CCXXXIII. THE EFFECT OF ADRENALINE ON EMBRYONIC CHICK GLYCOGEN *IN VITRO* AS COMPARED WITH ITS EFFECT *IN VIVO*¹

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MUCH less appears to be known of the mode of action of adrenaline on the breakdown of liver glycogen than of its mode of action on muscle glycogen. In muscle it has been shown that adrenaline initiates a breakdown of glycogen to lactic acid not only in an isolated perfused limb but in pieces of excised muscle *in vitro* [Hegnauer & Cori, 1934]. Lately Nachmansohn *et al.* [1936; 1937] have shown that adrenaline will act even on a muscle "brei", increasing the rate of formation of lactic acid and causing an accumulation of hexosemonophosphate, which seems to indicate that adrenaline may be acting here as a specific accelerator of one of the enzymes taking part in the phosphorylated glycogen glycolysis system in muscle.

With respect to its action on liver glycogen it is generally accepted that adrenaline produces a rise of blood sugar by breaking down preformed liver glycogen to glucose. But attempts to demonstrate the effect of adrenaline as a direct accelerating action on the liver glycogenase enzyme, converting glycogen into glucose, have so far proved unsuccessful [quoted Hodgson, 1936].

The various possibilities of the mode of action of adrenaline on liver glycogen and blood sugar seem to be:

(1) A direct action: (a) on the glycogenase enzyme independent of the cell structure, though this would appear unlikely in view of the experiments quoted by Hodgson; or (b) on the intact living liver cell, either breaking down a spatial separation of enzyme and substrate in the cell as Lesser [1920] suggested, or increasing the permeability of the cell wall to glucose, thereby causing a shift in the equilibrium between glycogen and glucose in the cell.

(2) An indirect action not demonstrable in the isolated living cell inasmuch as the final picture in the whole animal might be the resultant of a number of different actions of adrenaline. For example, the inhibition of glucose utilization under the influence of adrenaline reported by Cori & Cori [1928; 1929] and Colwell & Bright [1930] might account in part for the rise of blood sugar, while the constriction of the intrahepatic vessels as shown to occur with adrenaline by Clark [1928], Emery & Griffith [1931] and McMichael [1932] might be the cause of relative hepatic anaerobiosis and thus of the breakdown of liver glycogen.

In the hope of being able to throw some light on the question as to whether the action of adrenaline on liver glycogen is direct or indirect, an investigation was made of the effects of adrenaline and anaerobiosis on the glycogen content of cultures of embryonic chick liver where the conditions are not complicated by

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the presence of nerves and blood vessels. In the present paper the results of this investigation are described, together with a study of the glycogen content of the liver of the embryo chick during development and a comparison of the effects of adrenaline on its liver and muscle glycogen.

METHODS

Tissue culture methods and histology. 11- to 16-day-old chick embryo liver was cultured in "hanging drop" cultures by the technique described by Doljansky [1929] with certain modifications. The tissue was cut into cubes about 1 μ l. in size, which were planted on the surface of a firm plasma clot, containing 0.1%, 1% or 2% sugar. The cultures were incubated at 38° with the liver tissue resting on the surface of the clot, and experiments performed on them after 4-6 days. By this time the cultures had grown out, partly by spreading and partly by cell division, into thin sheets of epithelial cells with a slight thickening at the centre, and their glycogen content could easily be seen by staining methods.

The glycogen content of the cultures was ascertained histologically in the following way: The cultures were stained first by the I₂ vapour method [Lewis, 1921] and the intensity and position of the glycogen staining noted; they were then fixed in 98% alcohol and subsequently stained with Best's Carmine, haematoxylin or thionine being used as counter-stains. The agreement between the two different methods was satisfactory in all cases.

Reagents. All the adrenaline solutions used were made up from the pure crystalline solid (B.D.H. Epinephrine). Small quantities were weighed out and dissolved in an equivalent weight of *N* HCl, and diluted with Tyrode or Pannet and Compton solution immediately before using.

Glycogen determinations. Glycogen in embryo chick liver and muscle was determined by the method of Good *et al.* [1933], the sugar formed by hydrolysis being estimated by Somogyi's methods (Somogyi [1926]: for very small quantities of sugar, Somogyi [1937]). The liver and muscle samples were taken immediately after removing the embryo from the egg, and were dropped at once into weighed tubes of 30% KOH.

RESULTS

1. *Growth and glycogen content of liver cultures.* Liver cultures were grown from 6- to 16-day-old chick embryos and in all cases abundant glycogen was found in the cells, both in the centre of the explant and in the cells at the edge of the outgrowing epithelial sheet. The cultures usually remained healthy and retained their glycogen for 5 to 7 days after implantation without the medium being renewed in any way. Nordmann [1929], who made an extensive study of the growth of liver cells *in vitro*, claimed that all glycogen was broken down during the implantation and was only built up again by the liver cells at the end of the first 24 hr. But glycogen was seen histologically 6 hr. and 19 hr. after implantation, and chemical estimations by Heatley's [1933] micro-method of pieces of embryonic liver, cut up to the size of culture implants and left to stand in saline for 1 hr., showed glycogen contents of 0.315 to 2.17% (dry wt.).

The percentage of sugar in the medium did not have much effect on the glycogen content of the cultures, but those grown in a 0.5% or 1.0% sugar medium remained healthy and retained their glycogen longer than those grown in a medium to which no sugar was added.

For experiments with adrenaline, cultures of liver from 11- to 15-day-old embryos grown in a 0.5% or 1.0% sugar medium were used 4-6 days after implantation.

2. *Effect of adrenaline on liver cultures.* Groups of cultures were chosen as much alike as possible in size and extent of growth. To one group a drop of adrenaline solution was added, while to the control group a drop of Tyrode solution was added, and the cultures incubated at 38°. After varying periods of incubation they were tested histologically for glycogen. Solutions of adrenaline in concentrations of from 1 : 100 to 1 : 1,000,000 were employed and the pH of the solution was always adjusted to the same as that of the Tyrode solution used for the controls. The cultures were stained for glycogen after 5, 10, 15, 20, 30 and 40 min. and 7, 11 and 16 hr. The adrenaline-treated cultures, even those left for 16 hr. with a drop of 1 : 100 adrenaline solution, showed no signs of necrosis or destruction of the epithelial sheet, and in no case was their glycogen content diminished in comparison with the controls.

As Blaschko *et al.* [1937] and others have shown that liver tissue is one of the most active in destroying adrenaline in the presence of O₂, it was possible that, in spite of the strong doses used, the adrenaline was being oxidized to inactive compounds before it could diffuse into the cells. The drop of adrenaline was therefore sucked off and renewed every $\frac{1}{2}$ hr. for 3 hr., various concentrations of adrenaline being employed. There was no diminution of the glycogen content in the culture so treated, and it seemed unlikely that all the adrenaline added would have been oxidized before it could exert its effect.

In view of the possibility that in the absence of the pituitary the glycogenolytic action of adrenaline might be impaired [Corkill *et al.* 1933; Cope & Marks, 1934; Bachman & Toby, 1936], an alkaline extract of ox anterior pituitary was prepared, according to the technique described by Cope & Marks [1934]. The fresh glands were ground up with acetone, the acetone removed by filtration and evaporation in air and the acetone-free tissue extracted with saline. This extract was added to cultures, both alone and together with adrenaline, in several doses for 40 min. No reduction in their glycogen contents, as compared with the controls, was seen histologically. In all about 50 cultures were treated with adrenaline under the various conditions described.

It seemed therefore that under the conditions of the experiments adrenaline had no direct effect on the glycogen of isolated liver cells.

There was, however, an important possibility to be investigated before these results could be taken as an indication that the glycogenolytic action of adrenaline was not a direct one. This possibility was that the young chick embryos from which these cultures were made might not respond to a dose of adrenaline *in vivo* with a fall of liver glycogen.

Vladimirov [1931] injected very large doses of adrenaline (1 ml. 1 : 100 in some cases) into the air sac of the hen's egg and obtained evidence that the blood sugar was raised in the 19- and 20-day, and occasionally in the 14- and 15-day embryo, though not in the embryo younger than 14 days. But the values for the blood sugar level of normal and adrenaline-treated embryos all varied enormously and the adrenaline was given usually in doses of 0.5–1 ml. The absorption of such quantities of fluid he reports earlier in the paper as causing a fall of blood sugar in the younger embryos, due to a dilution of the blood. This might easily conceal any slight rise of blood sugar that would be produced by the breakdown of liver glycogen in, say an 11-day embryo. In such an embryo the quantity of liver tissue, and therefore of liver glycogen, present is very small compared with the volume of blood circulating in the whole egg.

Estimations of the liver glycogen, rather than of the blood sugar, of such small embryos would therefore be more likely to reveal any glycogenolytic action which adrenaline might have.

Accordingly an investigation of the effect of adrenaline on the liver glycogen of chick embryos was undertaken, and at the same time its effect on muscle glycogen was studied.

3. *Effect of adrenaline on the glycogen of chick embryos.* As the literature contained no values for the liver glycogen content of the chick embryo earlier than the 14th day of incubation, estimations were first made of liver glycogen of normal embryos from the 8th to the 21st day (see Fig. 1). Appreciable quantities of glycogen are present from the 8th to the 12th day (0.2 to 0.9%), followed by a sudden rise on the 13th or 14th day. The figures for each day of incubation show very wide variations, but the curve drawn through the average values shows the same rise up to the 19th day and subsequent fall at hatching as Vladimirov [1930] found.

The effect of adrenaline on the liver and muscle glycogen of 1-, 14- and 18-day-old embryos was then determined in the following way. A number of eggs, containing embryos of the same age, was divided into two groups, usually of about 6 to 10 eggs each. A small hole was made in the shell over the air sac, and with a hypodermic syringe 0.1 ml. 1:1000, and in a few cases 0.1 ml. 1:50,000 adrenaline was injected into the air sacs of one group, and 0.1 ml. Tyrode solution at the same pH was injected into the air sacs of the control group. The hole in the shell was sealed up carefully with paraffin wax and the eggs were incubated for 40 to 60 min. air sac uppermost. During this time all the injected solution was adsorbed and the surface of the air sac usually appeared white and dry. The embryos showed no ill effects after this treatment. They were removed from the eggs and their livers rapidly transferred to weighed tubes of 30% KOH. Large strips of pectoral muscle were taken for the muscle glycogen determinations. In the case of the 18-day embryos samples from 1 or 2 chicks were used for each determination, in the case of 14-day embryos samples from 1 to 4 chicks, and in the case of 11-day embryos samples from 5 to 7 chicks. Altogether 174 chicks were used: 46 18-day, 67 14-day and 61 11-day.

As can be seen from Fig. 2, the glycogen contents of the livers of 11-, 14- and 18-day embryos treated with adrenaline were much reduced as compared with the saline-injected controls. It appeared therefore that adrenaline caused a mobilization of liver glycogen even in the youngest embryos from which liver cultures were made, and a lack of action *in vivo* could not therefore be the cause of the lack of action *in vitro*.

An investigation of the effect of adrenaline on embryonic muscle glycogen was of interest because of the findings of Needham & Nowinski [1937] that embryonic chick tissue was unable to glycolyse glycogen, being deficient, or partly so, in four of the essential enzyme components of phosphorylating glycolysis.

Fig. 1. Liver glycogen in chick embryos. Numbers denote the number of embryos used for a determination, if that number was greater than one.

One of these enzymes was that responsible for the initial esterification of glycogen.

In Nachmansohn's [1937] experiments with frog muscle "brei" and adrenaline, and in Hegnauer & Cori's [1934] experiments with whole muscles placed in adrenaline solution, an accumulation of hexosemonophosphate was noted. It seems reasonable to suggest therefore that adrenaline might exert its accelerating effect at this initial step in lactic acid formation (*i.e.* the phosphorylation of glycogen). As slices of muscle from a 15-day chick embryo were unable to

Fig. 2.

Fig. 2. Effect of adrenaline on embryonic chick liver glycogen.
 • saline-injected embryos; × adrenaline-injected embryos.

Fig. 3.

Fig. 3. Effect of adrenaline on embryonic chick muscle glycogen.
 • saline-injected embryos; × adrenaline-injected embryos.

glycolyse glycogen in Needham & Nowinski's experiments, it might be anticipated that adrenaline would fail to cause a breakdown of muscle glycogen before the 15th day. Fig. 3 shows that no significant difference between the muscle glycogen values of the adrenaline-treated and saline-treated 11- and 14-day embryos was obtained, whereas on the 18th day of development there was a quite well defined reduction in muscle glycogen under the influence of adrenaline. No experiments have been done to ascertain whether a "brei" of 18-day embryo muscle can produce lactic acid from glycogen, but it would appear from these results that the enzyme, or enzymes, on which adrenaline acts to cause a breakdown of muscle glycogen to lactic acid are present by the 18th day of development.

In the 11- to 14-day embryo the liver glycogen, but not the muscle glycogen, is reduced by adrenaline.

The failure of adrenaline to cause any breakdown of glycogen in the cultures of 11- to 14-day embryonic liver was therefore in contrast to its effects *in vivo*. There might of course be some condition, not fulfilled in the culture experiments,

which would enable a glycogenolytic effect of adrenaline to be demonstrated *in vitro*, but in view of the other possibilities of adrenaline's mode of action on liver glycogen it seemed of importance to study the effect of anaerobiosis on the glycogen of liver cultures.

4. *The effect of anaerobiosis on liver cultures.* Cultures were subjected to an anaerobic atmosphere in special flasks as illustrated in Fig. 4. The coverslip on which the culture was grown was placed on the plate-glass sheet *P*, and held in

Fig. 4. Cross-section of apparatus employed for the subjection of cultures to an anaerobic atmosphere. *C*=culture on coverslip. *G*=ground glass surfaces. *P*=plate-glass sheet. *S*=sealing compound. *T*=glass taps. *W*=saline to keep atmosphere moist.

position with a little vaseline. A rubber grease was used between the ground-glass surfaces at *G*, and a sealing compound applied around the edges to complete the air-tight joint. The flasks were connected in series and a stream of N_2 and CO_2 mixture (95% N_2 and 5% CO_2) passed through them for 1 hr. The taps were then turned off and the flasks placed in the incubator. The culture could be observed microscopically at any stage of the procedure through the plate-glass sheet *P*.

Cultures were tested for glycogen after 1 to 6 hr. anaerobiosis at 38°. 10 hr. anaerobiosis were found to cause the death of all cultures so treated; the epithelial sheet appeared broken up and the individual cells rounded off. But cultures submitted to periods of 1-6 hr. anaerobiosis were found to remain healthy and to exhibit changes in their glycogen content.

Out of 45 cultures tested after 1-6 hr., 21 whose sister cultures all contained large quantities of glycogen were rendered completely glycogen-free.

DISCUSSION

The experiments reported here on embryonic chick muscle glycogen gave results which are in agreement with the work done by Needham and his collaborators. However, the results of the experiments which show a breakdown of glycogen in cultures of 11- to 14-day embryonic liver under anaerobiosis and a reduction in liver glycogen in the intact embryo's liver after injection of adrenaline are somewhat difficult to reconcile with their findings. They used "breis" of whole 3- to 13-day-old chick embryos and found no glycolysis of added glycogen, although lactic acid was formed rapidly from added glucose. From the results with tissue cultures and intact embryos reported in this paper it appears, however, that there is present in the liver, though not always in muscle, an enzyme system capable of breaking down glycogen. In order to explain the difference between these results and those of Needham *et al.* it must be assumed either (a) that this enzyme system can attack glycogen which is actually in the liver cells of the embryo but not that added to the surrounding medium, or (b) that the glycogen is breaking down to a substance which is neither glucose nor lactic acid. The former suggestion would seem the more likely.

The difference between the effect of adrenaline on the liver glycogen of chick embryos *in vivo* and *in vitro* appears to indicate that the glycogenolytic action of adrenaline is not a direct one on the liver cells but is dependent on the presence

of factors obtained only in the whole body. Against this assumption, however, is the possibility that liver cells in a culture may be in a somewhat different metabolic state from those in the intact embryo. For example, cultures which have had their medium repeatedly renewed over a period of about 14 days cease to store any glycogen, owing probably to an abnormally high rate of proliferation induced in them, as Doljansky [1930] suggests.

Although the cultures used in the experiments here reported still retained their glycogen and were capable of utilizing it under anaerobiosis, it is possible that even during their 4 or 5 days of life in an artificial medium the cells had become altered in some way which would affect the action of adrenaline. For instance, a certain definite reduction potential of the various cell components might be necessary to enable adrenaline to exert its effect, and a shift in this potential, due to a change in environment, might thus render it inactive as a glycogenolytic factor.

SUMMARY

1. Cultures of 11- to 14-day chick embryo liver, containing large quantities of glycogen easily observed by staining methods, were prepared. Adrenaline added to these cultures under a variety of conditions had no effect on their glycogen content.

2. Yet the chick embryo on the 11th, 14th and 18th days of incubation responded *in vivo* to doses of adrenaline with a well-marked fall of liver glycogen.

3. Although no reduction of muscle glycogen in the chick embryo under the influence of adrenaline was observed on the 11th and 14th days, there was a definite reduction on the 18th day of development. This result is in agreement with the results of Needham *et al.*, who found that the enzyme system in muscle, upon which adrenaline might exert its effect, was not present in the early stages of development.

4. Subjection of liver cultures to anaerobic conditions caused the complete breakdown of glycogen in a large number of cultures tested after 1-6 hr. anaerobiosis.

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