Effect of Oxygen Pressure on Glycogen Synthesis by Rat-Liver Slices

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1. Glycogen synthesized by rat-liver slices 0.5 mm. thick incubated at 1 atm. oxygen pressure in Hastings medium with glucose was localized in the cells of the periphery of the slice. Cells of the interior of this slice do not synthesize glycogen.
2. Inner cells of thin slices (about 0.3 mm. thick) can synthesize glycogen when such slices are incubated under the same conditions, but oxygen pressures higher than 1 atm. are required if inner cells of slices 0.5 mm. or more thick are to be able to synthesize glycogen.
3. Localization of newly synthesized glycogen in rat-liver slices incubated in Hastings medium with glucose does not depend on glucose concentration.
4. Calculation of the minimum oxygen pressure required to synthesize glycogen gives values between 0.09 and 0.17 atm.
5. The advantages of high oxygen pressures for the study of the synthesis of glycogen and other compounds that require ATP are discussed.

Mammalian liver homogenates show a poor capacity to incorporate [^{14}C]glucose into glycogen (Figueroa, Pfeifer & Niemeyer, 1962; Banerjee & Ganguli, 1962), but liver slices incubated in Hastings medium show a higher rate of incorporation (Hastings, Teng, Nesbitt & Sinex, 1952; Niemeyer & Figueroa, 1956; Cahill, Hastings, Ashmore & Zottu, 1958; Figueroa & Pfeifer, 1964). For this reason most of the studies of glycogen synthesis in mammalian liver have utilized the slice technique. Deane, Nesbitt, Buchanan & Hastings (1947) showed that the cells in the slice are not uniform in their capacity to synthesize glycogen. The new glycogen was localized at the periphery of the slice whereas the cells of the interior did not exhibit glycogen synthesis. There was, in addition, disappearance of mitochondria at the interior of the slice. The localization of the new glycogen was attributed to an adequate supply of oxygen and nutrients in the cells located nearer the surface of the slice.

The present work deals with the effect of oxygen pressure on the localization of glycogen synthesized by rat-liver slices. It is shown that the amount of glycogen is directly proportional to the oxygen pressure, and that with higher-than-normal oxygen pressures the inner cells of the slice behave similarly to the cells of the periphery.

EXPERIMENTAL

Animals. Male albino rats from our Institute strain, weighing about 200 g., were used. They were killed by

Fig. 1. Sealed cylinder used for the incubation of slices at high O_2 pressures. A, Valve of bicycle tyre where gas is introduced; B, manometer; C, valves that isolate the cylinder from the manometer and valve A; D, brass cylinder; E, glass flask where slices are incubated; F, screwed cover; G, rubber rim that permits the cover to close hermetically. The arrows indicate the direction of gas flow.

decapitation after 24 hr. without food but with free access to water. The liver was quickly excised and put in ice-cold 0.154 M-KCl.

Fig. 2. Transverse sections of rat-liver slices fixed and stained for glycogen by the periodate-Schiff technique. All of them, except (a), were incubated for 45 min. in Hastings medium containing glucose (0.03M) and with different O_2 pressures. (a) Non-incubated liver slice (glycogen content, 0.10%); magnification $\times 32$. (b) Slice incubated with O_2 at 0.84 atm. (glycogen content, 0.62%); glycogen is absent in the interior of the slice at the right-hand side, which is a thick part; the left-hand side, which is thinner, is full of glycogen; magnification $\times 32$. (c) Slice incubated with O_2 at 0.18 atm. (glycogen content, 0.12%); glycogen is confined to a rim about 0.08 mm. deep; cells along the very edge contain no glycogen, probably owing to damage; magnification $\times 180$. (d) Slice incubated with O_2 at 0.50 atm. (glycogen content, 0.22%); glycogen occurs in a rim about 0.16 mm. deep; magnification $\times 180$. (e) Slice incubated with O_2 at 0.84 atm. (glycogen content, 0.34%); the depth of glycogen reaches about 0.18 mm.; magnification $\times 180$. (f) Slice incubated with O_2 at 1.39 atm. (glycogen content, 0.58%); the depth of glycogen reaches about 0.28 mm.; magnification $\times 180$. (g) Slice incubated with O_2 at 2.7 atm. (glycogen content, 0.85%); the slice is 0.52 mm. thick; magnification $\times 90$.

Incubation of the slices. Liver slices were made free-hand with a razor blade and put in an oxygenated and cold solution of 0.154M-KCl. The slices were incubated in 25 ml. flasks by using the bicarbonate medium I of Hastings *et al.* (1952). Glucose was added to give a final concentration of 0.03M unless otherwise stated. The medium was equilibrated with various gas mixtures. For incubations at pressures over 1 atm. a sealed cylinder (described below) was used. The flasks were put in a water bath at 37° for 45 min. At the beginning and at the end of the incubation period two flasks were removed, one for chemical determination of glycogen and the other one for histological examination.

Gas mixtures. (a) An O₂+CO₂ (95:5) mixture passed through water. The mixture had a total pressure of about 720 mm. Hg (average atmospheric pressure in Santiago). The partial pressure of O₂ was calculated to be 0.84 atm. by subtracting the partial pressure of water vapour at 37° and the pressure of CO₂.

(b) An O₂+N₂+CO₂ (20:75:5) mixture passed through water. This gives an O₂ pressure of 0.18 atm.

(c) An O₂+N₂+CO₂ (59:36:5) mixture passed through water. This gives an O₂ pressure of 0.50 atm.

(d) Several mixtures with O₂ pressures over 1 atm. The cylinder used for this kind of experiment was washed with an O₂+CO₂ (95:5) mixture at atmospheric pressure and then it was injected with pure O₂ to increase the pressure to that desired.

Apparatus used to incubate slices at pressures higher than 1 atm. For incubation experiments at pressures above atmospheric pressure the flask containing slices was placed in a brass cylinder that could be sealed hermetically. This cylinder was equipped with a pressure gauge to indicate the internal pressure and a valve of a bicycle tyre through which the gas entered. The changes in pressure were achieved by means of a bicycle pump arranged in such a manner that O₂ or other gas could be pumped into the sealed cylinder. Two internal valves isolated the interior from the manometer and the tyre valve to prevent any leakage of gas. To wash the cylinder with gas at atmospheric pressure the manometer and the tyre valve were loosened. A schematic representation of the apparatus is given in Fig. 1.

Glycogen determination. Glycogen was isolated by digesting the slices with 1 ml. of 30% (w/v) KOH in a boiling-water bath for 30 min. Then 0.1 ml. of 2% (w/v) Na₂SO₄ and 3 vol. of ethanol were added to precipitate the glycogen. It was left standing for at least 2 hr. in the cold room and centrifuged down, dissolved in water and precipitated again with ethanol. The isolated glycogen was determined by the method of Montgomery (1957).

Histological methods. For histological examination, slices of one of the flasks of each experimental condition were fixed in a solution containing 80 ml. of ethanol saturated with picric acid, 10 ml. of 40% formalin and 10 ml. of ethanol. It was left standing at 2–3° for 2 hr. and then 2 hr. at room temperature. The fixed slices were embedded in paraffin wax and sectioned transversely at 6μ. Glycogen was detected by the periodate-Schiff method (Pearse, 1961). In some cases a study of mitochondria was made. The tissue slice was fixed in Zenker formal fluid for 24 hr., post-chromated in 3% (w/v) K₂Cr₂O₇ at 37° for 3 days, washed and embedded in paraffin wax to be sectioned and stained by the Mallory's ferric haematoxylin method (Langeron, 1949).

RESULTS

Figs. 2(a) and 2(b) show transverse sections of liver slices before and after incubation in Hastings medium with gas phase oxygen+carbon dioxide (95:5). This corresponds to oxygen at 0.84 atm. In confirmation of data of Deane *et al.* (1947), glycogen synthesis is limited to a rim nine or ten cells deep in the periphery of the slice. Inner cells of slices 0.5 mm. thick, corresponding approximately to 30 rows of cells, i.e. the optimum thickness recommended by Fuhrman & Field (1945) for the study of oxygen uptake, have no capacity to synthesize glycogen.

If thinner slices are used (about 20 rows of cells)

Fig. 3. Effect of O₂ pressure on the depth of the peripheral rim of liver slices that are able to synthesize glycogen when incubated with glucose in Hastings medium. Experimental details are given in the text.

Fig. 4. Effect of O₂ pressure on glycogen synthesis of rat liver slices incubated for 45 min. in Hastings medium. Points linked by a line correspond to slices from the same liver. Other experimental details are given in the text.

Fig. 5. Transverse sections of rat-liver slices fixed and stained for glycogen by the periodate-Schiff technique. They were incubated in Hastings medium with O_2 at 0.84 atm. (a) Slice incubated with glucose (0.03 M) for 10 min. (glycogen content, 0.16%); magnification $\times 95$; (b) Slice incubated with glucose (0.03 M) for 20 min. (glycogen content, 0.19%); magnification $\times 95$. (c) Slice incubated with glucose (0.03 M) for 45 min. (glycogen content, 0.34%); magnification $\times 190$. (d) Slice incubated with glucose (0.02 M) for 45 min. (glycogen content, 0.24%); magnification $\times 190$. (e) Slice incubated with glucose (0.05 M) for 45 min. (glycogen content, 0.38%); magnification $\times 190$. (f) Slice incubated with glucose (0.10 M) for 45 min. (glycogen content, 0.42%); magnification $\times 190$.

the cells of the central part of the slice are able to synthesize glycogen. This is shown in Fig. 2(b). The slice has a thin part and a thick part. The thin part at the left is full of glycogen and the central zone of the thick part at the right has no glycogen. Slices full of glycogen were always obtained when liver slices less than 20 cells thick were incubated with glucose. It is noteworthy that the two or three first rows of cells at the periphery of the slice do not synthesize glycogen (Deane *et al.* 1947); this may be due to damage of the cells during slicing and incubation.

Figs. 2(c)–2(f) show the effect of oxygen pressure on the capacity of the cells of the slice to synthesize new glycogen. The depth of the rim of active cells that is shown in the histological section depends directly on oxygen pressure (Fig. 3). With an oxygen pressure of 2.7 atm. the central part of a slice about 35 cells thick becomes active (Fig. 2g). There is also a good correlation between the net synthesis of glycogen as measured by chemical method and the oxygen pressure (Fig. 4).

Figs. 5(a)–5(c) show histological sections of liver slices incubated with glucose and removed at different periods of time from 10 to 45 min. The depth of the rim reaches a maximum. Fig. 5(a), representing the section of a slice after 10 min. of incubation, has a rim about the same depth of a slice that was incubated for 45 min. (Fig. 5c). The intensity of the periodate–Schiff reaction, however, shows that with more prolonged incubation the slice has more glycogen, which agrees with chemical determinations. It has been suggested that the inability of the cells of the inner part of the slice to synthesize glycogen is due not only to lack of oxygen but also to a low concentration of glucose, which could be the result of a rather slow diffusion of the nutrient (Deane *et al.* 1947). To test this hypothesis, liver slices incubated with different concentrations of glucose were examined. The microscopic sections are shown in Figs. 5(d)–5(f). The depth of the rim containing newly synthesized glycogen is independent of glucose concentration. However, the intensity of the periodate–Schiff reaction increases with augmented concentration of glucose. The histochemical reactions confirm that the net glycogen synthesis measured by chemical methods is proportional to the concentration of glucose (Cahill *et al.* 1958; Niemeyer, 1955).

An important factor that influences glycogen synthesis by liver slices is the relative proportions of K^+ and Na^+ in the incubation medium (Hastings *et al.* 1952). We found that, in slices incubated with Na^+ instead of K^+ , less glycogen was deposited in the active zone. In the Na^+ -containing medium the depth of the active zone was slightly less than in the K^+ -containing medium, confirming the data of Deane *et al.* (1947).

DISCUSSION

When liver slices prepared in a standard way and of such a thickness to permit optimum oxygen uptake are incubated in oxygen at 1 atm. pressure their inner cells cannot synthesize glycogen. On the other hand, all the cells from liver slices incubated at higher oxygen pressures can synthesize glycogen. The use of such higher oxygen pressures for the incubation of liver slices may bring several advantages. Let us consider a slice that is 35 cells thick (0.6 mm.) and is incubated at normal oxygen pressure. The wall of the tube of active cells, which forms a rim in the histological section of the slice, has a thickness of about ten cells. Only 57% of the cells of the slice are active if one neglects a few cells at the ends of the slice. By incubating the slice under an oxygen pressure higher than 1 atm., the relative proportion of active cells may be increased to 86%. Higher percentages of active cells cannot be obtained owing to damage to the two or three rows of peripheral cells. With a slice that is 20 cells thick and appears full of glycogen under 1 atm. oxygen pressure only, 75% of the cells of the slice are active owing to the fact that the damaged cells of the periphery represent a higher proportion of the total number of cells. These slices are rather thin, being about 0.35 mm. thick, and they are difficult to prepare and tear easily on agitation.

The behaviour of slices incubated under higher oxygen pressures probably gives a better indication of the synthetic activity of the tissue *in vivo*. This is not only due to a greater percentage of active cells, but also to the following consideration. In the slices incubated at normal pressure of oxygen, the interior cells not only do not synthesize glycogen but glycogen breakdown is also occurring. When liver slices of fed rats (high glycogen content) are incubated in a Krebs medium, the glycogen content of the tissue decreases during the incubation period (Niemeyer & Figueroa, 1956). This glycogen breakdown occurs in the inner cells of the slice and not apparently in the periphery. Therefore the net glycogen synthesis produced during the incubation of liver slices measures not the real capacity of the tissue to synthesize glycogen but rather the amount of glycogen synthesized by the peripheral cells minus the amount of glycogen broken down by the central cells.

The necessity of oxygen for glycogen synthesis arises from its dependence on ATP. Inhibition of ATP synthesis by 2,4-dinitrophenol inhibits glycogen synthesis in rat liver (Niemeyer, Figueroa & Gonzalez, 1956; Figueroa & Pfeifer, 1962). Disappearance of mitochondria in denervated muscle makes the tissue incapable of synthesizing glycogen (Carafoli, 1964). Histological examination of our liver slices incubated with glucose showed that the

zones that do not synthesize glycogen exhibit an almost complete disappearance of mitochondria but that they are conserved in the zones where glycogen is formed. These facts agree with those of Deane *et al.* (1947) and support the hypothesis that the necessity of oxygen for glycogen synthesis is dependent on mitochondria. The use of high oxygen pressures for the incubation of liver slices may be of value in biosynthesis studies on other substances that are dependent on ATP production.

The boundary between the active cells of the periphery and the inactive cells of the interior of the slice suggests that the cell needs a certain oxygen pressure to synthesize glycogen below which no synthesis occurs at all. According to Fuhrman & Field (1945), the maximum rate of oxygen consumption of rat-liver slices occurs with a slice thickness of 0.55 mm., little change in oxygen uptake occurring in the thickness range 0.48–0.62 mm. The maximal thickness that a tissue slice may attain without limitation of respiration of most interior cells through inadequate oxygen supply may be calculated by the equation (Warburg, 1928):

$$d' = \sqrt{8C_o \cdot \frac{A}{D}} \quad (1)$$

where C_o is the oxygen concentration (in atmospheres) outside the slice, D the diffusion constant in the tissue and A the rate of oxygen uptake expressed in ml./min./ml. of tissue; d' represents the thickness of the tissue slice (cm.). Applying eqn. (1) to the oxygen uptake found by Fuhrman & Field (1945), namely 5×10^{-2} ml./min./ml., and a value of D equal to 1.7×10^{-5} ml./min./cm.², a value of 0.059 cm. is obtained for d' , which agrees very well with the value determined experimentally. This agreement assures validity to the constant D for liver since its value is based on that obtained by Krogh (1919) with diaphragm. Field (1948) recommends 0.48–0.62 mm. as the best thickness of rat-liver slices for studying respiration in an atmosphere of oxygen. The equation (Warburg, 1928):

$$C_1 = C_o - \frac{A}{D} \cdot \frac{(d')^2}{8} \quad (2)$$

gives the concentration of oxygen (C_1) in the centre of the tissue slice. The other letters have the same meaning as in eqn. (1). Applying eqn. (2), an oxygen pressure of zero at the centre of the slice is obtained with a slice thickness of 0.52 mm. This agrees with the assumption that oxygen uptake is maximal in the cell with an oxygen pressure very close to zero. Eqn. (2) can also be used to calculate the oxygen pressure that exists in the boundary between cells that synthesize glycogen and cells

that do not. By using the value of A found by Fuhrman & Field (1945) and the value of D applied by the same authors, this critical pressure varies between 0.09 and 0.17 atm. This seems to be a limiting oxygen pressure, since under this pressure no glycogen is synthesized. This is in contrast with oxygen uptake, which is maximal with an oxygen pressure very close to zero. The difference is easily explained if we consider that glycogen synthesis depends on oxidative phosphorylation in the mitochondria. It could be that oxygen consumption in the mitochondria occurs at very low oxygen pressures whereas oxidative phosphorylation has a limiting oxygen pressure, the same as that found for glycogen synthesis. On the other hand, oxidative phosphorylation is a more fragile mechanism than oxygen consumption, as has been shown for isolated mitochondria (Lehninger, 1951). Therefore damage of mitochondria produced in the zones of very low oxygen pressure may become manifest only in the oxidative-phosphorylation mechanism.

It may be argued that oxygen may interfere in the process studied. Actually, oxygen at elevated pressure may have a harmful effect on the metabolism of mammalian tissue *in vitro* or in the activity of the enzymes. Oxygen uptake of rat-liver slices decreases with exposure to high oxygen pressure (Stadie, Riggs & Haugaard, 1945b). The same effect is observed in rat-brain slices or homogenates. The inhibition of brain metabolism by high oxygen pressure was similar with glucose, fructose, pyruvate or lactate as substrate (Stadie, Riggs & Haugaard, 1945a).

Many enzymes, especially those that depend on thiol groups for activity, are more or less inactivated by oxygen pressures at 1 atm. or higher. Others are generally resistant (Haugaard, 1946). These effects on tissues or homogenates *in vitro* require oxygen pressures higher than those used in the present work, except for the toxic effect of oxygen on the enzyme systems that oxidize glucose or pyruvate, where 1 atm. pressure is necessary (Haugaard, Hess & Itskovitz, 1957). On the other hand, the period of time required to harm the tissues or enzymes is always longer than 1 hr. The incubation period we used was only 45 min. Nevertheless, if there is some harmful effect of higher oxygen pressure on the mechanism of glycogen synthesis it is more than compensated for by the work of the increased number of active cells, since the net glycogen synthesis is greater with higher oxygen pressure.

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