryos from the primitive streak stage through the ten to fifteen somite stages on a simple defined medium to which was added the substrate to be tested, such as glucose, pyruvate, etc. He then compared the growth and functional development of the heart with other developing organ systems in the presence or absence of the various potential energy sources. In the study of mammalian embryos there exists at least two drawbacks to the use of this system. The first and most serious being that mammalian embryos of comparable age have not been cultured successfully on a simple defined medium. Secondly, assuming culture could be accomplished, a problem exists in the interpretation of results when various embryonic organs and tissues are cultured together - as for the intact embryo. With this technique, when a specific organ is under investigation, one is never sure that the organ in question is capable of utilizing the added substrate; it is entirely possible that other tissues in the system are taking up the substrate, processing it and contributing some metabolite(s) of that compound to a pool which is available to the organ under study.

This problem can be overcome by isolating the organ from the rest of the embryo and studying its growth and development while cultured in a defined media. The embryonic heart lends itself especially well to this manipulation in that it is fairly easy to separate from contiguous tissues. In addition, it has a built-in indicator of function - its contractile automaticity.

In spite of these obvious advantages surprisingly little has been done with isolated embryonic hearts. Burrows (1921) early noted that

isolated chick embryonic heart cells were capable of maintaining contraction in complex media when exposed to a nitrogen atmosphere for five to six hours. Guidotti and Foa (1961) used isolated chick hearts in their investigations of the development of an insulin sensitive system and also employed such preparations as a tool for studying cell permeabilities. More recently, Roberts (1966) and Roberts et al. (1965a, 1965b) have investigated isolated heart activity from three- and five-day old chick embryos. Their approach has been directed at demonstrating the development of neuro-humoral sensitive receptor sites in embryonic hearts, touching but briefly on energy metabolism. Several investigators (Webb and Hollander, 1956; Kardesch et al., 1958; and Yang, 1963) have reported unsuccessful attempts at maintaining activity in isolated hearts from mammalian embryos, especially under anaerobic conditions. Gunberg (1967) has conducted preliminary investigations using intact yolk sac-embryo preparations and a few isolated embryonic hearts. His results have indicated that cardiac contractility and yolk sac circulation could be maintained by single metabolites. The investigations to be presented were stimulated by questions which arose from the aforementioned experiments and were undertaken to obtain some understanding of the energy metabolism of the early embryonic mammalian heart, information which is not available in the current literature.

Literature Review

Early investigations into the function of embryonic hearts were primarily concerned with sites of origin and the development of electrical activity. The approaches taken included direct observation of the heart-forming primordia in cultures of developing chick embryos by Patten and Kramer (1933) and observation of hanging drop cultures of rat embryos by Goss (1937, 1938, 1942). These investigators were able

to identify which portions of the primitive hearts were early activation centers and they were able to observe the development of the wave-like spread to adjacent areas of the heart. Interest in the electrical activity of embryonic heart cells led to the development of a technique by Pollack (1931) for obtaining electrocardiograms in chick embryos. Hoff et al. (1939) used this technique and an improved amplifying device to extend the electrocardiographic studies to include the fifteen somite chick embryo. Goss (1940), using the techniques developed by Lewis (1919) for demonstrating cross-striations in embryonic heart muscle, found that functional activity, in the form of contractions, actually preceded the development of cross-striations.

Attempts at elucidating the metabolic activities of the developing heart began with Burrows (1921) and Lewis (1924) who studied the effects of anaerobiosis and temperature changes, respectively, on the contractile rate of isolated chick embryo hearts. Nutritional requirements and metabolic pathways were the objects of study in the experiments of Spratt (1948a, 1948b, 1949a, 1949b, 1950a, 1950b) who cultivated chick blastoderms on basal media (buffered-Ringer agar) containing different concentrations of various potential energy sources. From his observations Spratt was able to infer which substrates best supported growth and differentiation of the embryo as a whole as well as comparing growth of specific organ systems such as heart and brain. By using inhibitors of glycolysis (iodoacetate and sodium fluoride) and inhibitors of the respiratory chain (cyanide, azide and nitrogen), Spratt was able to compare the relative dependence of the brain and heart on anaerobic and aerobic metabolism of glucose. Duffey and Ebert (1957) used Spratt's approach in studying the effects of the metabolic inhibitors Antimycin A and sodium fluoride on the heart-forming areas of the chick. More

recently, Roberts (1966) and Roberts et al. (1965a, 1965b) have employed heart rate as the parameter of measurement in investigations utilizing isolated chick hearts. Their experiments were concerned primarily with the demonstration of receptor sites for certain neuro-humoral agents and touched but briefly on energy metabolism.

Similar approaches to the study of developing metabolic pathways in mammalian embryos have been limited to the earlier stages, due to the difficulties encountered in attempts at culturing the later (beating heart) stages in simple media. Brinster (1965a, 1965b, 1965c, 1966, 1967a, 1967b, 1967c), and Brinster and Thompson (1966) have studied growth and development of preimplantation to blastocyst stages of mouse, rat and rabbit embryos, concentrating on the emergence and relative importance of pathways involved in energy metabolism. Thompson (1966) has extended this line of investigation to include studies on the affects of specific metabolic inhibitors on early mouse embryo development. Fridhandler et al. (1967) and Daniel (1967) in the rabbit and Wales and Biggers (1966) in the mouse have made additional contributions in determining metabolic patterns of preimplantation mammalian embryos.

Recently, New (1967) and Shepard (1969) have related heart rate in cultured rat embryos to differences in both oxygen tension and age of the embryo. These studies, carried out on complex media, are the only ones dealing with cardiac metabolism in "in vitro" mammalian embryos of the beating heart stages.

Statement of Problem

To date little has been reported in the area of early embryonic metabolism - especially as concerns postimplantation mammalian embryos. The objective of these investigations is to add to the scanty information

available on early embryonic rat metabolism, dealing specifically with energy metabolism in the developing heart.

Heart rate in isolated hearts has been used as an indicator of metabolic function by several investigators. Heart rate has been chosen as the measurable parameter in the following studies of energy metabolism in isolated embryonic rat heart.

Answers are to be sought to the following questions:

1. Which substrates can be taken up and metabolized by the isolated heart so as to produce enough energy to maintain contractile activity?

2. Are there differences in substrates utilized by the hearts at different stages of development?

3. Can anaerobic glycolysis provide enough energy to maintain the maximum heart rate (under the conditions provided) in the eleven-day old heart? In the twelve-day old heart? In the thirteen-day old heart?

4. Does the TCA cycle vary in its importance as a metabolic pathway in the hearts from embryos of different ages?

5. What effects, if any, do a number of metabolic inhibitors have on heart rate?

MATERIALS AND METHODS

A. Animal Care and Breeding

Sprague-Dawley rats, obtained from Simonsen Laboratories, were used exclusively for this study. The females were ordered at a weight of one hundred eighty to two hundred grams and bred at a weight of approximately two hundred twenty to two hundred forty grams. Males were purchased weighing three hundred grams and kept as active breeders for approximately one year. A total of nine males and one hundred and twelve females were used over a three year period. All males, virgin and bred females were maintained in the same quarters - a room with the temperature held constant at approximately 70-75° F. The light cycle in this room was inverted, the dark period lasting from 10:00 A.M. to 10:00 P.M., in order that timed breeding could be carried out during the hours most convenient for the investigator. A diet of Purina rat chow plus supplemental greens (twice weekly) was given to all animals. With the exception of one outbreak of what appeared to be a particularly virulent viral pneumonia, resulting in sacrifice of all afflicted animals, the animals remained healthy during their sometimes lengthy stay in the aforementioned animal room.

Vaginal smears were obtained routinely at 9:30 A.M. each morning. Those that were determined to be in the proper stage of the estrus cycle, according to the method of Blandau et al. (1941), were placed with a male and left for a two hour period. After this time period the females were removed and checked for vaginal sperm and/or copulation plugs. Positive findings were taken as evidence for successful mating and the midpoint in the two hour interval was taken as the time of copulation. In this way aging of the resultant embryos was accurate to within plus or minus one hour. For example, eleven-day embryos referred to in this study

had a copulation age of precisely 264 ± 1 hours and twelve-day embryos
288 ± 1 hours. Timing the breeding in such a way led to a very
uniform size of embryo as determined by somite count. Eleven-day
embryos were fifteen to seventeen somites (plus or minus one), with
very few exceptions. The twelve-day embryos were twenty-seven to
twenty-nine somites (plus or minus one), again with very few exceptions.
Litters of embryos taken at ages older than twelve-days were more dif-
ficult to stage by the somite method. The ages of the embryos will,
in the remainder of this paper, be referred to in numbers of days after
copulation.

B. Removal and Isolation of Embryonic Hearts

On the specified day the pregnant females were anesthetised with
Nembutal (sodium pentobarbital, 50 mg/cc) at a dosage of 1 ml/kg body
weight. All injections were given intraperitoneally, in the ventral
midline, near the rib cage. The embryos, within their decidual capsules,
were removed by laparotomy and cesarean section. The whole implants,
still within deciduae, were placed in a watch glass containing normal
saline at room temperature and held there until ready for dissection
(a period of five to thirty minutes). In preparing to dissect out the
hearts, the implants were removed one by one from the watch glass and
transferred to a sterile disposable petri dish, also containing normal
saline. Here, with the aid of an A-O stereomicroscope and jeweler's
forceps, the embryos were freed of their surrounding membranes and the
pericardium was removed from the heart area. Under increased magnifi-
cation the hearts were severed at both sino-atrial and truncal ends
with sharpened jeweler's forceps. The thus isolated hearts were then
transferred to the appropriate solutions by pipette for subsequent
study, the procedures of which will now be described under separate

headings.

C. Incubation Technique and Apparatus

After pipetting the isolated heart from the saline solution the organ was transferred to a glass insert (part of a specially designed chamber to be described below) which in turn was bathed with the incubating medium. The incubating medium consisted of standard Krebs-Ringer's (see Appendix, A) to which was added streptomycin and penicillin to retard microbial growth, and phenol red, a pH indicator. Substrates to be tested were added as isosmotic solutions (300 mOsm) after withdrawing an equal volume of fluid from the incubating chambers. The sterility of the culture medium was checked at two different time periods during the course of an experiment, once at the onset and again at the termination. The check consisted of plating enriched agar, blood agar and Sabaroud's agar plates with a loop of medium from the chambers. Only background levels of bacterial colonies were seen after incubation of the plates - occasionally a staphylococcus strain with a rare colony of coliform bacilli. These results showed that antiseptic measures were effective in controlling contamination by microorganisms.

The chamber itself (Plate 1) consisted of a heavy Pyrex cylinder with a flanged top, upon which rested the lid. The lower portion of the chamber contained a ground glass female fitting designed to receive a tapered air-stone apparatus (labeled GAS INFLOW in the diagram). To the air-stone was attached rubber tubing leading from an Erlenmeyer flask half-filled with distilled water, through which was bubbled the appropriate gas mixture (Plate 2). The gas passed through the inlet and was broken up into many small bubbles by the air-stone. Gas that did not go into solution bubbled through the medium to collect in the

top of the chamber until the pressure reached a level which forced the excess gas out the exhaust port (Plate 1).

In order to make direct observations into the chamber (through the stereomicroscope), it was necessary to overcome the problem of condensation of water vapor on the glass lid. A satisfactory result was obtained when the lids were brought to a temperature slightly above that of the water vapor. The heating of the lid was accomplished by passing an electric current through a circuit consisting of two gold posts for contacts soldered to a very thin layer of tin oxide. The tin oxide was baked onto the lid as a ring limited to the periphery, or that area resting upon the flange, and, therefore, did not interfere with visualization. The voltage applied to the contacts was 110 a.c. passed through a Variac variable resistance rheostate set at approximately eighteen volts.

As stated previously, the hearts rested in small glass inserts (see Plate 1) which were suspended from a glass disc containing holes slightly greater in diameter than the diameter of the inserts. The disc was held in place about one-quarter of the way down from the chamber top by resting upon an indentation that circumscribed the chamber wall. The inserts were removable and contained small (0.5 mm diameter) holes that perforated the glass. Free exchange between the fluid inside the insert and that outside the insert was thus ensured. By this means it was possible, in effect, to bathe the small embryo hearts with 500 cc of fluid without displacing them. It was also possible to remove the lid from the chamber, add a particular compound, replace the lid and note any changes in heart rate. Any desired change in gas phase was easily effected, simply by changing the gas supply leading to the water trap. It was repeatedly observed that the altered

gas phase rapidly came into equilibrium with the medium and thus with the heart cells. Based on cardiac function it can be stated that this equilibrium was reached in four to five minutes after a gas change.

The media-filled chambers containing the isolated embryonic hearts were kept in a thermostatically controlled ($37 \pm 1.5°$ C) circulating water bath (Plate 2). The water bath was made of plexiglass in the form of a rectangular box (36 in. x 14 in. x 6 in.). The box rested on a plate-glass shelf which was elevated from the table top by two wooden blocks, one at either end. The space between plate-glass and table top was sufficient to accommodate a light source which could be adjusted while viewing the heart through the stereoscopic microscope until the best image was obtained. The time taken for ten beats of each heart was measured with the Lab Chron (by Labline, Inc.) electric timer. The time taken for ten beats was then converted into the number of beats per minute and recorded as such.

By having three incubation chambers constructed, it was possible to run three different types of media or gas phases with the embryonic hearts from one litter. With most litters being made up of from twelve to fifteen embryos, the three groups run simultaneously would contain four to five embryos per group.

After each experiment the chambers were cleaned with Biodegradable soap and stored in benzalkonium chloride (1:1000) or sixty-percent ethyl alcohol until next used.
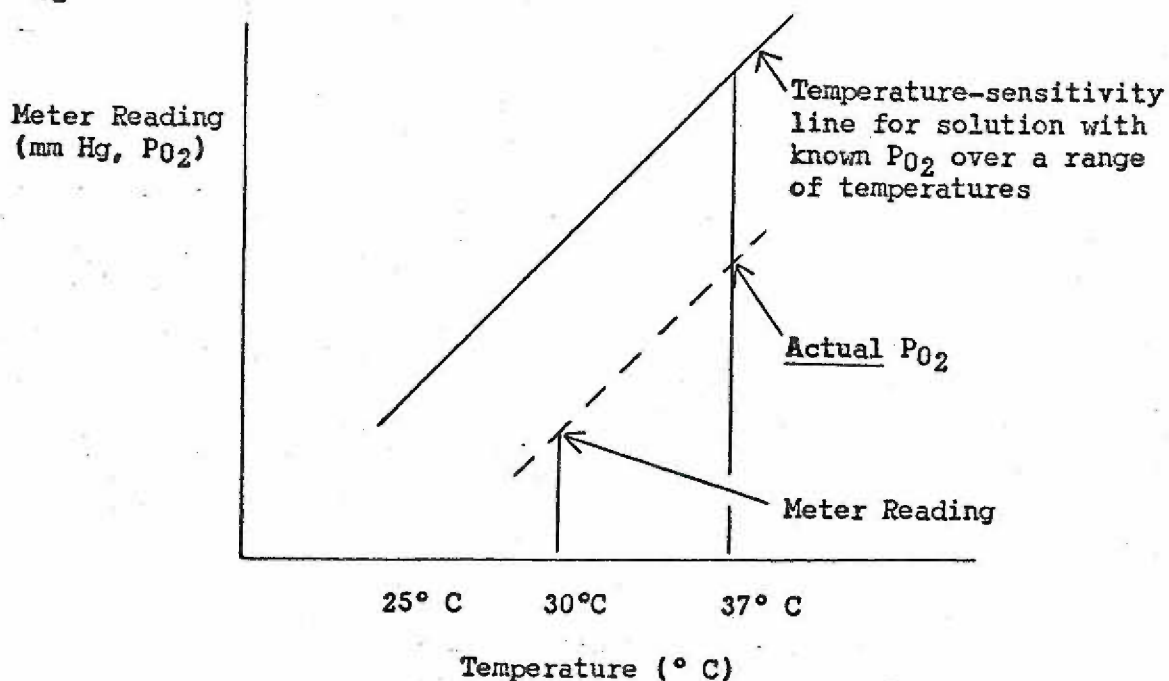
D. Oxygen Electrode Technique

The rate of oxygen consumption was measured using a Beckman model 160 oxygen micro electrode and physiological gas analyzer. The technical aspects of their operation are covered in detail in an instruction manual published by the Radiometer Company (Radiometer technical bulletin

#E5044/D61S). The principle on which the electrode operates is briefly described in the Appendix, B.

In order to measure the oxygen consumption of isolated hearts, it was necessary to construct a special chamber to house the electrode and the solution containing the hearts. A small block of lucite was drilled with a large hole perpendicular to the long axis of the block (Plate 3). The large perpendicular hole held the electrode snugly and application of heated sticky wax to the electrode-block junction ensured a tight seal. The tip of the electrode extended into the chamber, which contained the solution plus embryo hearts. Two smaller holes, drilled obliquely also communicated with the chamber. The smaller holes were used for the introduction of the hearts after temperature equilibration of the solution was attained. The entire volume of the chamber plus side arms was 0.65 ml after filling the chambers and introducing the hearts. The side arms were plugged with plastic stoppers and sealed with sticky wax and the entire apparatus was then immersed in the same thermostatically controlled water bath that contained the incubating chambers. The electrode chamber, fastened to a ring stand, was manually agitated throughout the time course of each study. Readings were taken from the P_{O_2} meter and from a thermometer attached to the chamber. Because there was some fluctuation in temperature of the water bath, it was necessary to make appropriate adjustments to the P_{O_2} read on the meter to obtain actual P_{O_2}'s (i.e., P_{O_2} at 37° C) as the oxygen electrode is very sensitive to temperature changes. This meant that some sort of compensation for temperature change must be made. Construction of a graph (see below) relating Temperature (in degrees Centigrade) to Meter Reading (in mmHg) was made by first calibrating the amplifier while the electrode was immersed in a solution of known P_{O_2} at 37° C, then reducing

the temperature of said solution to, say, 25° C. After allowing the meter reading to stabilize, this value was recorded and a line was drawn between the two points (meter reading at 37° C and meter reading at 25° C). The slope of the line connecting the two points was a measure of the variation of electrode sensitivity with temperature changes. To obtain <u>actual</u> values for P_{O_2}'s at temperatures other than 37° C, one plots the value obtained from the meter reading at the proper temperature interval on the abscissa. By then laying a ruler parallel to the temperature-sensitivity line and passing through this new point, an intercept with a line dropped perpendicular to the 37° C meter reading point is obtained. This intercept marks <u>actual</u> P_{O_2}. The following example illustrates this method for temperature compensation, with the dotted line being the parallel line drawn to obtain <u>actual</u> P_{O_2}.

Meter Reading
(mm Hg, P_{O_2})

Temperature-sensitivity line for solution with known P_{O_2} over a range of temperatures

Actual P_{O_2}

Meter Reading

25° C   30°C   37° C

Temperature (° C)

Readings were thus taken and converted to _actual_ P_{O_2} by use of the graph. The total change in P_{O_2} between onset and termination of an experiment (a one hour period) was converted to volume of oxygen consumed by the formula:

$$\dot{V} = \frac{\propto \text{ x } \dot{V} \text{ x } P}{760}$$

where $\dot{V}$ = volume of oxygen consumed
  $\propto$ = Bunsen solubility coefficient (0.024 for Krebs-Ringer's)
  V = volume of solution in chamber
  P = total change in P_{O_2} (in mm Hg)

Oxygen consumption is commonly expressed in $\mu L O_2$/Hr/mg dry weight. The dry weight of each group (usually four hearts run together constituted a group) was measured in the following manner: after each run the electrode chamber was raised above the water level, the seal around the electrode broken and the electrode transferred to a standardization flask. The hearts were then pipetted from the chamber and rinsed in distilled water before being placed on pre-weighed strips of aluminum foil. The excess water was drawn off the foil by a micro-tipped pipette and the heart-containing foil was placed in a dessicator. After several days in the dessicator, the foil and dried hearts were removed and weighed on a Misco quartz-helix microbalance (calibrated to 10 micrograms). By subtracting the weight of the foil from the total, the weight of each group of hearts was derived, and oxygen consumption was then expressed in terms of volume consumed per hour per milligram dry weight.

RESULTS

A. Substrate Utilization

1. Hexoses

Four simple (non-phosphorylated) hexoses were investigated to ascertain if the isolated heart preparations could utilize them as a source of energy for contraction. The four hexoses tested were glucose, galactose, fructose and mannose. Comparisons were made between glucose and the other three hexoses. The procedure for comparison involved separating the hearts from a litter of embryos into three groups (three incubation chambers, as described under Materials and Methods). One group was incubated in Krebs-Ringer's bicarbonate plus glucose, a second group in Krebs-Ringer's bicarbonate plus one of the other hexoses (galactose, fructose or mannose) and the third group (control) in Krebs-Ringer's bicarbonate (K-R salts) alone. This procedure was repeated with at least three litters of embryos for each comparison study. The data were then gathered and combined from the different litters. In this way functional variation in litters would be reflected in each group within a comparison study. Substrate utilization was taken to be positive when the mean heart rate of that group was significantly greater than that for the K-R salts control group.

For the glucose-galactose and glucose-fructose comparison studies heart rate was measured following a one hour period in nitrogen (95% N_2, 5% CO_2) after which the gas phase was changed to include oxygen (60% O_2, 35% N_2, 5% CO_2) and heart rate was recorded at hourly intervals for three hours thereafter. The data reported from these studies came from twelve-day embryos. A similar pattern of substrate utilization (although with lower values for mean heart rate) was seen for hearts from eleven-day embryos.

For the glucose-mannose comparison studies the hearts were left in nitrogen for a two-hour period before changing the gas phase to include oxygen. The data reported here were accumulated from investigations done on hearts from eleven-day embryos.

The results of the hexose utilization studies are presented in graphic and tabular form in figures 1-5 and their accompanying tables. All statistical analyses were done using the probabilities from the table of distribution of t of Fisher and Yates (1963).

a. <u>Glucose vs. Galactose vs. K-R salts</u> (Figure 1 and tables)

After one hour in an anaerobic environment it was noticed that there arose a very significant spread between the mean heart rates of the three different groups. The glucose group had the highest heart rate followed by the galactose group with a mean heart rate of approximately one-half that of the glucose group. The control (K-R salts) group showed no measurable heart rate.

After introducing oxygen into the incubation media a number of changes took place. The glucose group showed a slight rise in mean heart rate for hour-two which dropped off to slightly below initial values by the end of hour-four. Both the galactose and K-R salts groups showed a marked jump in mean heart rate after the introduction of oxygen. At hour-two no difference existed between the galactose and control groups, however, both were significantly lower than the glucose group. The heart rate fell off rapidly for the K-R salts group by hour-three and remained at zero for hour-four as well. The galactose group showed a lesser decline in mean heart rate over the ensuing two hour period.

These results showed that the addition of glucose or galactose to Krebs-Ringer's solution allowed for a faster heart rate than that attained by hearts in Krebs-Ringer's solution alone, both under

Table 1-1. Mean heart rate (in beats per minute) for glucose, galactose and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Mean $\pm$ standard deviation			
			Hr. 1	2	3	4
glucose	10	3	177$\pm$15	181$\pm$13	174$\pm$16	176$\pm$19
galactose	13	3	87$\pm$11	131$\pm$26	131$\pm$20	112$\pm$11
salts	11	3	0	130$\pm$23	0	0

Table 1-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$).

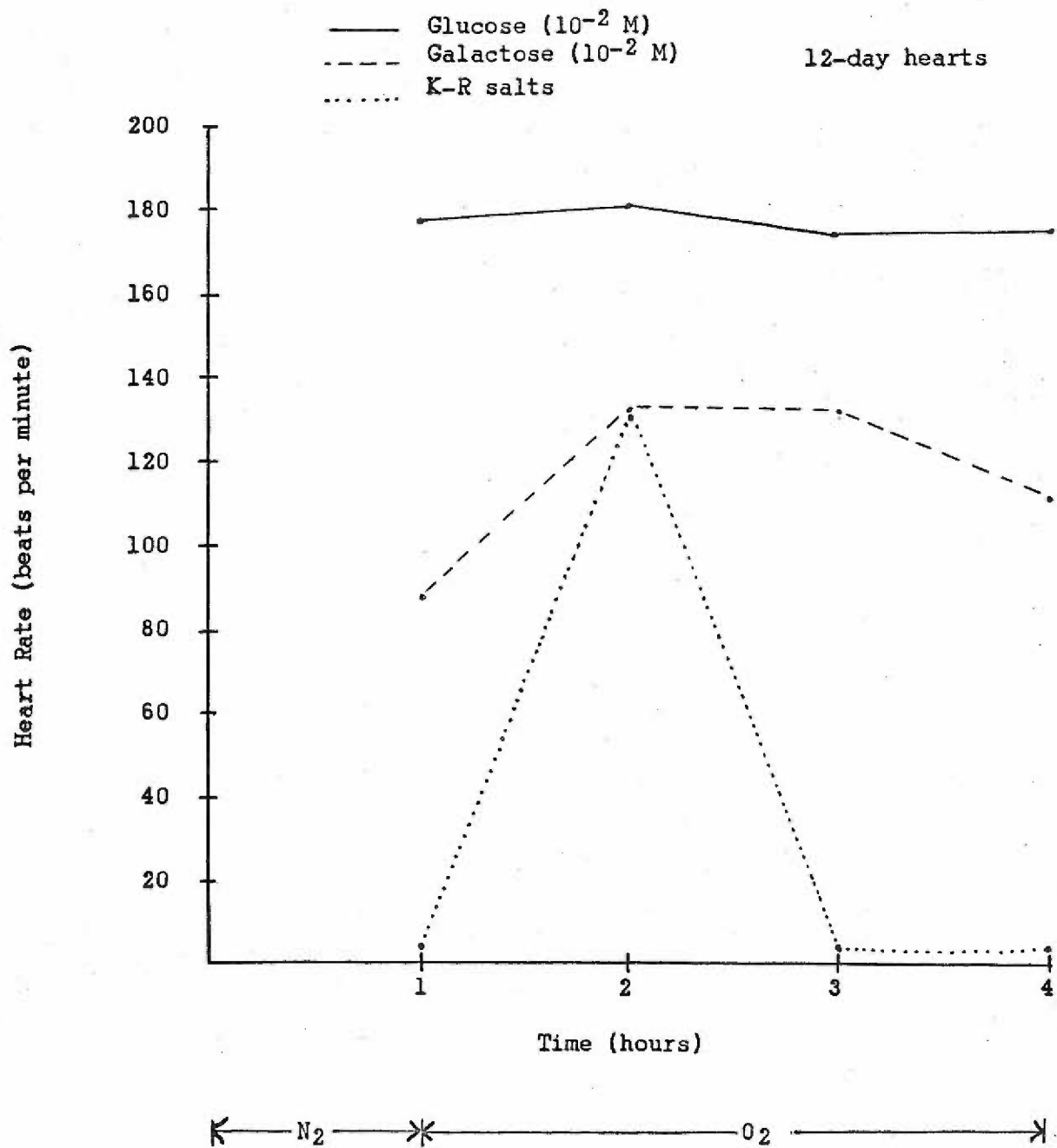| Comparison	Hr. 1	2	3	4
glu vs. salts	++	++	++	++
glu vs. gal	++	++	++	++
gal vs. salts	++	−	++	++

Figure 1

aerobic and anaerobic conditions. In addition, it was noted that glucose is superior to galactose as a source of energy for contraction, especially in an anaerobic environment.

    b. <u>Glucose vs. Fructose vs. K-R salts</u> (Figures 2, 3, 4 and tables)

    The results from the fructose studies proved to be complex in nature. After one hour in nitrogen the glucose group exhibited a heart rate significantly greater than that for either the fructose or the K-R salts group (Figure 2). When the gas phase was changed to include oxygen the marked difference between glucose and fructose disappeared to be replaced by a difference which was not significant (P> 0.05) for the remaining three hour period. The mean heart rate for the glucose group was markedly different from the K-R salts group over the entire time period except after the first hour in oxygen (hour-two). The mean heart rate of the fructose group was greater than that for the K-R salts group throughout the experiment, but the difference was not significant (P> 0.05) until after three hours of incubation. In addition, it was noted that the variation about the mean in the fructose and K-R salts groups was larger than expected.

    Closer analysis of the data led to the observation that within this large sample there existed two separate populations. The separation developed between groups of hearts coming from the litters of females who were bred with different males. All the embryos coming from females bred with one particular male (male #1) had hearts that utilized fructose to a much lesser degree than glucose under both anaerobic and aerobic conditions (Figure 3). This then constituted an isolated population.

    In contrast, the general population was made up of embryos coming from females who were bred by a variety of males other than male #1. When the utilization of fructose was compared with that of glucose in
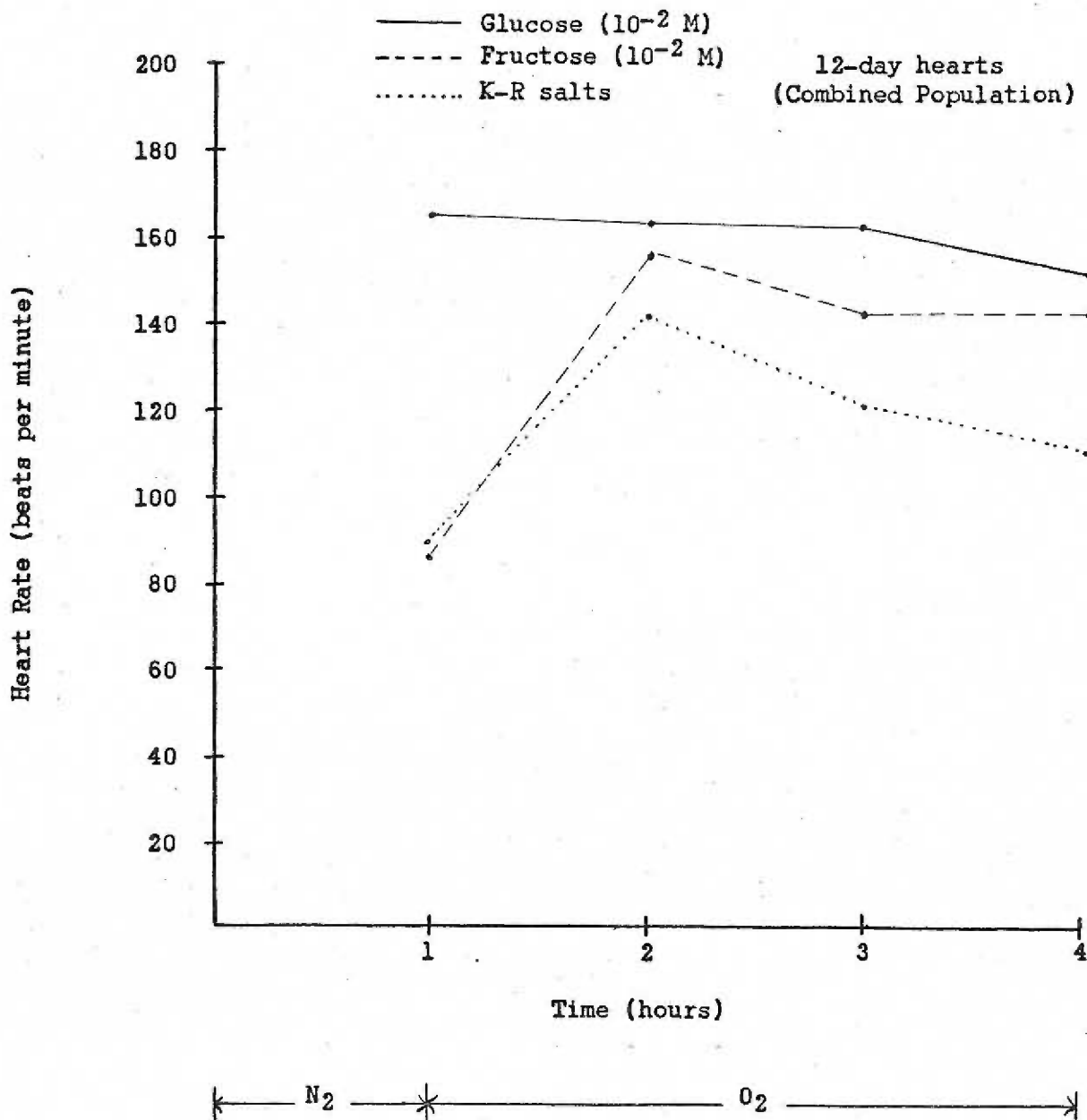
Table 2-1. Mean heart rate (in beats per minute) for glucose, fructose and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
glucose	24	6	165±12	163±16	162±18	152±14
fructose	23	6	85±9	155±35	142±24	142±25
salts	20	6	89±12	141±29	121±18	110±15

Table 2-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$).

Comparison	Hr. 1	2	3	4
glu vs. salts	++	+	++	++
glu vs. fru	++	−	+	−
fru vs. salts	−	−	+	+

Figure 2

Table 3-1. Mean heart rate (in beats per minute) for glucose, fructose and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
glucose	12	3	162±8	165±16	168±12	152±10
fructose	11	3	84±3	122±16	121±16	118±7
salts	10	3	71±0	111±9	106±10	0

Table 3-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the P< 0.05 level. A double plus (++) indicates statistical significance at the P< 0.01 level. A dash indicates no statistical significance (P> 0.05).

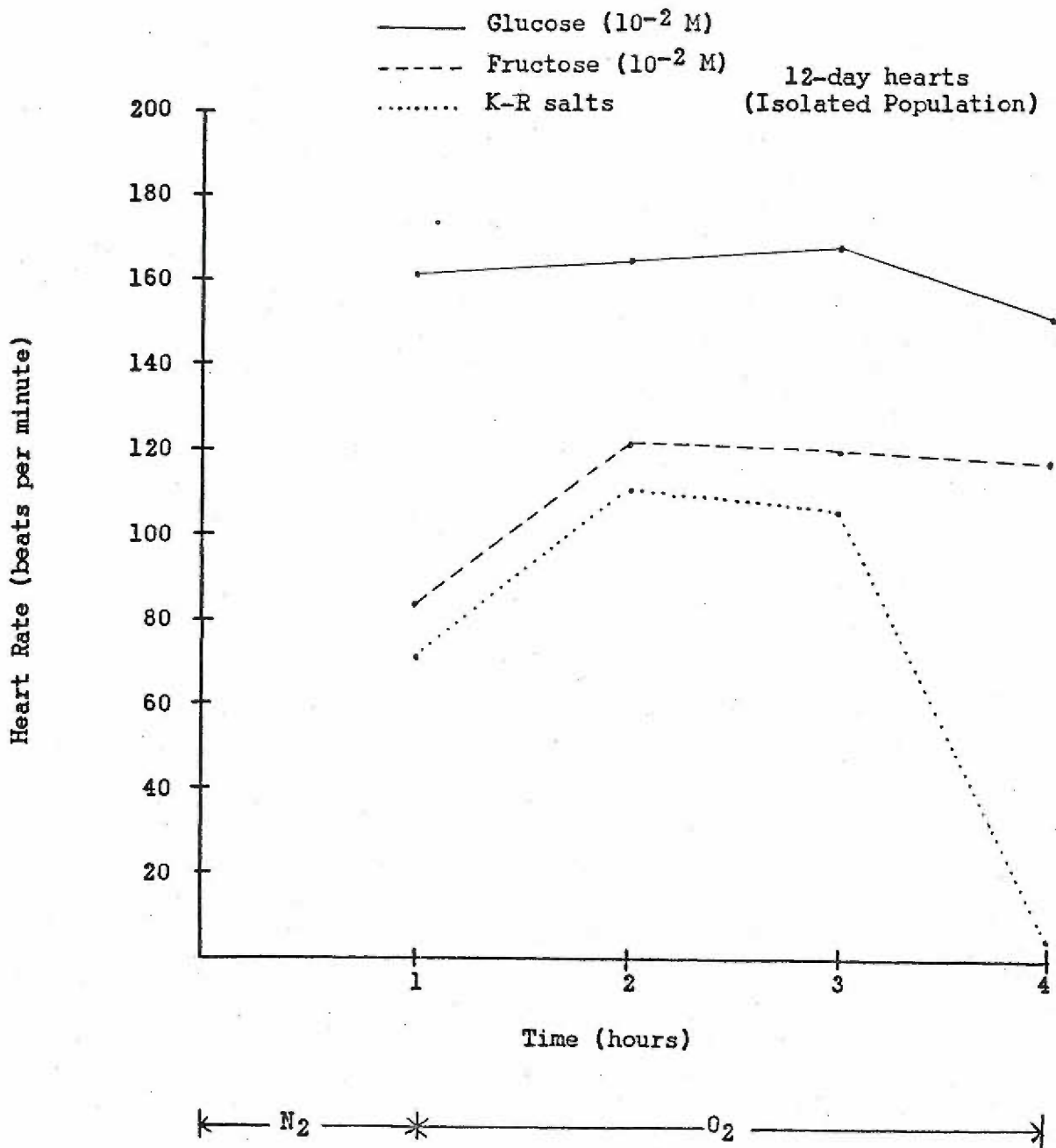| Comparison	Hr. 1	2	3	4
glu vs. salts	++	++	++	++
glu vs. fru	++	++	++	++
fru vs. salts	−	−	+	++

Figure 3

Table 4-1. Mean heart rate (in beats per minute) for glucose, fructose and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
glucose	12	3	169±15	160±18	156±22	153±17
fructose	12	3	85±10	180±20	158±15	160±14
salts	10	3	92±10	159±14	129±16	112±15

Table 4-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the P< 0.05 level. A double plus (++) indicates statistical significance at the P< 0.01 level. A dash indicates no statistical significance (P> 0.05).

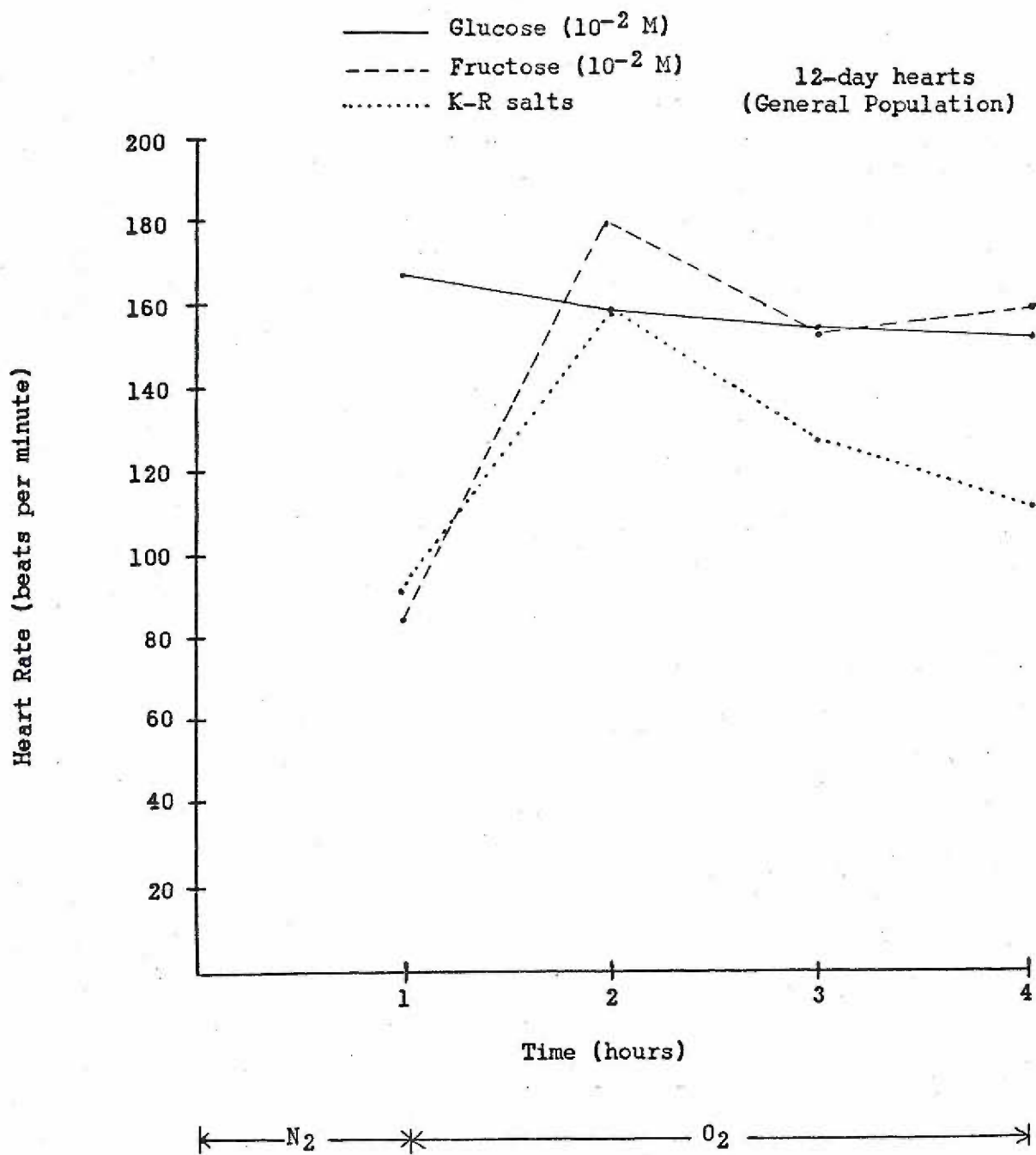| Comparison	Hr. 1	2	3	4
glu vs. salts	++	–	++	++
glu vs. fru	++	+	–	–
fru vs. salts	–	+	++	++

Figure 4

the general population it was noted that a marked difference existed
when the hearts were subjected to an anaerobic environment, but in the
presence of oxygen there proved to be no difference in utilization of
the two hexoses (Figure 4).

The results from this series of experiments showed that in both
populations fructose was not metabolized anaerobically. In the general
population, aerobic metabolism of fructose was equal to that of glucose.
In the isolated population, aerobic metabolism of fructose was found
to be markedly less than that for glucose.

  c. Glucose vs. Mannose vs. K-R salts (Figure 5 and tables)

A comparison of mannose with glucose showed that mannose could
be utilized anaerobically, but not as well as glucose (hours 1 and 2
in Figure 5). With the shift to an aerobic environment, the difference
in utilization between mannose and glucose disappeared.

  2. Aerobic Glucose Utilization vs. Anaerobic Glucose Utilization
   in Eleven-, Twelve-, and Thirteen-day Isolated Hearts (Figures
   6, 7 and 8 and tables)

Comparisons were made between mean heart rate in glucose and oxygen
and in glucose and nitrogen for three different ages of embryos:
eleven-, twelve-, and thirteen-days old.

For the eleven-day old embryo it was observed that the mean heart
rate attainable in glucose and nitrogen was approximately the same as
that attainable in glucose and oxygen (Figure 6). Any differences
between the groups were not significant.

The twelve-day old embryonic heart demonstrated a significant dif-
ference in heart rate attained in the two gas phases (Figure 7). The
glucose and oxygen group maintained a mean rate approximately thirty
points greater than the mean rate for the glucose and nitrogen group.

Table 5-1. Mean heart rate (in beats per minute) for glucose, mannose and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Hr. 1	2	3
glucose	12	3	154±10	143±12	146±19
mannose	12	3	115±7	102±9	151±19
salts	12	3	0	0	0

Table 5-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the P< 0.05 level. A double plus (++) indicates statistical significance at the P< 0.01 level. A dash indicates no statistical significance (P > 0.05).

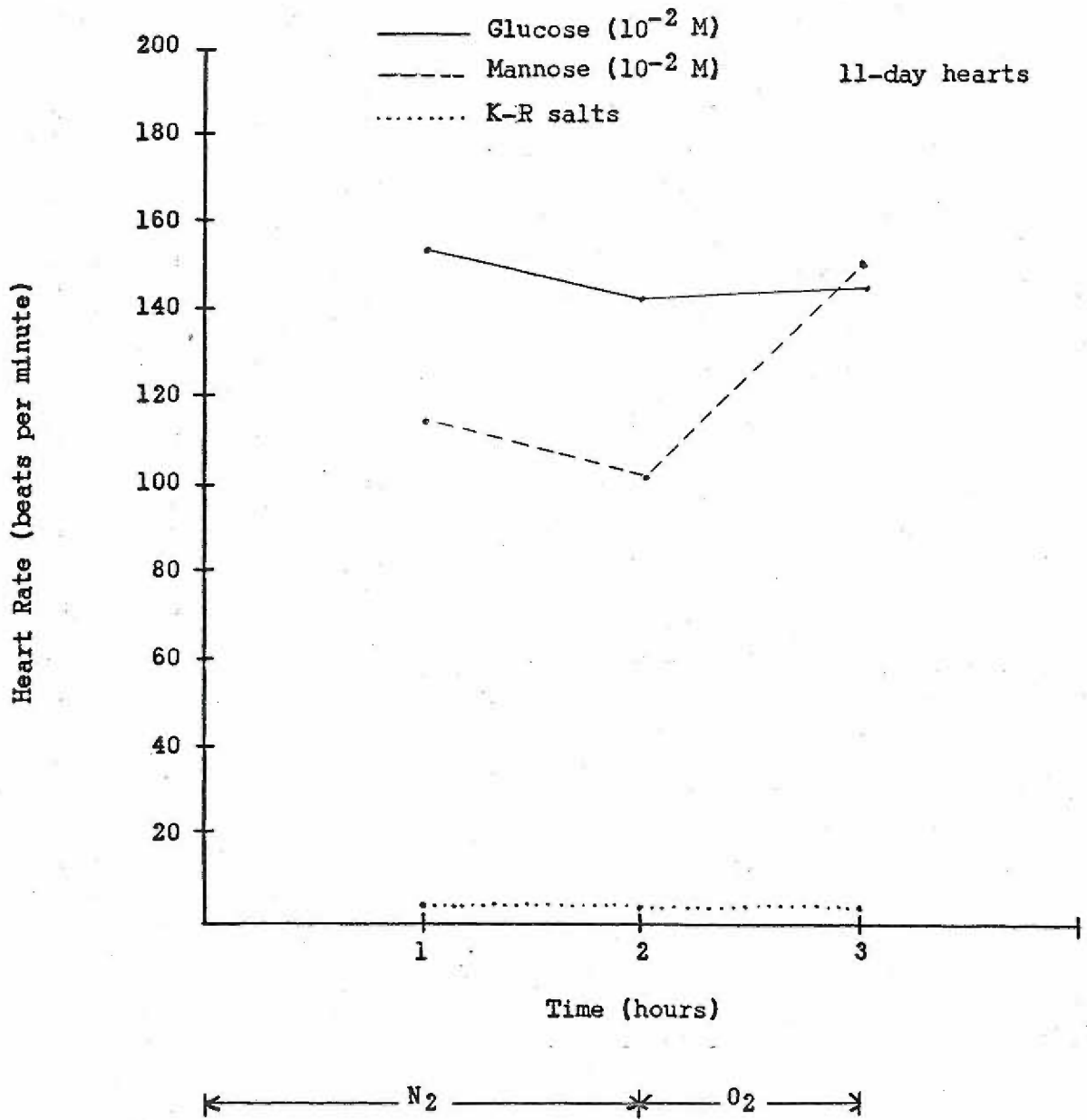| Comparison	Hr. 1	2	3
glu vs. salts	++	++	++
glu vs. man	++	++	-
man vs. salts	++	++	++

Figure 5

Table 6-1. Mean heart rate (in beats per minute) for glucose and oxygen (glu, O_2) and glucose and nitrogen (glu, N_2) at the different time periods. 11-day hearts.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
glu, O_2	10	2	148±15	146±16	137±12	136±14
glu, N_2	10	2	146±10	143±14	136±10	131±14

Table 7-1. Mean heart rate (in beats per minute) for glucose and oxygen (glu, O_2) and glucose and nitrogen (glu, N_2) at the different time periods. 12-day hearts.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
glu, O_2	11	3	200	193±9	194±6	185±7
glu, N_2	11	3	172±23	164±19	165±17	159±12

Table 8-1. Mean heart rate (in beats per minute) for glucose and oxygen (glu, O_2) and glucose and nitrogen (glu, N_2) at the different time periods. 13-day hearts.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
glu, O_2	7	2	192±10	186±7	180±20	170±12
glu, N_2	7	2	131±12	139±18	132±16	125±16

Tables 6-2, 7-2, 8-2 combined. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$).

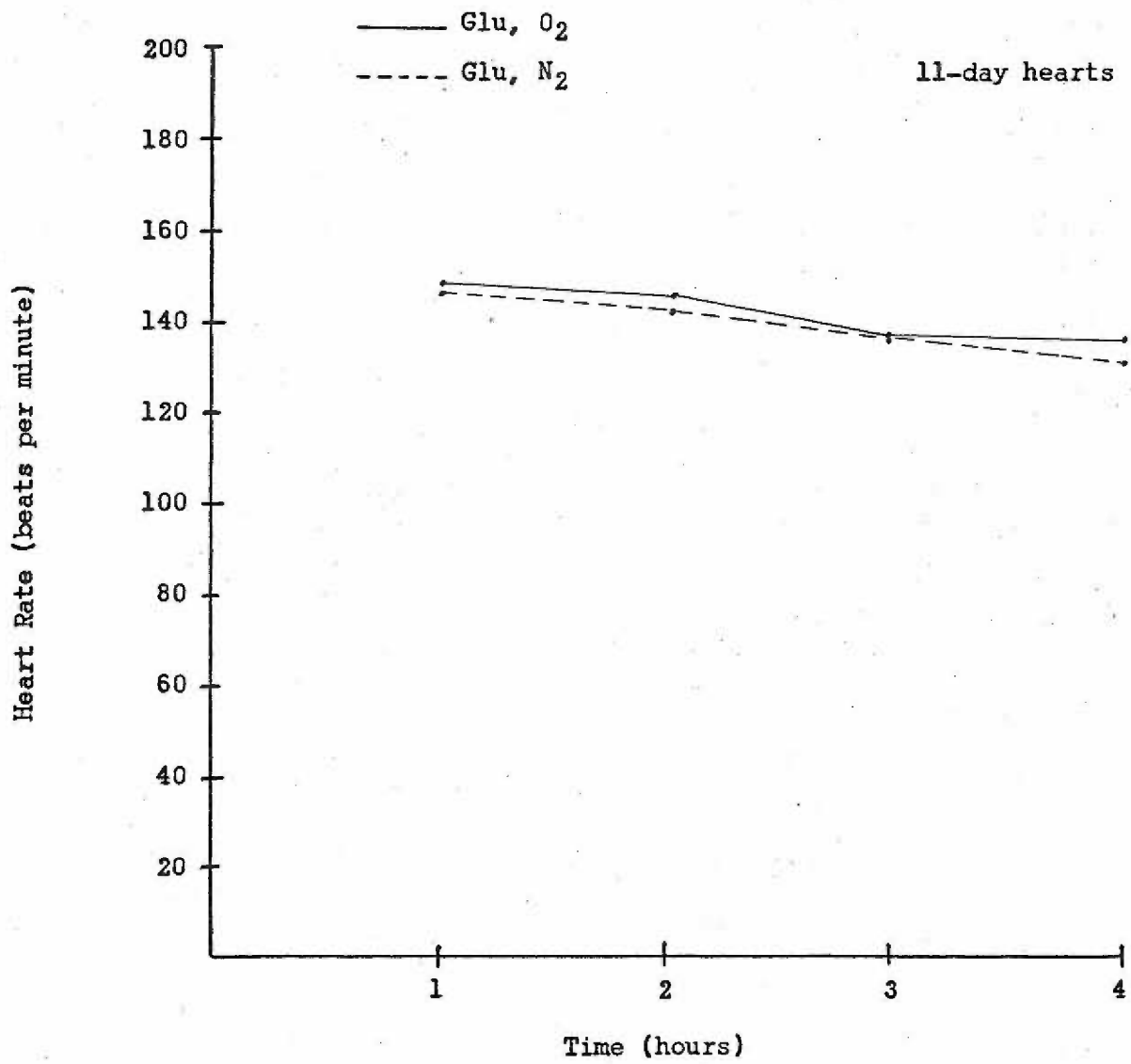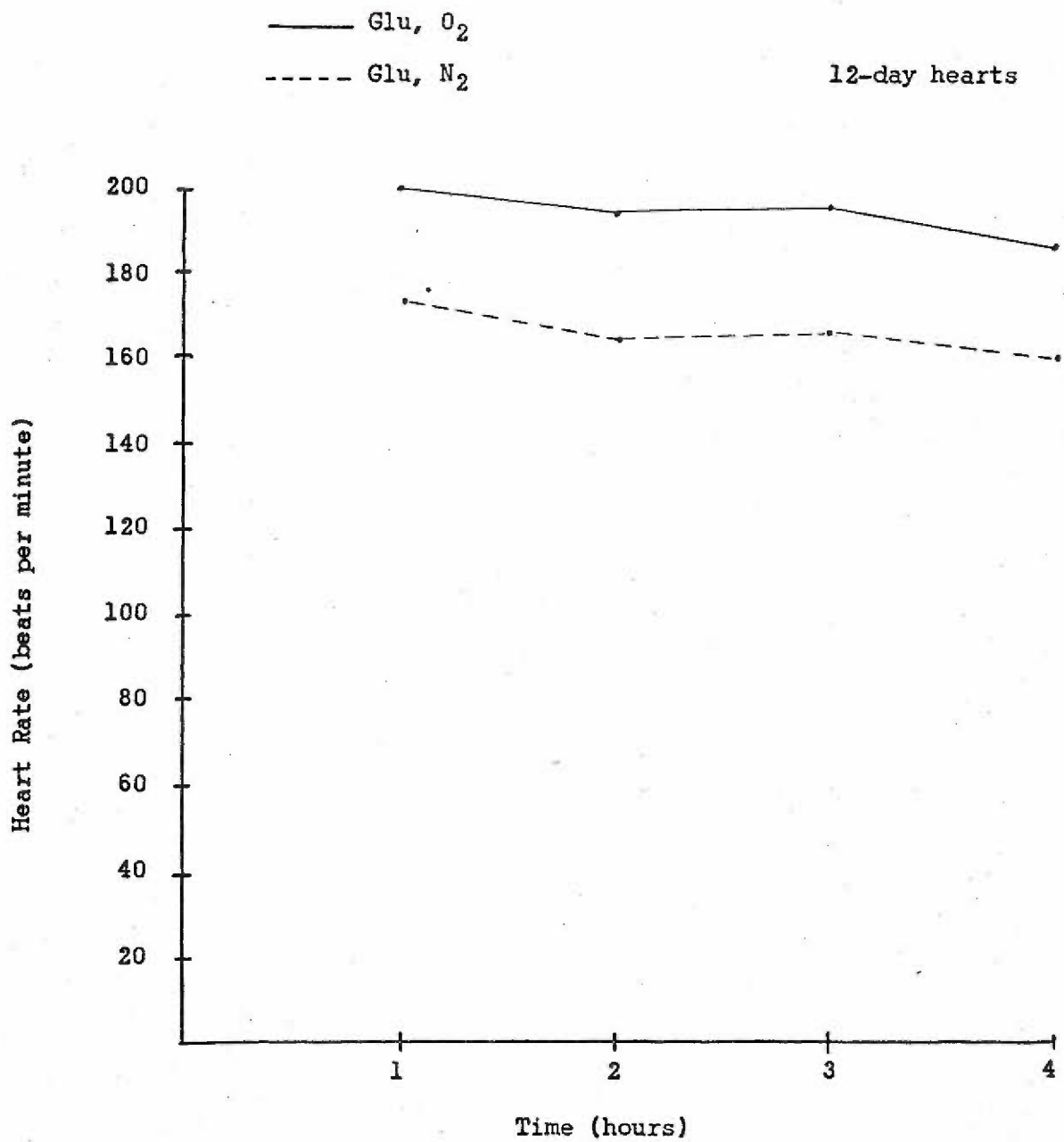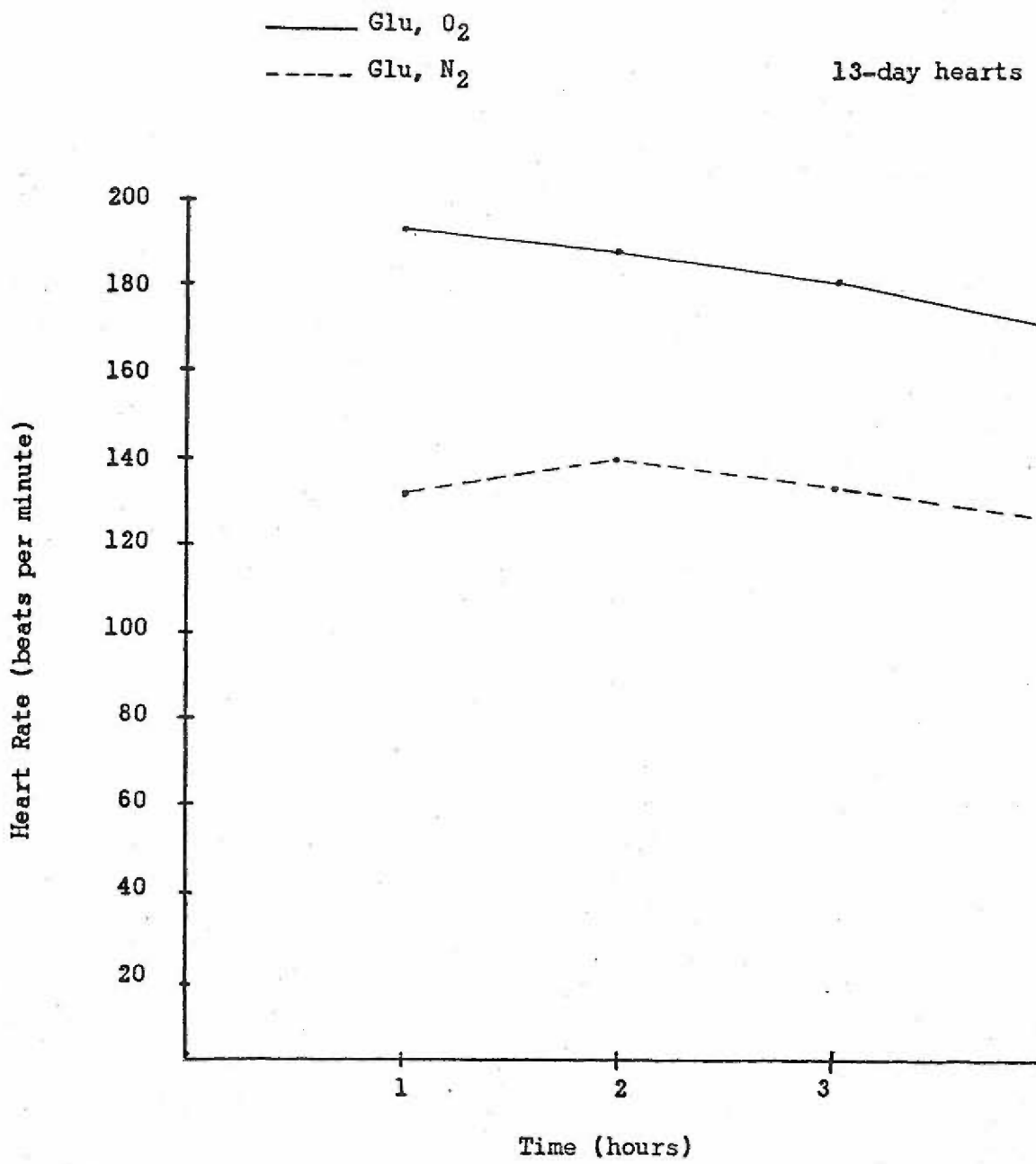| Comparison	Hr. 1	2	3	4
glu O_2 vs. N_2, 11-day	-	-	-	-
glu O_2 vs. N_2, 12-day	++	++	++	++
glu O_2 vs. N_2, 13-day	++	++	++	++

Figure 6

Figure 7

Figure 8

In the thirteen-day old embryo heart the difference in rate between the glucose and oxygen group and the glucose and nitrogen group was even more accentuated than the difference noted for the twelve-day hearts. The glucose and oxygen group maintained a mean rate approximately fifty points greater than the mean rate for the glucose and nitrogen group (Figure 8).

3. Phosphorylated Compounds

Three monophosphorylated hexoses (glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate) were tested under a variety of conditions and in a variety of concentrations. Since the data came from experiments that varied greatly in design, graphic presentation was not feasible. It was noted, nonetheless, that in six different experiments with twenty-eight isolated hearts from both eleven- and twelve-day embryos there was no apparent utilization of any of these three phosphorylated hexoses.

In contrast to this, it was found that the double phosphorylated hexose, fructose-1,6-diphosphate, was utilized as an energy source by eleven- and twelve-day hearts. Again this information came from a variety of differently designed experiments so graphic presentation was not attempted. However, the data in Figure 16 demonstrates that the addition of fructose-1,6-diphosphate (5×10^{-3} M) to trypan blue inhibited hearts, reverses the apparent metabolic block produced by that compound. It should be noted that $\propto$ -glycerophosphate in a concentration of 3×10^{-2} M was found to produce the same effects as fructose-1,6-diphosphate in similar preparations and, therefore, was also regarded as being utilized by the isolated hearts.

4. Ketone Bodies (Figure 9 and tables)

Utilization of the ketone bodies acetoacetate and β-hydroxybutyrate

Table 9-1. Mean heart rate (in beats per minute) for ketone bodies (acetoacetate and β-hydroxybutyrate) and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Hr. 1	2	3	4
				Mean ± standard deviation		
ketone bodies	11	3	89±19	160±14	157±12	135±15
salts	11	3	89±16	156±19	137±17	110±13

Table 9-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$).

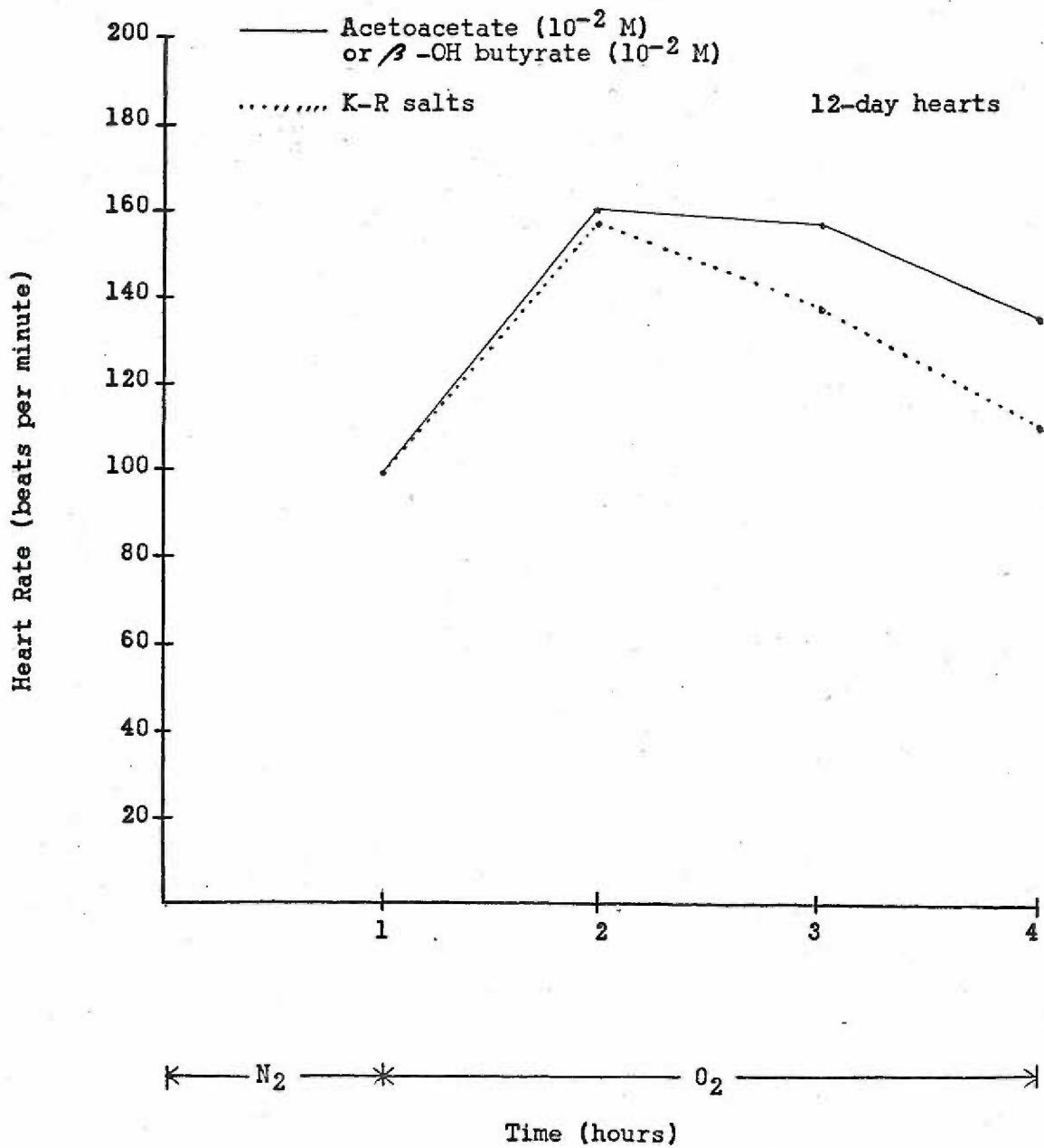| Comparison	Hr. 1	2	3	4
ketone vs. salts	−	−	+	++

Figure 9

was tested for in both eleven- and twelve-day embryo hearts. Eleven-day embryonic hearts were negative as far as ketone body utilization was concerned.

In twelve-day embryonic hearts, however, the results were positive. As might be expected, no utilization of ketone bodies under anaerobic conditions was observed (Figure 9). With the introduction of oxygen into the incubation chambers, a significant ($P< 0.01$) difference between the mean heart rate for the ketone body group and the control group arose by the end of the experimental period.

5. Amino Acids (Figures 10 and 11 and tables)

Three amino acids (L-alanine, hydroxy-L-proline, and L-valine) were tested for utilization by the embryonic hearts from eleven- and twelve-day embryos.

In the eleven-day embryonic hearts none of the three amino acids proved to be effective in maintaining contraction, either anaerobically or aerobically.

Twelve-day old hearts were unable to utilize any of the amino acids as a source of energy for contraction under anaerobic conditions. With oxygen, however, it was noted that both L-alanine (Figure 10) and hydroxy-L-proline (Figure 11) could be used by the twelve-day hearts. L-valine was not utilized by the twelve-day hearts as an energy source under either anaerobic or aerobic conditions.

6. TCA Intermediates and Pyruvate (Figures 12, 13 and 15 and tables)

Three intermediates of the tricarboxylic acid cycle were tested for an ability to maintain heart activity in the isolated hearts from eleven-, twelve-, and thirteen-day old embryos. The three intermediates tested were succinate, isocitrate and malate.

As was expected, none of the TCA intermediates were utilized by the

Table 10-1. Mean heart rate (in beats per minute) for L-alanine and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Mean ± standard deviation Hr. 1	2	3	4
L-alanine	9	2	91±9	187±18	151±14	136±19
salts	9	2	88±10	171±20	129±17	106±12

Table 10-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the P< 0.05 level. A double plus (++) indicates statistical significance at the P<0.01 level. A dash indicates no statistical significance (P> 0.05).

Comparison	Hr. 1	2	3	4
ala vs. salts	−	−	+	++

Figure 10

Table 11-1. Mean heart rate (in beats per minute) for hydroxy-L-proline and K-R salts control at the different time periods.

Substrate	No. in group	No. of litters	Mean $\pm$ standard deviation			
			Hr. 1	2	3	4
OH-pro	10	3	91$\pm$10	178$\pm$17	164$\pm$15	144$\pm$7
salts	10	3	90$\pm$17	150$\pm$15	127$\pm$16	108$\pm$14

Table 11-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$).

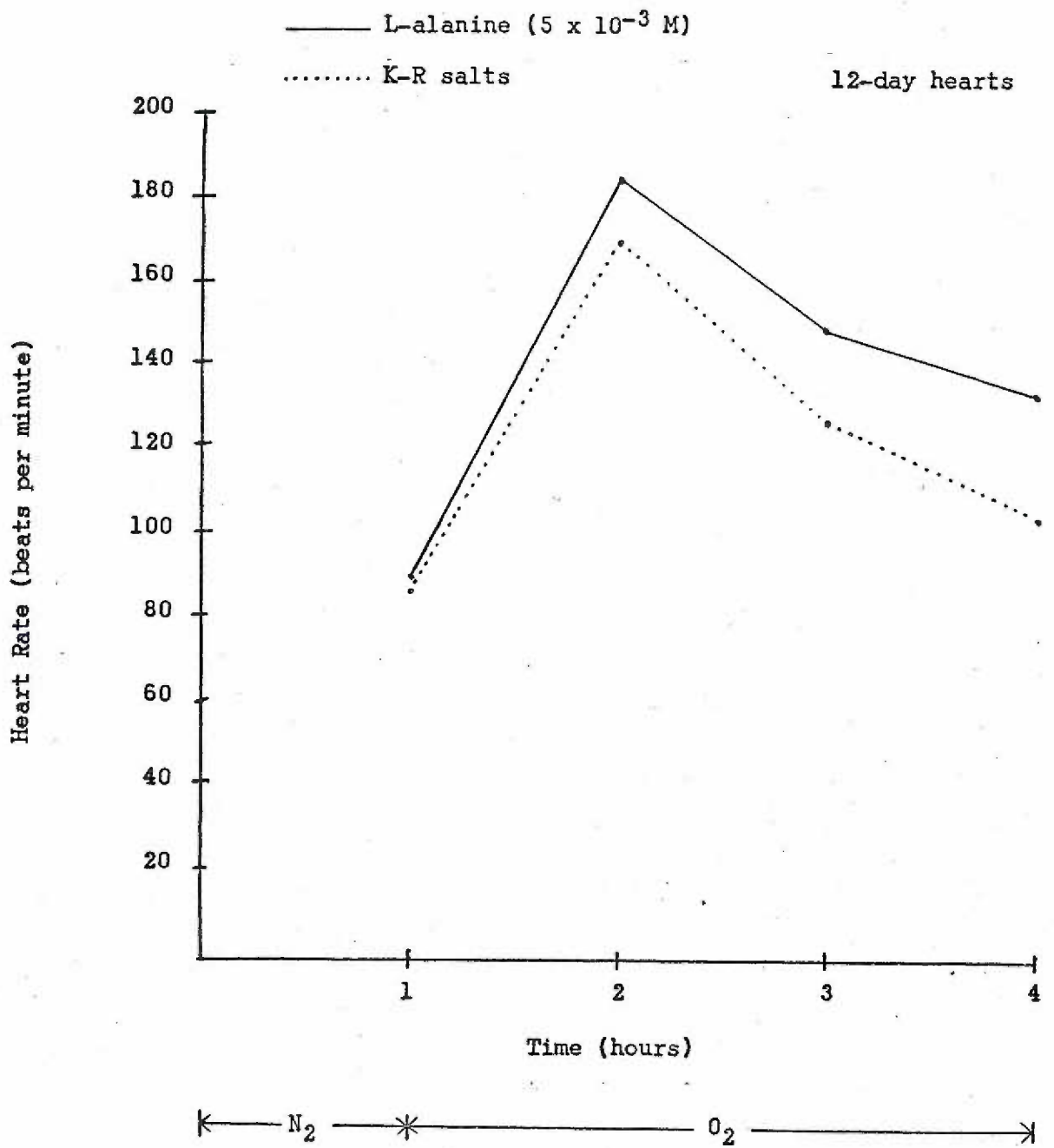| Comparison	Hr. 1	2	3	4
OH-pro vs. salts	–	++	++	++

Figure 11

Table 12-1. Mean heart rate (in beats per minute) for isocitrate and K-R salts at the different time periods.

Substrate	No. in group	No. of litters	Mean ± standard deviation Hr. 1	2	3	4
isocitrate	11	3	82±15	168±12	150±8	121±12
salts	11	3	89±16	156±19	137±17	110±13

Table 12-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$).

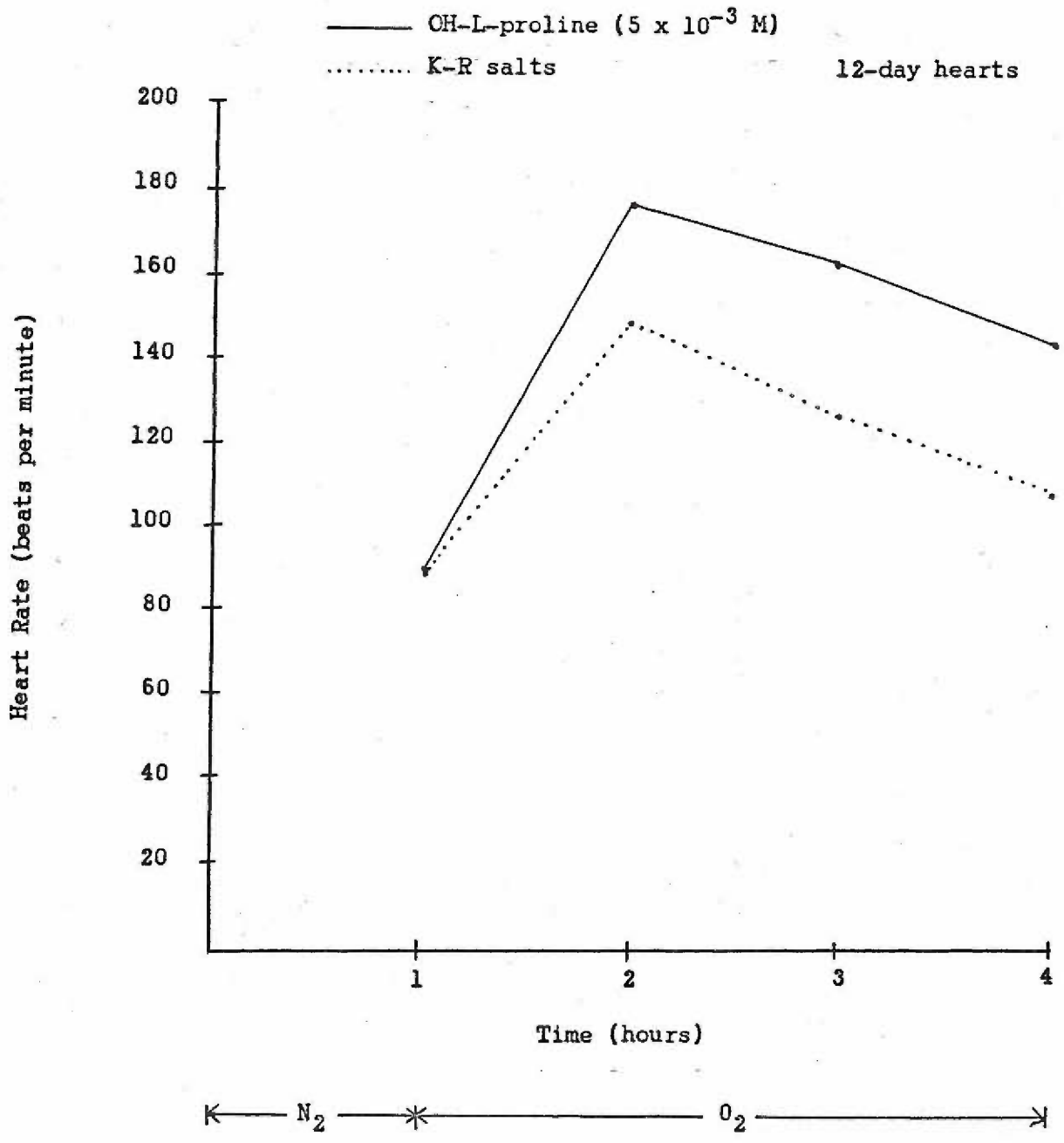| Comparison	Hr. 1	2	3	4
isocit vs. salts	–	–	+	–
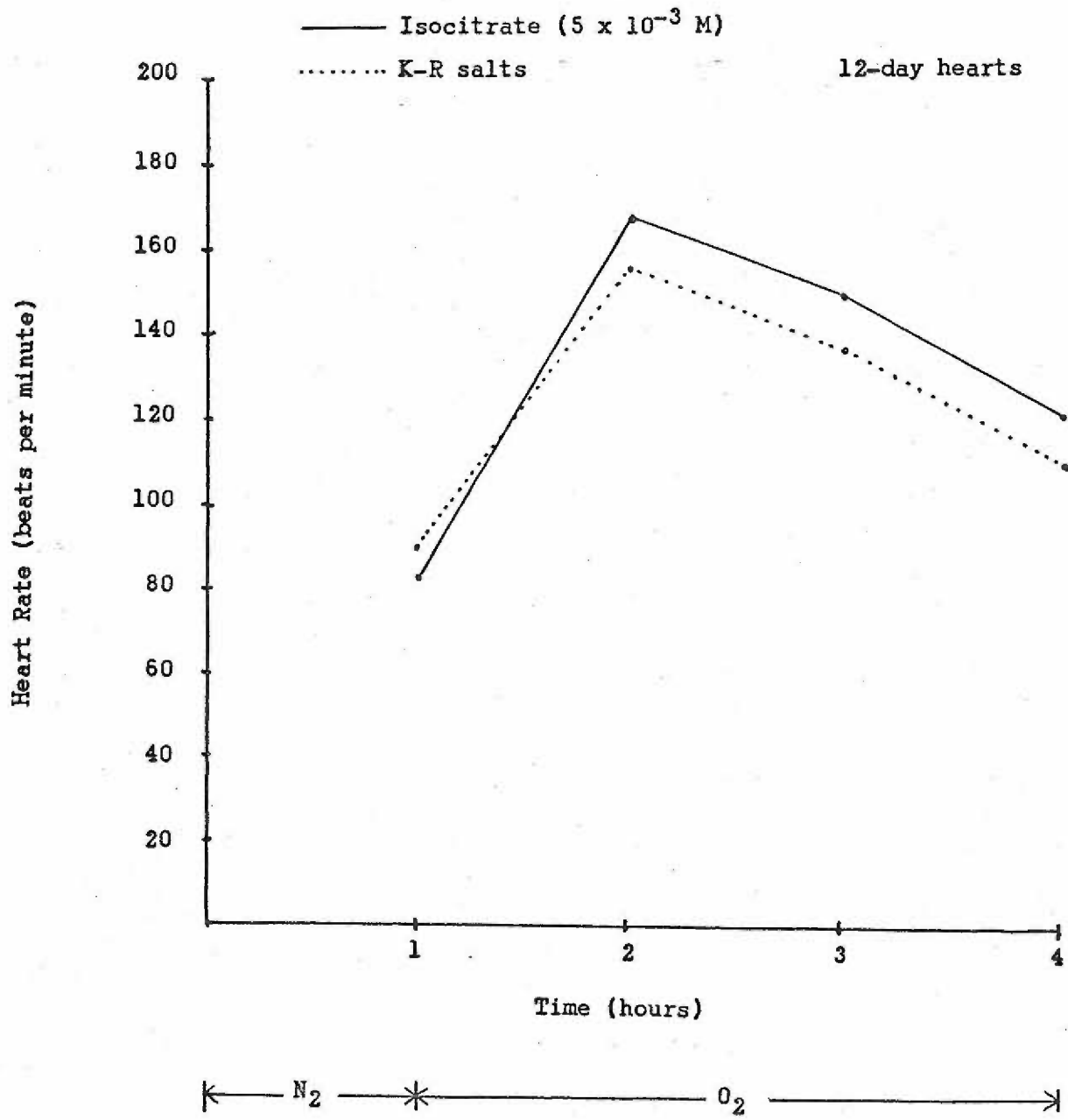
Figure 12

Table 13-1. Mean heart rate (in beats per minute) for succinate
and K-R salts at the different time periods.

Substrate	No. in group	No. of litters	Mean $\pm$ standard deviation			
			Hr. 1	2	3	4
succinate	10	3	81	172$\pm$16	155$\pm$18	122$\pm$11
K-R salts	10	3	90$\pm$17	150$\pm$15	127$\pm$16	108$\pm$12

Table 13-2. Statistical analysis of differences between comparison
groups. One plus (+) indicates a statistically significant dif-
ference at the $P < 0.05$ level. A double plus (++) indicates statis-
tical significance at the $P < 0.01$ level. A dash indicates no
statistical significance ($P > 0.05$).

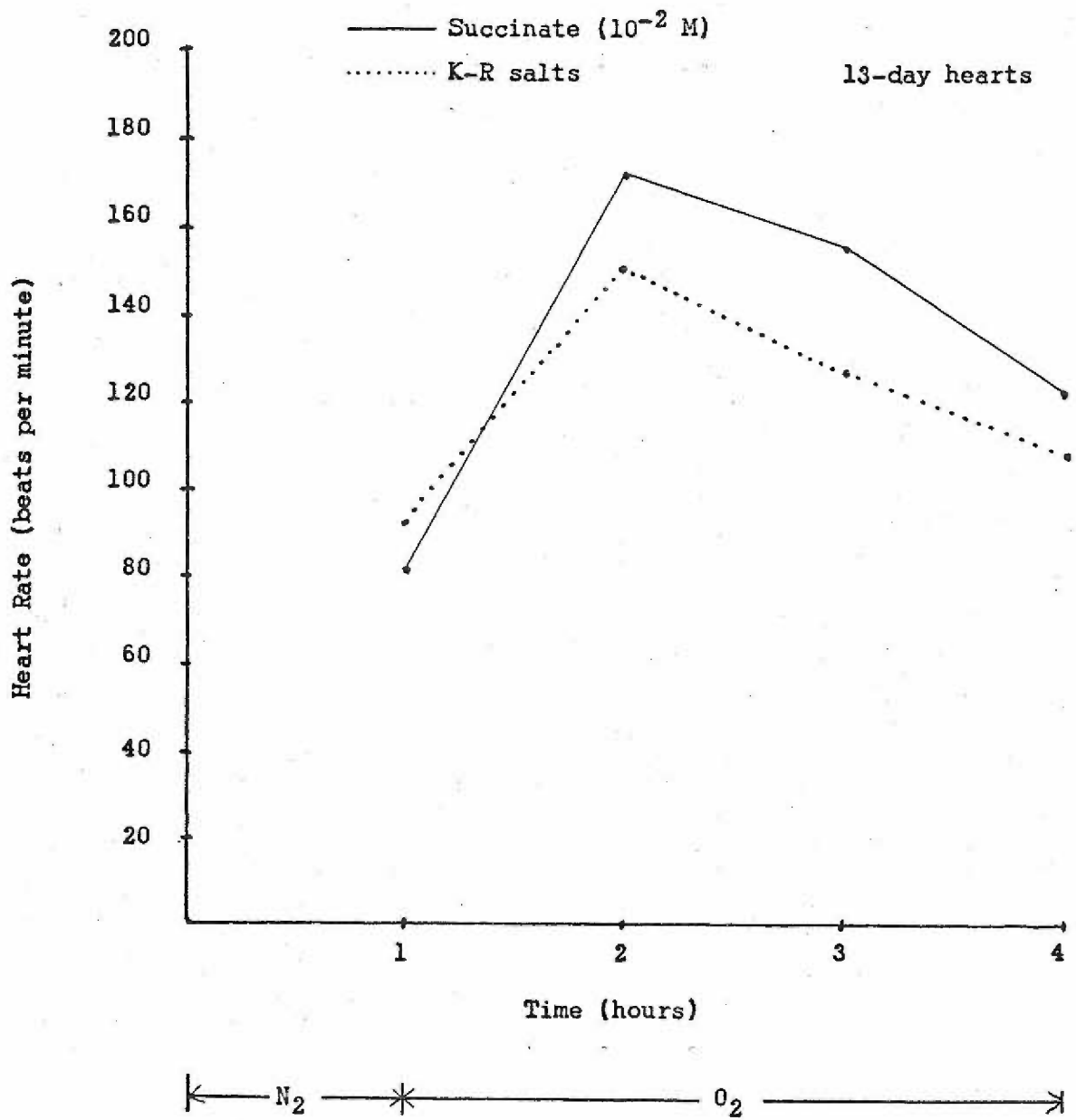| Comparison	Hr. 1	2	3	4
succinate vs. salts	–	++	++	+

Figure 13

hearts as a source of energy for contraction under anaerobic conditions. In addition, it was noted that the eleven-day hearts could not utilize any of the three intermediates provided them in oxygen.

Aerobically, the twelve-day old hearts were negative for succinate and malate at all concentrations attempted (5×10^{-4} M through 1×10^{-2} M). Isocitrate, at a concentration of 5×10^{-3} M, may or may not have been utilized. It was observed (Figure 12) that after one hour in nitrogen and two hours in oxygen the difference between the isocitrate group and the control group was significant ($P < 0.05$). One hour later, however, the difference was no longer observable. This was rather unusual in that ordinarily the difference between a positive substrate group and the control group increased with time. For this reason the interpretation of whether or not isocitrate was utilized was left open.

The thirteen-day old hearts were negative for isocitrate and malate but positive for succinate at a concentration of 10^{-2} M (Figure 13).

Pyruvate, although not an intermediate in the TCA cycle, is known to be an important precursor to cycle intermediates. It was found that under aerobic, but not anaerobic, conditions pyruvate was utilized as an excellent source of energy in hearts from both eleven-day and twelve-day embryos. Figure 15, A and B, shows the reversal of iodoacetic acid inhibition in eleven-day and twelve-day hearts. Additional information from a variety of other experiments also indicated pyruvate utilization by hearts of this same age.

7. Summary of Substrate Utilization

The substrates tested were graded on their ability to provide energy for contraction. A four plus (++++) system was chosen whereby the substrate which was capable of maintaining the highest contraction

rate for that age was accorded the four plus value. The other substrates were ranked in comparison. A negative (–) value indicates non-utilization by the hearts. A question mark (?) indicates a question as to interpretation of results. A cross (X) indicates insufficient data on which to make a judgement.

Substrate	11-day		12-day		13-day	
	N_2	O_2	N_2	O_2	N_2	O_2
glucose	++++	++++	+++	++++	++	++++
mannose	++	++++	++	++++	X	X
galactose	+	++	+	++	X	X
fructose 1	–	++	–	++		
fructose 2	–	++++	–	++++	X	X
glu-1-PO$_4$	–	–	–	–	X	X
glu-6-PO$_4$	–	–	–	–	X	X
fru-6-PO$_4$	–	–	–	–	X	X
fru-1,6-diPO$_4$	++++	++++	+++	++++	X	X
α-glucero-PO$_4$	++++	++++	+++	++++	X	X
acetoacetate	–	–	–	+++	–	++
β-OH butyrate	–	–	–	+++	–	++
L-valine	–	–	–	–	X	X
L-alanine	–	–	–	+++	X	X
OH-L-proline	–	–	–	+++	X	X
succinate	–	–	–	–	–	+++
isocitrate	–	–	–	?	–	–
malate	–	–	–	–	–	–
pyruvate	–	+++	–	+++	X	X

fructose 1 = those litters from females bred with male #1 (isolated population)
fructose 2 = those litters from females bred with a variety of males (general population)

B. Oxygen Consumption

The measurement of oxygen consumption in isolated eleven-, and twelve-day old embryonic hearts was carried out as described under Materials and Methods. The mean heart rate of each group of hearts placed in the chamber for oxygen consumption measurements was recorded before and after measurements were taken.

In the following table the first column represents mean heart rate of the groups of hearts before measurements of oxygen consumption were made. The substrate column indicates the exogenous source of energy (if any) that was available to the hearts during measurements. $\dot{V}O_2$ is oxygen consumption in μLO_2 consumed per hour per milligram dry weight. The number of hearts in the electrode chamber during measurements is recorded in the next to last column.

Heart Rate (bpm)	Substrate	$\dot{V}O_2$	No. of Hearts	Age of Embryo		
177± 7	Glucose (10^{-2} M)	6.73	4	twelve-days old		
169±16	"	6.50	4	"	"	"
144±27	"	6.00	4	"	"	"
116±15	"	4.95	5	"	"	"
0	Glucose (10^{-2} M) + I.A.A. (3×10^{-5} M)	0	4	"	"	"
155±11	K-R salts	4.66	4	"	"	"
138±24	"	4.28	4	"	"	"
111±21	"	4.10	4	"	"	"
140± 7	Glucose (10^{-2} M)	6.67	3	eleven days old		
135±13	"	6.53	5	"	"	"
139± 7	Fructose (10^{-2} M)	6.76	3	"	"	"
167± 5	K-R salts	5.27	3	"	"	"
142± 4	"	4.83	3	"	"	"
117± 8	"	4.31	4	"	"	"

Because of the variations in heart activity it was difficult to cross-compare the oxygen consumption data determined for the different aged hearts. Certain observations were made, however, which are as

as follows: (1) for each age embryo the amount of oxygen consumed varied directly with the mean heart rate of that group; (2) the cessation of cardiac contraction induced by poisoning with iodoacetic acid led to a cessation of oxygen consumption by those hearts; (3) the oxygen consumption in eleven-day old hearts was greater than that for twelve-day old hearts although the mean heart rates were lower.

C. Metabolic Inhibitors

Several known metabolic inhibitors were used in order to determine what effect, if any, inhibition of specific metabolic processes had on heart rate. One inhibitor of oxidative metabolism (nitrogen) has already been presented and will not be presented again. The other inhibitors studied and the concentrations employed are as follows: (1) an inhibitor of glycolysis, 3×10^{-5} M Iodoacetic Acid (I.A.A.); (2) an inhibitor of the TCA cycle, 1.5×10^{-2} M malonate; (3) an uncoupler of oxidative phosphorylation, 1×10^{-4} M 2,4-dinitrophenol (2,4 DNP); (4) an inhibitor with an uncertain site of action, 1×10^{-5} M trypan blue.

1. Inhibition of Glycolysis vs. Inhibition of TCA Cycle, Eleven-day vs. Twelve-day (Figure 14, A and B, and tables)

A comparison was made between heart rate in eleven-day and twelve-day old hearts after the administration of iodoacetate (3×10^{-5} M) and malonate (1.5×10^{-2} M). Three groups of hearts were first allowed to equilibrate in K-R salts for one hour prior to addition of one of the inhibitors to two of the chambers. The third group remained uninhibited and served as the control.

It was noted that in both ages addition of iodoacetate resulted in complete depression of heart activity after two hours. This depression was reversed by the addition of pyruvate (1×10^{-2} M) to the medium.

The addition of malonate had no apparent effect on heart rate in the hearts from eleven-day old embryos (Figure 14, A), whereas malonate significantly depressed heart activity in the hearts from twelve-day old embryos (Figure 14, B).

2. An Uncoupler of Oxidative Phosphorylation (Figure 15, A and B and tables)

Table 14, A-1. Mean heart rate (in beats per minute) for K-R salts, salts plus iodoacetate (I.A.A.) and salts plus malonate at the different time periods. 11-day hearts.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
I.A.A.	11	3	152±15	107±12	0	143±17
malonate	11	3	155±16	149±13	137±16	122±10
K-R salts	11	3	162±12	149±15	137±15	126±18

Table 14, B-1. Mean heart rate (in beats per minute) for K-R salts, salts plus iodoacetate (I.A.A.) and salts plus malonate at the different time periods. 12-day hearts.

Substrate	No. in group	No. of litters	Mean ± standard deviation			
			Hr. 1	2	3	4
I.A.A.	10	3	197±6	119±18	0	119±27
malonate	10	3	197±5	157±19	120±18	114±15
K-R salts	9	3	199±2	187±16	161±16	150±21

Table 14, A-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$). 11-day hearts.

Comparison	Hr. 1	2	3	4
I.A.A. vs. salts	−	++	++	−
malonate vs. salts	−	−	−	−
I.A.A. vs. malonate	−	++	++	+

Table 14, B-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$). 12-day hearts.

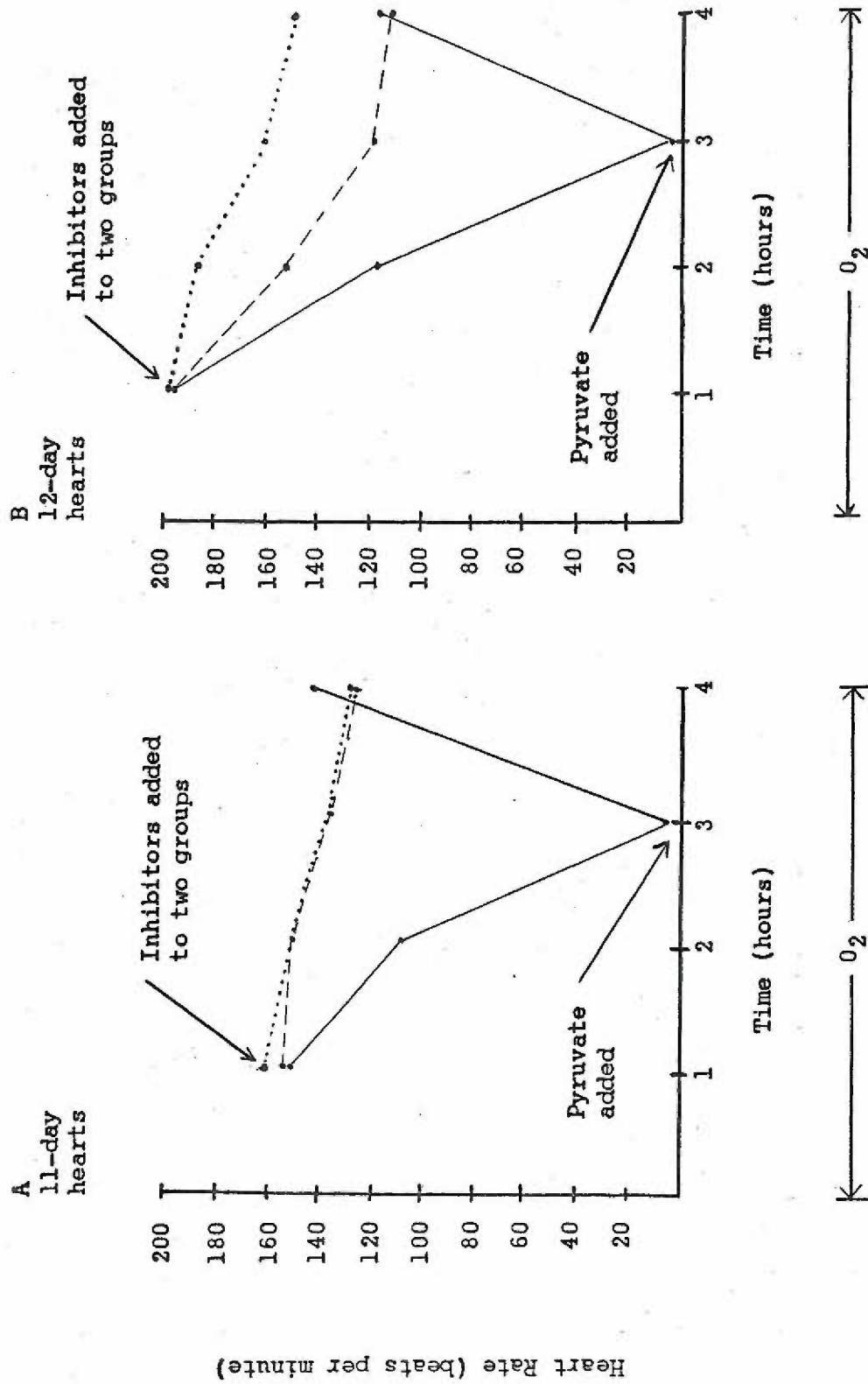| Comparison	Hr. 1	2	3	4
I.A.A. vs. salts	−	++	++	+
malonate vs. salts	−	+	++	++
I.A.A. vs. malonate	−	++	++	−

Figure 14

Table 15, A-1. Mean heart rate (in beats per minute) for a glucose solution plus 2,4-DNP and either oxygen (glu, O_2) or nitrogen (glu, N_2) at the different time periods. 11-day hearts.

Substrate	No. in group	No. of litters	Mean $\pm$ standard deviation			
			Hr. 1	2	3	4
glu, O_2	12	2	150$\pm$18	106$\pm$19	93$\pm$13	91$\pm$10
glu, N_2	11	2	153$\pm$18	107$\pm$13	87$\pm$9	90$\pm$14

Table 15, B-1. Mean heart rate (in beats per minute) for a glucose solution plus 2,4-DNP and either oxygen (glu, O_2) or nitrogen (glu, N_2) at the different time periods. 12-day hearts.

Substrate	No. in group	No. of litters	Mean $\pm$ standard deviation				
			Hr. 1	$1\frac{1}{2}$	2	3	4
glu, O_2	11	2	170$\pm$12	0	99$\pm$17	115$\pm$17	104$\pm$14
glu, N_2	11	2	180$\pm$21	0	0	0	0

Table 15, A-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$). 11-day hearts.

Comparison	Hr. 1	2	3	4
glu, O_2 vs. glu, N_2	−	−	−	−

Table 15, B-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$). 12-day hearts.

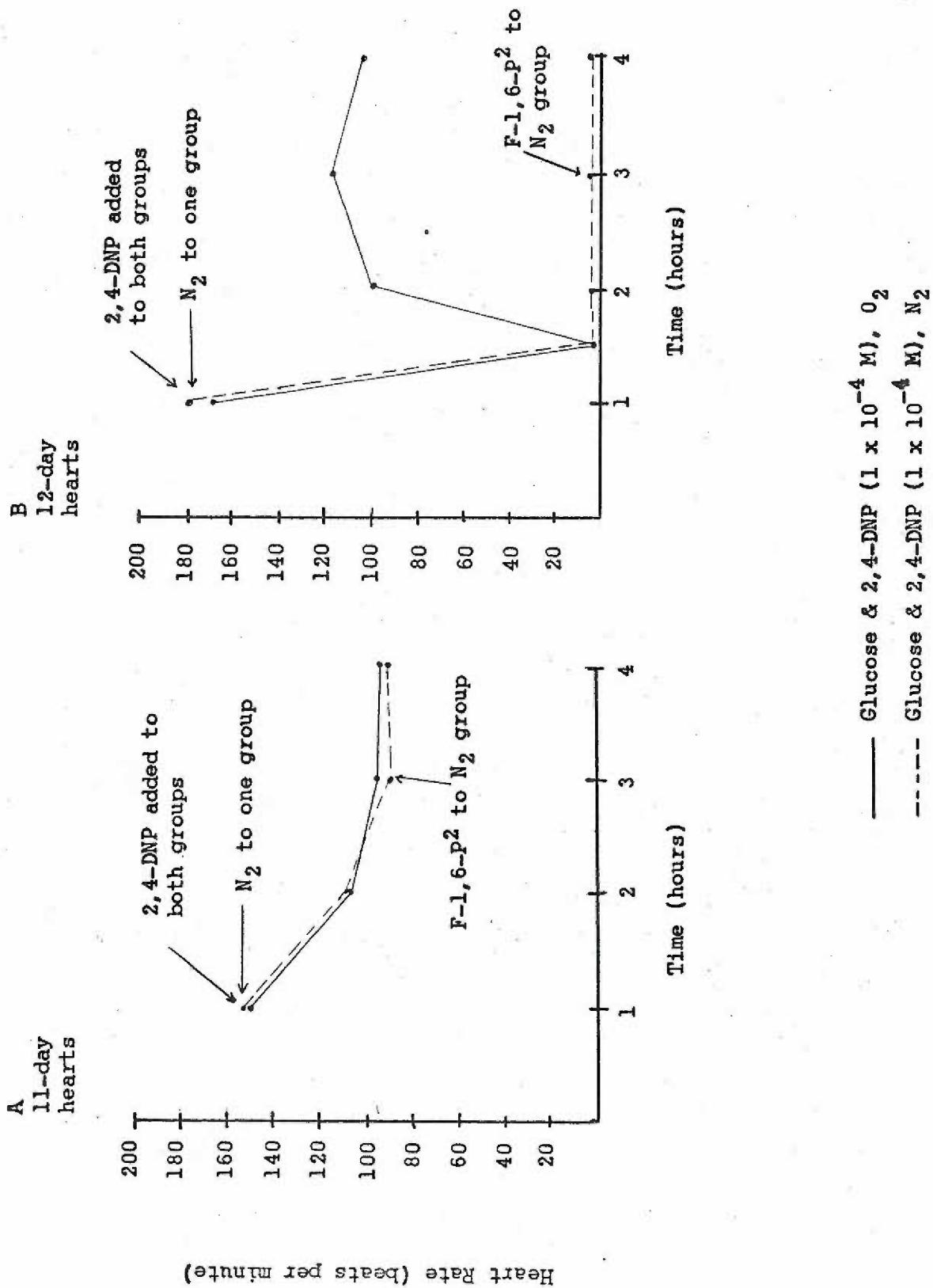| Comparison	Hr. 1	$1\frac{1}{2}$	2	3	4
glu, O_2 vs. glu, N_2	−	−	++	++	++

Figure 15

A comparison between eleven- and twelve-day old hearts after the addition of an uncoupler of oxidative phosphorylation, 2,4-DNP (1×10^{-4} M), was made. Two groups of hearts were allowed to equilibrate for one hour in an oxygenated glucose solution. The inhibitor was added to both groups after this time. One of the groups of hearts remained in oxygen while the other group was switched to an anaerobic environment. After two hours (hour-three) of anaerobiosis, fructose-1,6-diphosphate (5×10^{-3} M) was added to the medium.

The eleven-day old hearts showed a depression of heart rate after addition of 2,4-DNP (Figure 15, A). No difference between the aerobic and anaerobic group was noted. Neither group had a fall in heart rate to zero over the four hour period.

In contrast, the twelve-day hearts showed a complete cessation of heart rate in less than thirty minutes after the addition of 2,4-DNP (Figure 15, B). The anaerobic group remained at zero for the full course of the experiment. The aerobic group showed a partial recovery of function after one hour to approximately 50% initial rate.

The addition of fructose-1,6-diphosphate to the anaerobic group did not improve the heart rate for either age.

3. Trypan Blue as a Metabolic Inhibitor (Figure 16 and tables)

Two groups of eleven-day old embryonic hearts were allowed to equilibrate in an oxygenated glucose solution for one hour. One of the groups also contained the dye trypan blue at a concentration of 1×10^{-5} M. After the initial hour in oxygen, the gas phase was changed to nitrogen for both groups for the remainder of the experiment.

It was observed that after one hour in oxygen no difference in mean heart rate existed between the two groups. After changing to an anaerobic environment, however, a marked separation between the two

groups was observed with the trypan blue group being the lower of the
two (Figure 16).

After two hours in nitrogen (hour-three) fructose-1,6-diphosphate
(5×10^{-3} M) was added to both groups. A recovery in heart rate of the
trypan blue group to within the range of that for the control group
was noticed. It was observed in other experiments that without the
addition of fructose-1,6-diphosphate the heart rate in the trypan blue
group continued to decline and did not recover spontaneously. It
should be also noted that α-glycerophosphate in a concentration of
3×10^{-2} M had the same effect as fructose-1,6-diphosphate in reversing
the trypan blue block. The same pattern (with different heart rate
values) was observed for twelve-day old hearts under similar conditions.

4. Inter-group Comparison of No Substrate Controls

A comparison of the results obtained from the no-substrate control
groups led to the observation that for most groups of controls the mean
heart rates during the first hour (anaerobic environment) was approx-
imately one-half to two-thirds the value of the experimental. With
the introduction of oxygen the mean rates rose and then tapered off
gradually with time. This was not the case for the galactose or man-
nose controls. The galactose control group was at zero in nitrogen,
recovered during the first hour in oxygen and then fell off to zero by
hour-three. The mannose control group was run for two hours in nitrogen
and then exposed to oxygen. The heart rates remained at zero throughout
the entire time period.

Table 16-1. Mean heart rate (in beats per minute) for a glucose control group (glu) and a group with glucose plus trypan blue (glu+t.b.) at the different time periods.

Substrate	No. in group	No. of litters	Mean $\pm$ standard deviation Hr. 1	2	3	4
glu	12	3	165$\pm$19	153$\pm$18	141$\pm$16	151$\pm$12
glu+t.b.	12	3	161$\pm$21	118$\pm$20	113$\pm$15	144$\pm$11

Table 16-2. Statistical analysis of differences between comparison groups. One plus (+) indicates a statistically significant difference at the $P < 0.05$ level. A double plus (++) indicates statistical significance at the $P < 0.01$ level. A dash indicates no statistical significance ($P > 0.05$).

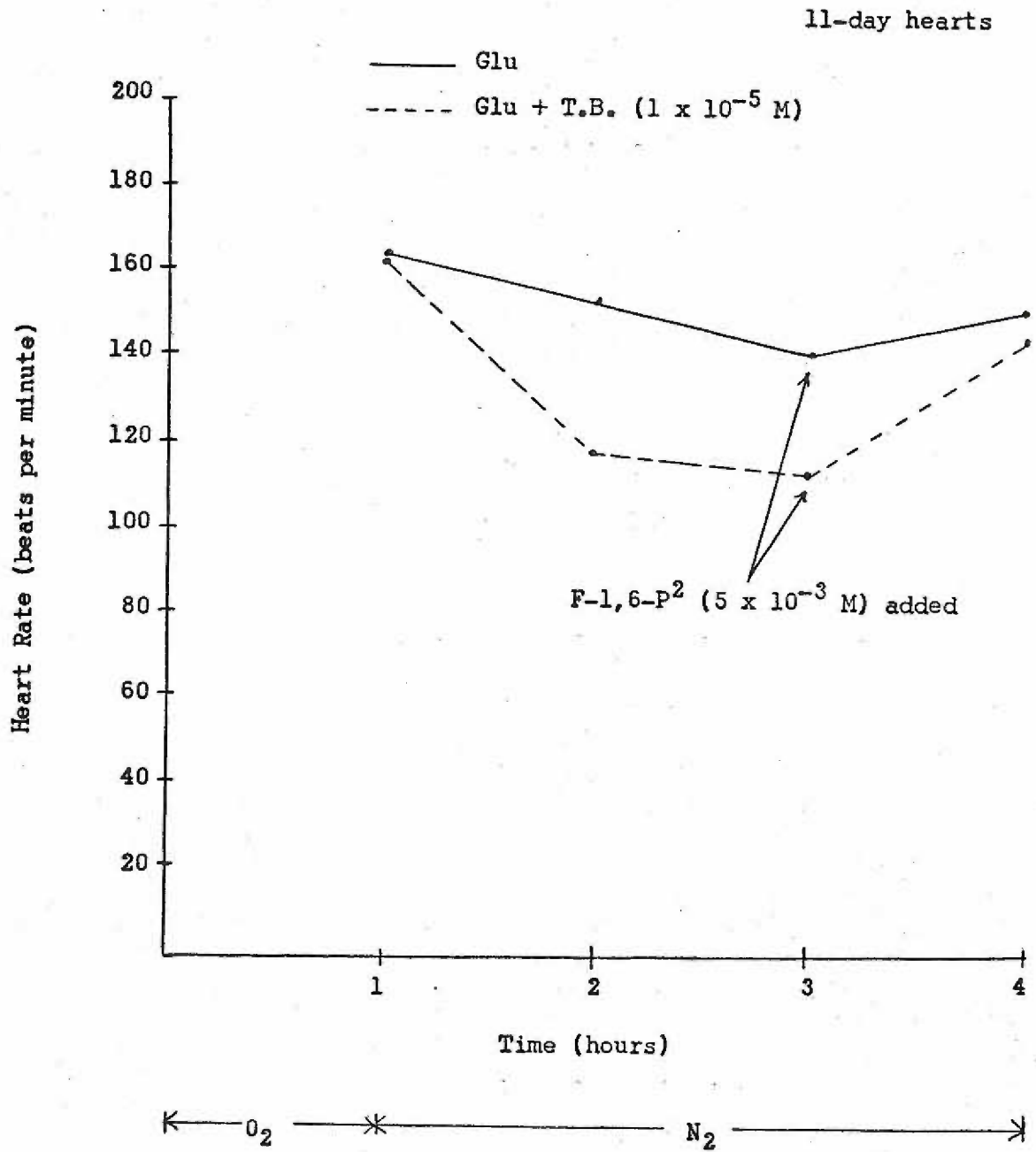| Comparison	Hr. 1	2	3	4
glu vs. glu+t.b.	–	++	++	–

Figure 16

D. Selection of Substrate, Oxygen and Metabolic Inhibitor Concentrations

Before discussing the results obtained in these investigations, it should be noted that preliminary studies were conducted to determine optimal concentrations of substrate, oxygen and metabolic inhibitors. The initial concentrations for the substrate studies were taken from reports in the literature dealing with similar preparations. These concentrations were then decreased until a minimum concentration was reached which still was capable of supporting maximum heart rate. Concentrations were also increased beyond initial dose levels in an attempt to extend the maximum rate. An increase in substrate concentration had no effect on heart rate within the range of the substrates tested with the exception of isocitrate. Isocitrate demonstrated a decrease in heart rate with concentrations greater than 5×10^{-3} M.

Preliminary studies were also conducted to determine the effect of oxygen concentration on heart rate. No differences in rates were noted among the three oxygen concentrations tested, 20%, 60% and 95%. The decision to employ 60% oxygen was made arbitrarily.

In selecting the optimal concentrations of the metabolic inhibitors employed in these experiments, the starting point was again taken from concentrations suggested in published reports. None of these concentrations were exceeded and all dosages reported in these investigations were lower than values reported for other studies. This was done in an attempt to further avoid the possibility of inhibition of pathways other than the one being tested.

DISCUSSION

These investigations have dealt with the embryonic heart as an entire organ. Heart rate was obtained by visually counting contractions of the tissue at a point distal to the anatomical location of the sinus venosus. Changes in heart rate observed may, therefore, represent the effects of an experimental regimen on that cell or cells responsible for generating the embryonic heart beat or upon the conducting tissue between the origin of impulse generation and that area contracting (contractile proteins). It is recognized that microelectrode data would be required in order to ascertain the specific anatomical site(s) of substrate and inhibitor action. Therefore, the site of action responsible for the negative chronotropic effects observed in those preparations subjected to the absence of substrate, to metabolic poisons or to noneffective metabolites cannot be deduced from the experiments presented. Although it can be postulated that a reduced heart rate is a measure of the functional activity of a particular pacemaker cell(s), it seems inappropriate at this time to eliminate the possibility that other components of cardiac function might not be involved. Consequently, the following discussion pertains to the metabolic pathways which appear essential for the maintenance of maximum heart rate in isolated embryonic rat hearts only insofar as visible contraction of the whole organ is concerned and it is not the intent of the author to delineate the cell or cells responsible for this organ's response.

A. Glycolysis

The data presented in the foregoing sections indicates that the younger the heart the more functionally dependent it appears to be on the Embden-Meyerhof pathway for its energy. Stated another way, the older

hearts (12-, and 13-day) appear to become more demanding in their energy requirements and cannot derive enough energy from glycolysis alone to maintain their maximum heart rate. This statement gains support after a consideration of the data from the experiments dealing with a comparison of anaerobic vs. aerobic glucose utilization in the 11-, 12-, and 13-day isolated hearts. It was found that in the 11-day heart the maximum rate attainable was the same for both aerobic and anaerobic glucose utilization. In the 12- and 13-day hearts the maximum rate was found in hearts provided glucose and oxygen, whereas anaerobic glycolysis was less efficient as an energy source in these older hearts. Similar observations have come from work done on developing chick heart by Spratt (1950a) and Duffey and Ebert (1957) who demonstrated that cardiac morphogenesis and pulsation could be accomplished when chick explants were cultured in the presence of various respiratory inhibitors. Their work on the early chick heart is in agreement with the observations reported here for functional activity in the 11-day rat embryo heart. Roberts (1966) working with isolated chick hearts from older (3 and 5 days) embryos than those used by Spratt or Duffey and Ebert, found that in the presence of glucose, anaerobiosis depressed the heart rate of her isolated preparations to approximately 80% of that of the aerobic group. This information is more directly comparable to the results reported here, since the same parameter (heart rate) was measured.

Although the above discussion suggests that glycolysis becomes less important as an energy producing pathway as the hearts develop, it must be pointed out that this decrease in importance is a relative one. The studies reported here on the effects of the glycolytic inhibitor,

iodoacetic acid, clearly show that inhibition of glycolysis leads to a stoppage of heart function, as determined both by heart rate and oxygen consumption, in both the 11- and 12-day hearts. That glycolysis remains the primary metabolic pathway until after birth has been suggested by several investigators using histochemical and biochemical assays (Chiquoine, 1957; DeHaan, 1965; Toth and Schiebler, 1967). These investigators point out that in the mammalian embryo, the myocardium remains rich in glycogen throughout gestation, suggesting the continued importance of glycolysis. The fetal and newborn mammalian hearts are also known to rely heavily on glycolysis for energy, especially during periods of hypoxia at parturition (Breuer et al., 1967; Dawes et al., 1959).

Differences in utilization of hexoses by developing chick brain were noted by Spratt (1950a) in his studies on the nutritional requirements of the developing chick embryo. He noted no differences, however, in the development of the heart when presented with any one of the four hexoses - glucose, mannose, fructose, or galactose. This was shown not to be the case for function in the developing rat heart. Of the four hexoses studied, only glucose was able to maintain a maximum heart rate, mannose was next best, with fructose and galactose being essentially incapable of maintaining heart rate under anaerobic conditions. Aerobically, glucose utilization appeared to be equaled by mannose and fructose (general population), whereas galactose and fructose (isolated population) were found to be less efficient as a source of energy for contraction. The differences in hexose utilization could be due to differences in levels of enzymes necessary for the degradation of these hexoses, or the differences could be due to variation in transport mechanisms across the membrane.

The discovery of the isolated population of fructose metabolizers was unexpected. Since the common denominator in this case was found to be a particular male bred to a variety of females, it can be stated that the decreased ability to metabolize fructose by his offspring probably represents a genetically determined inborn error of metabolism or a retarded stage of development. The mode of transmission cannot be determined from these studies. It would be interesting to follow this possibility further by developing a colony of offspring from this particular male.

Some phosphorylated compounds were able to provide energy for heart activity, whereas others were not. All three mono-phosphorylated hexoses proved to be negative, while the doubly phosphorylated hexose, fructose-1,6-diphosphate, and the phosphorylated triose, α-glycerophosphate, were both positive. The observation that phosphorylated compounds can cross the cell membrane is not new; such large molecules as ATP have been shown to cross cell membranes in an in vitro cell system to be utilized by the cell (Bloom, 1970). The findings reported here probably represent a difference in permeability of the cell to molecules of varying configuration rather than representing a selective permeability based on the size of the molecule.

B. Oxidative Metabolism

Again referring to the studies comparing heart rate of the 11-, 12- and 13-day preparations when incubated in glucose and oxygen as opposed to glucose and nitrogen, it became apparent that anaerobic glycolysis was sufficient to maintain the maximum heart rate in the 11-day hearts, but not in the older ones. This observation raised two questions:

(1) Since anaerobic glycolysis can maintain a maximum cardiac rate in the 11-day heart, does this imply that oxidative processes are not functional at this age?

(2) If oxidative metabolism is becoming more important in the older hearts, could other sources of energy (other than glucose or glycolytic intermediates) be utilized by these hearts?

The first question was answered by oxygen consumption experiments and by experiments dealing with substrate utilization and metabolic inhibitors. The oxygen consumption data showed that in a solution containing 20% oxygen the 11-day hearts did consume oxygen, in fact per milligram they consumed oxygen at a rate greater than the older hearts. This observation is in agreement with Sippel's (1954) results from experiments on homogenates of rat embryo heart of a comparable age. He found an increase in succinoxidase activity over the period of $10\frac{1}{2}$ days to $13\frac{1}{2}$ days. Along with this increase he noted a decrease in oxygen consumption. His explanation of this finding was that as the oxidative system becomes more efficient, one would expect to find a decrease in oxygen consumption. In addition to the oxygen consumption data, evidence for an intact oxidative system in the 11-day heart was supplied by the observation that pyruvate in oxygen, but not nitrogen, could provide energy for contraction. That the TCA cycle is not necessary for maximum heart function was also shown by the studies utilizing malonate in concentrations that are inhibitory to succinic dehydrogenase (Webb, 1966). It was, therefore, concluded that oxidative metabolism of glucose can be, and probably is, carried out normally, but if need be, anaerobic metabolism of glucose can provide enough energy to maintain contractile energy.

The second question, regarding utilization of sources of energy other than glycolytic intermediates in the older hearts, was answered in the affirmative. It was found that for the 11-day hearts the only substrate effective that feeds into the TCA cycle was pyruvate, an end product of glycolysis. Changing patterns were noted for the older hearts. Not only could pyruvate be utilized, but by day twelve the hearts could also use acetoacetate and β-hydroxybutyrate, both of which can enter into the TCA cycle through acetyl-CoA; two amino acids, hydroxy-L-proline and L-alanine, both of which can be metabolized to pyruvate; and possibly, although not unequivocally demonstrated in these investigations, an intermediate of the TCA cycle itself, isocitrate. By day thirteen the possibilities for energy sources were expanded by the observation that succinate could be utilized. That some of the pathways involved in the metabolism of these compounds might be important at this age of the rat heart has been suggested by other studies. Sippel (1954) demonstrated an increase in the succinoxidase system which leveled off at about day thirteen and one-half. Toth and Schiebler (1967), using histochemical techniques, displayed an increase in β-hydroxybutyric acid dehydrogenase by day thirteen. However, these investigators were unable to demonstrate, histochemically, an increase in succinic dehydrogenase.

That the function of older hearts is more dependent on oxidative metabolism was also suggested by response to the TCA cycle inhibitor, malonate. In 12-day hearts a marked depression in heart rate was noted, whereas no depression was noted for 11-day hearts. Additional evidence for the importance of oxidative metabolism was shown by the experiments using the uncoupler of oxidative phosphorylation, 2,4-DNP. Hearts from

11-day embryos were depressed somewhat, but by day twelve the response to the uncoupler was quite dramatic. A complete cessation of activity ocurred within one-half hour after exposure to the uncoupling agent. It was interesting to note that the group remaining in oxygen showed a slight rebound, whereas the nitrogen group did not. The rebound was probably due to the effect dinitrophenol has on increasing the rate of carbohydrate oxidation (West et al., 1967). The observation that the addition of fructose-1,6-diphosphate did not increase the heart rate in the hearts subjected to 2,4-DNP will become more significant when the action of trypan blue is discussed.

C. Trypan Blue as a Metabolic Inhibitor

The mechanism of action of the teratogen, trypan blue, has long been the subject of debate (see review by Beck and Lloyd, 1966). Most investigators of this agent are of the opinion that it elicits its teratogenic effect by disrupting yolk sac function, where it is concentrated in the visceral entoderm. Beck, Lloyd and Griffiths (1967) and Lloyd et al. (1968) postulated that trypan blue produces malformations in the embryo by interfering with the "embryotrophic" function of the yolk sac. They base their argument on evidence showing the dye to be inhibitory to several yolk sac hydrolases, including acid phosphatase.

Recently, Davis and Gunberg (1968) have reported that small amounts of trypan blue can be seen in the embryo under a dissecting microscope and in histological preparations. Their observation raises the question of whether or not trypan blue might have a direct action on embryonic tissues. That there might be a direct metabolic effect of trypan blue on embryonic heart was earlier proposed by Adams-Smith (1963). He suggested a possible direct interference with glycogen utilization

after observing an accumulation of glycogen granules in embryonic hearts from dye-treated mothers. He found no glycogen in his controls at this stage of development. This latter finding is at variance with the findings of other investigators and may be a partial explanation as to why his theory was not widely accepted.

The results from the studies reported in this paper show that the presence of trypan blue (in concentrations one-tenth to one-fiftieth of that used by Lloyd and his co-workers) in the incubation medium leads to a depression of glucose utilization as indicated by heart rate. The depression by trypan blue can be reversed by the addition of fructose-1,6-diphosphate or α-glycerophosphate to the medium. This information suggests that trypan blue interferes with glucose utilization somewhere before the level of fructose-1,6-diphosphate in the glycolytic pathway. The inhibition could be at the membrane level, preventing adequate uptake of glucose, but not interfering with transport of fructose-1,6-diphosphate. Another possible site of action of trypan blue could be at the enzyme level. As already stated, trypan blue is a known inhibitor of certain hydrolytic enzymes. Perhaps this agent also inhibits glycolytic enzymes. It is interesting to note here that certain Schistosomicidal agents, the antimonials, are known to have as their site of action, within the parasite, inhibition of the enzyme phosphofructokinase (Bueding, 1959). Some similarities exist between the structure of trypan blue and stibophen, one of the antimonials.

Coffey et al. (1964) has shown in the early chick heart and Tanimura and Shepard (1969) in the rat embryo that the pentose phosphate pathway is highly active in the early stages of embryonic development. When considering the possible sites of action of trypan blue, this be-

comes increasingly important, for if trypan blue does interfere with glucose uptake, then not only would the glycolytic energy producing pathway be interfered with, but also the pentose shunt synthetic pathway as well. This could be of primary importance in explaining a direct action of this dye in producing cardiac malformations. It is quite possible, however, that trypan blue inhibition of a glycolytic enzyme alone could cause cardiac malformations by interfering with the primary energy producing pathway of the early embryonic heart. That interference with energy production in developing embryos can produce cardiac malformations has been proposed by Haring (1965) and explored with his work on hypoxia and cardiovascular anomalies. In the same vein, Runner (1959, 1965) has pointed out the importance of carbohydrate metabolism in the developing mammalian embryo and he suggested that interruption of that metabolism (directly or indirectly) by teratogenic agents might be responsible for developmental anomalies. The investigations reported here tend to support that hypothesis, for one teratogenic agent at least.

SUMMARY AND CONCLUSIONS

Energy metabolism as it relates to contractile rate in isolated rat embryo hearts from 11-, 12- and 13-day old embryos was studied. The isolated hearts were incubated in a simple defined salt solution to which was added a variety of substrates. Utilization of the added substrate was determined after comparing contractile rate of the hearts in an experimental medium with those in a control (no substrate) medium. The effects that inhibitors of specific metabolic pathways had on the hearts was investigated and oxygen consumption in the isolated hearts was measured using the oxygen electrode technique.

Analysis of the data from these three approaches to heart function, substrate utilization, metabolic inhibitors, and oxygen consumption, led to the following conclusions:

(1) Of the substrates tested for utilization by the 11-day hearts, only those compounds involved in the Embden-Meyerhof glycolytic pathway were capable of maintainingcardiac contraction at the maximum rate.

(2) Anaerobic glycolysis can provide sufficient energy to maintain a maximum heart rate in the 11-day isolated heart.

(3) Oxidation of pyruvate plus oxygen consumption data on the 11-day hearts suggested that aerobic glycolysis was functional in these organs.

(4) Although glycolysis remains of primary importance, the 12- and 13-day old hearts show a shift in dependence towards their metabolic pathways. This conclusion was based on the following observations:

(a) Anaerobic glycolysis is no longer sufficient to maintain a

maximum heart rate in vitro.

(b) A wide variety of non-glycolytic compounds can be used as sources of energy by these further developed hearts.

(c) Inhibitors of oxidative processes have an adverse effect on function of the 12- and 13-day hearts.

Results from other experiments utilizing the teratogenic agent, trypan blue, suggest that this dye interferes with glucose utilization in the embryonic heart. It was concluded that this interference could be at the membrane level, preventing adequate uptake of glucose into the cells, or at the enzyme level, inhibiting glucose catabolism. Depression of heart rate by trypan blue was reversed by the addition of fructose-1,6-diphosphate or α-glycerophosphate to the incubation medium.

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APPENDIX

A. Krebs-Ringer's bicarbonate solution

Component	Amount
NaCl	6.920 gm/L
KCl	0.353 "
$CaCl_2$	0.281 "
KH_2PO_4	0.162 "
$MgSO_4 \cdot 7H_2O$	0.295 "
$NaHCO_3$	2.102 "
Phenol Red	0.010 "
Penicillin G	100 u/ml
Streptomycin	50 μg/ml

B. The operating principle of the oxygen electrode

The PO_2 microelectrode is constructed of a 5×10^{-4} inch platinum wire cathode sealed in glass with only the tip exposed. The cathode is cemented within a silver tube which forms the anode of the electrode. A thin polyethylene membrane, permeable to gases, covers the tip of the electrode. Between the membrane and the electrode is an electrolyte solution which wets the tips of the platinum cathode and silver anode. The platinum cathode and silver anode are connected to a small battery so that the platinum wire forms the negative pole and the silver wire the positive pole. Oxygen (O_2) in the electrolyte solution (KCl) contacts the charged cathode and is reduced, i.e., each oxygen atom gains two electrons, forming $0 =$ ions. These ions then combine with hydrogen ions, H+, to form OH-. To balance the reduction of oxygen at the cathode, atoms at the anode are oxidized and lose an electron. The silver ions, Ag^+, combine with Cl- in the electrolyte, forming silver chloride which is deposited on the anode. In this type of electrode, reduction of oxygen at the cathode and oxidation of chloride at the anode allow a direct current to flow. The flow of current is directly proportional to the rate of oxygen reduction at the cathode and because the partial pressure of oxygen outside the membrane is the motive power in the

oxygen diffusion, the current is proportional to this pressure. The current is amplified and read on a meter which is calibrated to read directly in mmHg partial pressure of oxygen. The characteristic of the electrode is strictly linear, so that standardization against an oxygen-free solution and a solution with a known oxygen tension is all that is required.
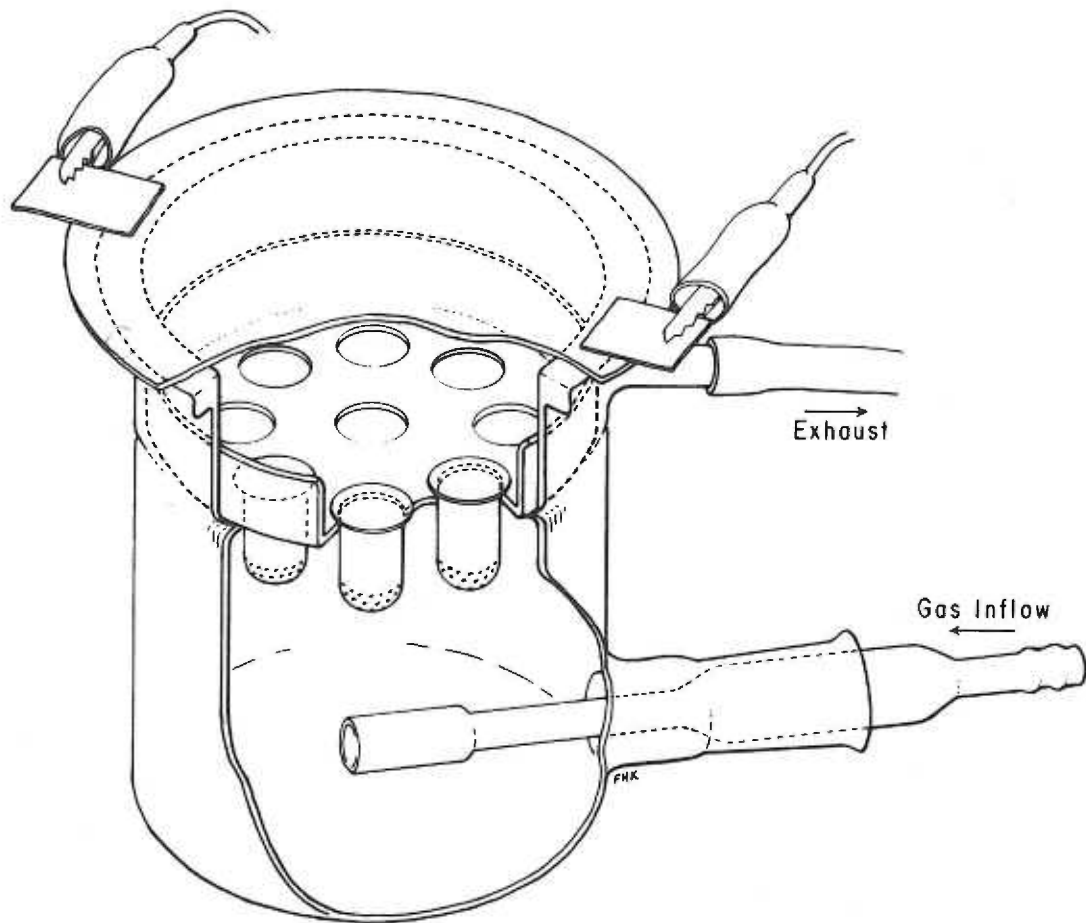
C. Plates

Exhaust
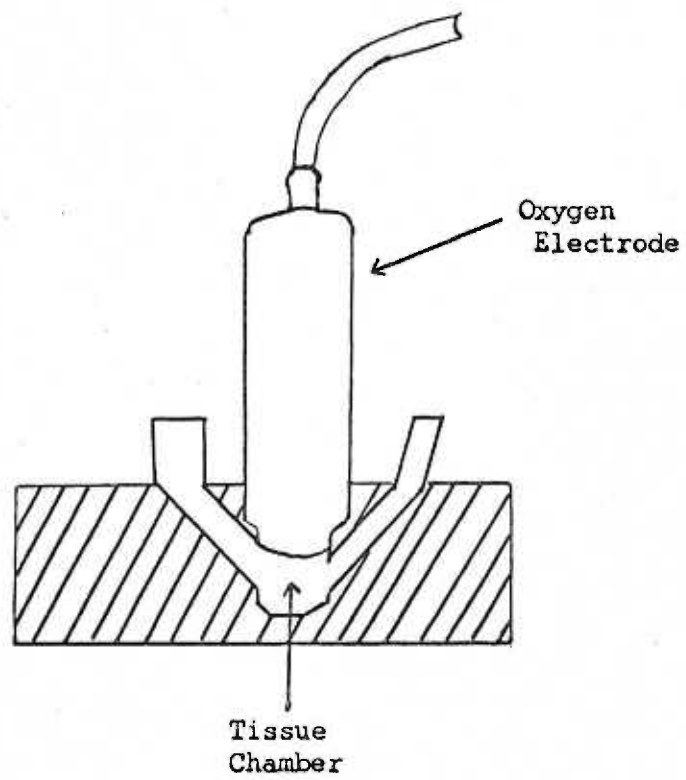
Gas Inflow

PLATE 1

Oxygen Electrode

Tissue Chamber

PLATE 3