J. Physiol. (1941) 100, 329–336

612.352.18:612.17
591.112:612.173.1:612.352.1

THE GLYCOGEN CONTENT OF THE FROG'S HEART

By F. DAVIES and E. T. B. FRANCIS

Departments of Anatomy and Zoology, University of Sheffield.
With a Chemical Section by L. B. Winter, *Department of Physiology*

(*Received* 28 *June* 1941)

For the mammalian heart (dog), Lewis [1925] has expressed as 'The Law of Cardiac Muscle' a correlation between the size (breadth), glycogen content and physiological properties of cardiac muscle fibres, namely, that the fibre size, glycogen content, and rate of conduction of the impulse for contraction increase in the order, nodal, ventricular, atrial, Purkinje tissue; while the length of systole and property of rhythmic contraction (when nourished under natural conditions) diminish in the same order.

There has, however, been some controversy as to whether the rapidly conducting Purkinje fibres in the mammalian bundle of His do actually contain more glycogen than the ordinary cardiac muscle. By histological study, after staining with either Best's alkaline carmine or iodine, Mönckeberg [1908], Nagayo [1908], Lewis & Rothschild [1915] and Ungar [1924] claimed that the Purkinje system is richer in glycogen. On the other hand, Buadze & Wertheimer [1928], by quantitative chemical estimations, found less glycogen in the Purkinje fibres than in the general myocardium (dog, goat, sheep) and thus concluded that the histological methods are unreliable. The later careful quantitative estimations of glycogen by Yamazaki [1929] on the horse and ox, by Yater, Osterberg & Hefke [1930] on the horse, and by Noll & Becker [1936] on the horse and calf, however, all agree that the bundle of His is richer in glycogen than the myocardium. Indeed, Noll & Becker employed the carmine and iodine histological tests alongside their quantitative chemical estimations, and found that the results agreed. These later studies thus substantiate the reliability of the histological methods.

So far as lower vertebrates are concerned, Clark *et al.* [1938] maintain that the number of chemical estimations that can be made on so small a quantity of tissue as that provided by frogs' hearts is limited, and these authors preferred to estimate the total reducing substances rather than the glycogen alone. Such estimations include both glycogen and lower carbohydrates, as well as some reducing substances which are not carbohydrates; for the sake of convenience, although as they point out it is not strictly accurate, they employ the term 'total carbohydrates' to cover all these substances. Since by an analysis of nine individual tortoise hearts they found [1933] only a small difference between the total carbohydrate content of the atria and ventricle (average—atria 1·5 %, ventricle 1·67 %) they concluded that 'one portion of the frog or tortoise heart, therefore, will serve as a fairly reliable control as regards the carbohydrate content of the remainder'. Various observers, using chemical methods, have noted considerable individual variation in the carbohydrate content of frogs' hearts. A seasonal variation also has been noted by Wertheimer [1933] and by Clark *et al.* who find more carbohydrate in the hearts of winter than summer frogs.

The present authors [Davies & Francis, 1941] have shown that there are no differences—apart from size—in the histological structure of the muscle fibres of the various chambers of the heart of either the salamander or frog (sinus, atria, ventricle, bulbus), as revealed by staining with haemalum and eosin, Van Gieson's acid fuchsin and iron haematoxylin, or with Bodian's activated protargol. They also demonstrated a similar absence of histological specialization of the musculature joining the chambers of these hearts, and concluded, contrary to the view commonly held, that the Purkinje system of the mammalian (and avian) heart is a neomorphic structure, and is not derived from more extensive junctional tissues of similar structure in lower vertebrate hearts.

The aim of the present investigation is to determine whether in one and the same frog's heart there are any differences in the glycogen content of the musculature of the several chambers or of that at the junctional sites.

Observations

For both the histological and chemical investigations the hearts were removed early in February from animals which had been kept in captivity for some months.

Histological

The hearts of 3 frogs (*Rana temporaria* L.) were rapidly removed from pithed animals and immediately placed in abs. alcohol. In this connexion it may be noted that while Evans [1934] showed that in the mammal (rat) asphyxia (anoxaemia) causes rapid reduction in the glycogen content of the heart, Clark *et al.* [1938] found that even prolonged manipulation (10–15 min.) under conditions of anoxaemia causes no appreciable diminution in the glycogen content of the frog's heart. The 3 hearts were embedded in paraffin and cut transversely in serial sections at 5μ thickness, and stained with alkaline carmine [Best, 1907], controlled by the saliva test and by sections of liver stained at the same time.

On visual inspection with the microscope, it was at once apparent that the glycogen content of the muscle fibres differ in the several chambers of the heart. The sinus muscle is not very rich in glycogen; the atrial muscle contains slightly more; the ventricular muscle is considerably richer than the atrial, while the bulbar muscle contains still more. Further, the glycogen content of the musculature at the sinu-atrial and atrio-ventricular junctions shows no appreciable difference from that of the neighbouring chambers. It thus appears that the glycogen content of the muscle fibres shows a progressive increase from the sinus to the bulbus.

Chemical estimation of glycogen

Alongside our direct histological observations, chemical investigation has been carried out in collaboration with our colleague, Dr L. B. Winter, of the Physiology Department, University of Sheffield. Two series of estimations were made, 6 animals being used for each.

First series. Each heart was rapidly removed from a pithed animal and placed immediately in an ice-cooled glass tube. The several chambers were successively separated from each other by section and, after removing adherent blood with filter paper, were weighed on a micro-torsion balance; the operation being so arranged that individual parts of the hearts were out of the ice-cooled tube for only a very brief period. In the case of the sinus and bulbus, owing to their small mass, the entire chambers were used, but in the case of the ventricles and atria, portions only of the walls were removed and weighed, the parts removed differing in each heart so that all parts of these chambers were represented in the final mass of tissue on which the estimation was made. After weighing,

the fragments were placed in ice-cooled, small centrifuge tubes and corked, as follows: tube A, 38 mg. bulbus; tube B, 38 mg. sinus; tube C, 39 mg. ventricle; tube D, 35 mg. atria.

Chemical estimation by L. B. WINTER

The tissue samples were handed to me in small centrifuge tubes, corked and cooled in ice: the tubes were numbered and the weight of tissue in each was stated, but I was unaware which tissue was contained in each tube until the estimations had been completed and the percentage of glycogen (as glucose) in each tissue calculated. For the estimation of glycogen the directions given by Osterberg [1929] were followed, with certain modifications. The weight of tissue was greater, and to each tube was added 0·2 c.c. of 60 % potash. After 3 hr. reflux under an air condenser at 100° C., the tubes were allowed to cool in the bath and to each were added 0·2 c.c. water and 1·2 c.c. strong alcohol: the contents were well mixed. Five days in a refrigerator were allowed for complete precipitation of the glycogen; by this means the precipitate was induced to adhere firmly to the bottom of the tubes, and the use of the centrifuge was always found to be unnecessary during the successive washings. Each day the supernatant fluid was carefully poured off, the tubes being inverted for a few minutes to ensure complete draining, and 2·0 c.c. 70 % alcohol were run in. After one washing each day for 5 days the alcohol no longer coloured phenolphthalein and the tubes were dried in vacuo over sulphuric acid. For the conversion of glycogen to glucose, 2·0 c.c. 2·2 % HCl were added to each tube, and reflux at 100° C. was carried out for 3 hr. After cooling, the exact amount of alkali was added to neutralize the acid; the contents were stirred, transferred to a 10 c.c. volumetric flask and the tube was washed out with small amounts of water. 4·0 c.c. portions were taken for duplicate glucose estimations by the method of Hagedorn and Jensen. Results: sinus, 0·15 %; atria, 0·62 %; ventricle, 1·52 %; bulbus, 0·22 %.

It will be observed that whereas these results agree with our visual observations as far as the increasing order of glycogen content of the sinus, atria and ventricle is concerned, there is a marked discrepancy in the case of the bulbus. This would appear to be due to the relatively large amount of connective tissue present in the bulbus, including the fibrous septum bulbi and valves, which would have the effect of diminishing the proportion of glycogen-containing muscle in the weighed amount of bulbar tissue, thus giving too low a figure for the chemical estimation. This idea is supported by the results obtained in the second series of estimations.

Second series. The procedures detailed above were repeated with another 6 frogs except that the septa were removed from two bulbi. The remaining four bulbar septa were not removed since, owing to the way these bulbi contracted on excision, the operation was deemed likely to require too much time and to involve too much traumatic injury. The two bulbi from which the septa were removed offered very favourable conditions and the operation required only a few seconds. Results: sinus, 0·26 %; atria, 0·78 %; ventricle, 1·85 %; bulbus, 0·73 %.

It will be seen that whereas in the case of the sinus, atria and ventricle these results are slightly higher than those of the first series, the percentage of glycogen in the bulbus in the second estimation is more than three times that found in the first experiment.

Graphical method of estimation

Owing to the difficulty of obtaining a valid estimate of the glycogen in the *musculature* alone in the bulbus by chemical means, we have estimated the *relative* amounts of glycogen in the musculature of the several cardiac chambers by a graphical method. The histological sections, stained with Best's carmine, were projected by means of an arc lamp and microscope on to transparent graph paper, ruled in millimetre squares, at a linear magnification of $\times 1480$. The outlines of small areas of cardiac muscle (in longitudinal section) from each chamber were traced and within these frames the outlines of the red-strained globules and small masses of glycogen were mapped out. By counting the number of squares covered by both the muscle and the glycogen a ratio was obtained which serves to indicate the relative amounts of glycogen present in the musculature of the various parts of the heart. (The reliability of this method is discussed below.)

By this means the following results were obtained, expressed as fractions in which the numerators represent the number of millimetre squares covered by glycogen and the denominators the total areas of cardiac muscle fibres outlined: sinus, 18,182/156,475 = 11·62 %; atria, 28,428/200,406 = 14·18 %; ventricle, 50,211/175,307 = 28·6 %; bulbus, 67,549/218,008 = 31·03 %. The musculature at the sinu-atrial and atrio-ventricular junctions was analysed in a similar manner and the following results obtained: S-A ring, 17,349/123,980 = 13·99 %; A-V ring, 41,120/145,935 = 28·2 %; A-V funnel, 31,120/127,895 = 24·3 %. Each of the above results represents the total of many tracings made from sections of different regions of the several cardiac chambers, so that the total represents a fair average of the glycogen content of the musculature of the chamber concerned. It was noted in making individual tracings of small areas of each chamber that, although there was a fair consistency in the amount of glycogen in each area, there was, however, considerable indiscriminate variation in the glycogen content of neighbouring fibres.

Discussion

Of the methods described above, chemical estimation is evidently the only one which provides an absolute figure for the amount of glycogen present in each cardiac chamber. Where, however, there is a considerable amount of connective tissue present (a circumstance only determinable by histological control), the result obtained does not represent the amount of glycogen present in the *musculature* of that chamber. Thus, in the present instance, where it is desired to compare the glycogen content of the *musculature* of the various cardiac chambers, the chemical method is not applicable on account of the varying extent to which connective tissue participates in the structure of the walls of the several chambers of the heart.

The graphical method, on the other hand, does enable a comparison to be made between the glycogen content of the muscle fibres from various parts of the heart. It is obvious that this method depends for its reliability upon the specificity of the carmine as a stain for glycogen. That this may be trusted is indicated by the work of Noll & Becker [1936], while we have ourselves controlled the staining of our material by the saliva test. Further, it must be pointed out that this method deals purely with relative areas, and not volumes, of glycogen and muscle. In any particular individual mass of glycogen outlined, there are parts which do not extend throughout the entire thickness of the 5μ sections, and high magnification reveals that these masses are aggregations of smaller globules. In plotting such masses on squared paper it is necessarily assumed that the areas are homogeneous and extend through the entire thickness of the section. These errors, however, are common to all chambers and therefore do not seriously affect the validity of the results so far as a comparison of the glycogen distribution in the various parts of the heart is concerned, although they do prevent an estimate being made of the absolute amount of glycogen present. On the other hand, the consistency of the results calculated from each of the numerous tracings made from individual cardiac chambers confirm the reliability of this method when used purely for comparative purposes.

With regard to the significance of the results obtained, it is evident that there is, in the frog's heart, a relationship between the glycogen content and the intrinsic rate of rhythmic contraction of the various chambers, in that, while this rate *decreases* in the order sinus, atria, ventricle, bulbus, the glycogen content of the muscle fibres *increases* in the same order. Whether this indicates a causal relationship between the

glycogen content and the intrinsic rhythmic rate of a cardiac muscle fibre, or is purely coincidental, remains to be determined. A further correlation can, however, be made in that the density of musculature and the work done increase in the same order as the glycogen content of the muscle fibres, suggesting that the increasing glycogen content is related to the greater energy demands for the work of contraction in the successive cardiac chambers.

Now with regard to the specialized conducting system of the mammalian heart, the Purkinje fibres contain more glycogen than the ordinary myocardium. This Purkinje system has been held by various workers to represent remnants of more extensive tissues of similar structure in the hearts of lower vertebrates. The present authors, however, as the result of a detailed histological study of the amphibian (salamander) heart, have elsewhere [1941] put forward the view that the cardiac conducting systems of homoiothermal vertebrates (mammals and birds) constitute neomorphic developments, and are not remnants derived from the lower vertebrate heart. They based their conclusions on the complete lack of any histological specialization in the musculature joining the several cardiac chambers. The absence of any accumulation of glycogen in the junctional muscle of the frog's heart affords additional evidence in favour of this view.

It should be emphasized that the present work is concerned solely with the *relative proportions* of glycogen present in the *musculature* of the various parts of one and the same heart at a given time, and not with the absolute quantities of glycogen in each chamber, or with the variations due to seasonal or other disturbances which are known to occur.

Summary

1. Visual examination of serial sections of frogs' hearts, stained by Best's carmine method, revealed a progressive increase in the glycogen content of the muscle fibres, chamber by chamber, from sinus to bulbus. Graphical analysis confirmed this. Chemical analysis of the separated chambers agreed with the visual and graphical results, except in the case of the bulbus, where the figures were much lower than anticipated; this is explained by the relatively large amount of connective tissue in the bulbus.

2. The glycogen content of the muscle fibres is correlated directly with the work done by the several cardiac chambers, and inversely with their intrinsic rhythmic rates.

3. The lack of accumulation of glycogen in the junctional muscle supports the authors' view that the specialized cardiac conducting systems of mammals and birds are not remnants of more extensive tissues of similar structure in lower vertebrate hearts.

REFERENCES

Best, F. [1907]. *Zbl. allg. Path. path. Anat.* **18**, 465–73.

Buadze, S. & Wertheimer, E. [1928]. *Pflüg. Arch. ges. Physiol.* **219**, 233–7.

Clark, A. J. *et al.* [1933]. *J. Physiol.* **77**, 432–46.

Clark, A. J. *et al.* [1938]. *The Metabolism of the Frog's Heart.* Edinburgh and London: Oliver Boyd.

Davies, F. & Francis, E. T. B. [1941]. *Philos. Trans.* In the Press.

Evans, G. [1934]. *J. Physiol.* **82**, 468–80.

Lewis, T. [1925]. *The Mechanism and Graphic Registration of the Heart Beat.* 3rd ed. London: Shaw.

Lewis, T. & Rothschild, M. A. [1915]. *Philos. Trans.* B, **206**, 181–226.

Mönckeberg, J. G. [1908]. *Untersuchungen über das Atrioventricularbündel im Menschlichen Herzen.* Jena: Fischer.

Nagayo, M. [1908]. *Verh. dtsch. path. Ges.* **12**, 150–3.

Noll, A. & Becker, M. [1936]. *Virchows Arch.* **296**, 443–7.

Osterberg, A. E. [1929]. *J. biol. Chem.* **85**, 97–108.

Ungar, M. [1924]. *Lotos.* Prague.

Wertheimer, E. [1933]. *Pflüg. Arch. ges. Physiol.* **232**, 120–39.

Yamazaki, K. [1929]. *J. Biochem.* **10**, 481–90.

Yater, W. M., Osterberg, A. E. & Hefke, H. W. [1930]. *Arch. intern. Med.* **45**, 760–71.