CCCVI. THE DISTRIBUTION OF GLYCOGEN IN THE REGIONS OF THE AMPHIBIAN GASTRULA; WITH A METHOD FOR THE MICRO-DETERMINATION OF GLYCOGEN.

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Linderstrøm-Lang and Holter [1931] have described a micro-technique of great beauty, which they have used, amongst other things, for the estimation of reducing sugars [1933]. With the help of this technique, the Pflüger [1904] method for the quantitative precipitation of glycogen was adapted to the estimation of small amounts of this substance. A number of difficulties were met with, and the experimental procedure is given below in some detail, as it was found that slight alterations in the method might cause considerable errors.

Until a short time ago it was maintained by histologists [*e.g.* Woerdemann, 1933] that whereas the neural plate of the amphibian gastrula was rich in glycogen, the mesoderm, which is formed by invagination of the neural plate ectoderm, contained almost none. It has been suggested by Pasteels [1935], however, that the mesoderm does in fact contain glycogen, but that it is washed out of the cells during the fixing process. This suggestion is supported by Pasteels by experimental evidence which is shortly to be published.

When the method for the micro-estimation of glycogen had been satisfactorily worked out, direct chemical measurements were made of the glycogen contents of various regions of the gastrula, which were isolated by dissection.

I. *Micro-estimation of glycogen.*

Principle. The tissue is weighed out into glass tubes measuring 5×120 mm. and digested by heating with potassium hydroxide. The glycogen is then precipitated by adding alcohol, and flocculated by heating to boiling-point [Good *et al.*, 1933]. It is then centrifuged down and washed twice with absolute alcohol, after which it is hydrolysed with acid, which is subsequently neutralised; the reducing sugar formed is then estimated by the method of Linderstrøm-Lang and Holter [1933].

Experimental procedure. 1. About 35μl. of 30% potassium hydroxide are measured into the small tube, which is stored in a CO_2-free atmosphere till the tissue has been weighed out.

2. The tissue is added and the tube is capped.

3. The tube is heated in a steam-bath for 20 min.

4. With the same pipette which has been used for measuring out the potash, 35μl. of water and then 105μl. of absolute alcohol are added. (Unless the alkaline solution is diluted before adding the alcohol, two layers are formed which will not mix.)

5. The contents of the tube are thoroughly mixed by rotating and at the same time stirring with a fine platinum wire.

6. The tube is immersed in the steam-bath for a few seconds to flocculate the glycogen, care being taken that it is removed before the contents spurt out.

7. The tube is centrifuged at 3500 r.p.m. for 15 min.

8. The clear centrifugate is removed and discarded by inverting the tube and drawing it off by means of a fine pipette.

9. The residue is washed by discharging about 100 μl. of absolute alcohol from a fine pipette on to the solid at the bottom of the tube.

10. The tube is centrifuged at 3500 r.p.m. for 5 min., and the centrifugate is removed as before.

11. The washing process is repeated once more.

12. After the final washing, the centrifugate is discarded, and the last traces of alcohol are removed from the residue by placing the tube in the steam-bath for a few minutes.

13. About 35 μl. of 0·6 N hydrochloric acid are then added in such a way that the deposit adhering to the upper parts of the tube is washed down. (This operation requires considerable care if it is to be effective, on account of the small volume of fluid used.)

14. A small cube of paraffin wax, weighing 20–30 mg., is added, and the tube is placed in the steam-bath for 2½ hours.

15. After the hydrolysis, the tube is removed and whilst still hot is inverted and rotated so that an even coating of wax is spread over the upper parts of the tube.

16. A stirrer, consisting of a small glass sphere filled with iron filings, is added, and the thin film of wax on the actual surface of the hydrolysate is broken by rotating the tube whilst it is held against an electromagnet.

17. A micro-drop of 0·05 % aqueous thymol blue is added and the contents are titrated against N NaOH till a bluish grey colour is obtained. N NaOH is used in order to keep the volume as small as possible, and the end-point is often overshot; this is easily adjusted, however, by adding dilute (0·1 N) hydrochloric acid from a finely drawn out capillary. When the tubes have been neutralised they are kept in a CO_2-free atmosphere.

Blanks are set up by taking 35 μl. of 0·6 N HCl, adding a drop of thymol blue, neutralising and adding 50 μl. of buffer before adding the iodine; the films of sulphuric acid and starch are formed as usual. (For details of the sugar estimation reference must be made to Linderstrøm-Lang and Holter [1933].)

Accuracy of the method. This was tested by a number of experiments, of which the following are typical.

15 μl. of approximately 1·0 % glycogen solution were accurately measured into the bottom of each of six tubes. The glycogen in three of them was hydrolysed directly, whereas the remaining tubes were heated for 20 min. with 30% potash, and the glycogen was precipitated in the manner described above before being hydrolysed. The sugar was then estimated in all tubes, the following thiosulphate titrations being obtained:

Hydrolysed at once		Hydrolysed after precipitation	
Titration	Vol. of thiosulphate ≡ reducing sugar	Titration	Vol. of thiosulphate ≡ reducing sugar
12·90	29·50	13·25	29·15
12·80	29·60	13·30	29·10
12·90	29·50	13·25	29·15

Mean blank value 42·40.

Thus there is a small loss of glycogen during the precipitation. It remained to determine in what way this loss is related to the total amount of glycogen present.

This was done by taking samples of equal volumes of different glycogen solutions of known relative concentration and estimating the glycogen in them by this method. The following results are typical:

Sample	Conc. of glycogen %	Titration	Vol. of thiosulphate ≡ reducing sugar
1	1·00	11·70	29·90
2	1·00	11·35	30·25
3	0·20	35·40	6·20
4	0·20	35·35	6·25
5	0·10	38·20	3·40
6	0·10	38·50	3·10
7	0·05	39·75	1·85
8	0·05	39·85	1·75

Mean blank value 41·60.

These results have been plotted in Fig. 1.

It will be seen that the points lie on a straight line which does not quite pass through the origin. The fact that the points lie on a straight line shows that the amount of glycogen lost during the precipitation is a constant percentage of the

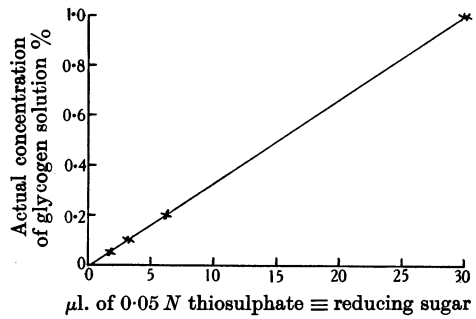

Fig. 1.

total amount present; the fact that the line does not pass through the zero of abscissae suggests that the blank value is too high, but only by about 0·4 μl. A correction is, of course, made for this.

The maximum error which has been observed in a series of parallel estimations is ±0·3 μl. of 0·05 *N* thiosulphate. Now 2·0 μl. of 0·05 *N* thiosulphate are equivalent to approximately 0·01 mg. of glycogen. Therefore there should be a maximum error of less than ±2 γ of glycogen.

The loss of about 1·5 % incurred during the precipitation does not affect the results, since it has been found most convenient to standardise the reagents by means of a control estimation of a solution of glycogen of known strength.

Whether the same degree of accuracy may be expected when working with tissues instead of with solutions of pure glycogen is a point which cannot be tested, since no glycogen-containing tissues are available which are sufficiently homogeneous for this purpose.

II. *Distribution of glycogen in the regions of the amphibian gastrula.*

A preliminary experiment was performed with the gastrula of the axolotl, but the remaining work was done on *Triton alpestris*. Each gastrula was divided into the following regions:

1. Neural plate ectoderm.
2. Ventral ectoderm.
3. Mesoderm.
4. Endoderm.

The embryos were all in the "late gastrula" stage, though some were definitely more advanced than others.

Treatment of tissue. With such small quantities as were used, it was quite out of the question to determine the wet weight of the tissue. On the other hand, care had to be taken that during the drying of the tissue (preparatory to obtaining its dry weight) no glycogen was broken down. The material equivalent to two or three embryos was used for each determination, and as each piece was dissected out it was dropped into absolute alcohol, a procedure calculated to stop effectively all enzyme action. When enough had been collected, the material was placed in a punt-shaped vessel of platinum and dried for 2 hours at 100° in a gentle stream of dry air. When cool, the punt was weighed on a micro-balance; some of the material was then removed, after which the punt was reweighed. The dried tissue was more or less compact, and did not adhere at all to the vessel in which it had been dried. The most satisfactory way of transferring the tissue to the glass tube is to touch it with a needle which has been dipped in vaseline and wiped almost clean, and then to detach it from the needle into the tube with a gentle tap.

The assumption has been made that from the time of excision of the tissue none of its glycogen has been lost. The absolute alcohol would fix the material practically instantaneously, and as it would have access to both sides of the tissue it is unlikely that there would be any appreciable loss of glycogen.

A more serious source of error lies in the fact that a certain amount of material will be dissolved from the tissues by the absolute alcohol, and thus give a low value for the dry weight of the material. It is probable that there will be more loss from the tissues of some regions than from others, due to the greater amount of alcohol-soluble substances. The exact loss could easily have been determined if sufficient material had been available.

Table I.

		Neural plate ectoderm		Ventral ectoderm		Mesoderm		Endoderm	
		Wt. of tissue mg.	Glycogen %	Wt. of tissue mg.	Glycogen %	Wt. of tissue mg.	Glycogen %	Wt. of tissue mg.	Glycogen %
Axolotl		1·13	17·1	—	—	1·16	11·1	—	—
		1·34	15·0	—	—	1·11	11·0	—	—
		1·25	15·0	—	—	—	—	—	—
Triton alpestris	1	0·43	19·7	—	—	0·54	12·4	0·93	8·8
	2	—	—	—	—	0·32	16·1	1·00	8·0
	3	0·40	18·6	0·75	15·8	0·36	10·1	1·80	5·5
		0·30	19·1	0·65	16·9	0·48	8·8	—	—
	4	0·76	17·5	0·59	15·1	0·67	10·9	1·15	6·5
		—	—	—	—	0·64	9·3	—	—
	5	0·33	23·6	0·79	16·5	—	—	1·34	7·2
	6	—	—	0·78	14·7	0·36	13·2	1·33	5·9

Results. These are given in Table I. There is a considerable scatter, due, partly, no doubt, to biological variation, but mainly to the extremely unfortunate fact that the indicator used for neutralisation after hydrolysis contained a small amount of alcohol, which reacted to some extent with the iodine to form iodoform. As the reagents were standardised against a glycogen solution of known strength, the error would be small, except in cases where the amount of tissue taken was smaller than usual; here a high value would be found. Subsequent experiments with this particular indicator showed that the error could not be greater than 10%, though it was too erratic for a correction to be applied. It is emphasised, therefore, that the results as they stand are slightly too high.

Discussion.

The results described above lend direct support to the view that the mesoderm of the amphibian gastrula contains a certain amount of glycogen. It is clear, on the other hand, that during invagination through the dorsal lip of the blastopore there is a distinct loss of glycogen, since the mesodermal roof of the archenteron contains less than the uninvaginated dorsal ectoderm (presumptive neural plate). The significance of this for our knowledge of the metabolism of the organisation centre and the liberation of the evocator may be considerable. A discussion of this aspect of the problem will be found in the paper of Waddington *et al.* [1935].

Summary.

1. A method is described for the estimation of glycogen in amounts of tissue of the order of 1 mg.; the probable error is less than $\pm 2\gamma$ of glycogen.

2. The amounts of glycogen in the various morphologically distinct regions of the amphibian embryo during gastrulation have been estimated. The dorsal ectoderm has most, the yolk endoderm least and the ventral ectoderm occupies an intermediate position. During invagination, and hence during the liberation of the evocator, there is a diminution of about 35% of the glycogen in the invaginating cell layers.

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