MICROMETHOD FOR THE ESTIMATION OF GLYCOGEN IN THE GENITAL ORGANS OF THE MOUSE

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Summary

(i) A micromethod has been described for the estimation of small amounts of glycogen in the vagina and uterus of the mouse.

(ii) The method involves digesting the tissue in KOH, precipitating with 60 per cent. ethanol, and extracting glycogen from the precipitate with 5 per cent. trichloroacetic acid. The glycogen is estimated with the anthrone reagent.

(iii) The method was satisfactory for estimating amounts of glycogen down to 5 μg in the presence of tissue hydrolysates made from the mouse vagina. A quantitative recovery of the glycogen was obtained.

I. INTRODUCTION

Various methods have been described for the determination of glycogen in small amounts of tissue and in tissue of low glycogen content, e.g. Good, Kramer, and Somogyi (1933); Zondek and Shapiro (1942); Boettiger (1946); Seifter *et al.* (1950); Walaas and Walaas (1950); van der Kleij (1951); Walaas (1952*a*, 1952*b*); Bowman (1953); Fong, Schaffer, and Kirk (1953); Kemp and van Heijningen (1954), and van der Vies (1954). The authors of all of these papers, except Fong *et al.* (1953) were concerned with estimating glycogen in amounts greater than 100 μg. The aim of the present work was to produce a method capable of estimating the very small amounts of glycogen which are present in the vagina and the uterus of the mouse. Experience has shown that, depending on the degree of stimulation by oestrogens, these quantities are aften as low as 5 μg and are rarely greater than 100 μg (Balmain, Biggers, and Claringbold 1956).

In most of the methods, the tissue is digested in strong potassium hydroxide solution and the glycogen is precipitated by the addition of ethanol. Recent workers have favoured the anthrone-reducing sugar method for the estimation of the precipitated glycogen, since the anthrone reagent reacts directly with polysaccharides, e.g. glycogen, and its use eliminates a hydrolytic stage. The method we have evolved follows these principles, and is modelled on the micromethod of Seifter *et al.* (1950). Various aspects of the method, including an examination of the "blank" due to interfering substances, have been studied using multifactor experimentation (Fisher 1949; Bennett and Franklin 1954).

II. EXPERIMENTAL

(*a*) *Preparation of Tissues*

Randomly-bred albino mice were used in this work. They were killed by dislocation of the spine, and the genital tract was then dissected out as rapidly as possible. After removal of extraneous fat, the vagina and the uterine horns were separated from the cervix, and the urethra was stripped from the vaginal wall.

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(*b*) *Estimation of the Glycogen by the Anthrone Reaction*

Glycogen was estimated colorimetrically with the anthrone reagent, following closely the method of Seifter *et al.* (1950). However, the volumes of reagents used were reduced in order to allow smaller quantities of glycogen to be estimated.

The anthrone reagent (0·2 per cent. solution in 95 per cent. sulphuric acid) is freshly prepared each day.

The glycogen is dissolved in 2·5 ml of distilled water or 5 per cent. trichloroacetic acid (TCA) and 5·0 ml of the anthrone reagent added. The tubes containing the glycogen solution are agitated in cold running water throughout the addition of the anthrone reagent; the reacted solutions are then heated for 10 min in a boiling water-bath, cooled, and the percentage transmission read at 620 mμ in a Coleman spectrophotometer.

Transmission readings between 15 and 60 per cent., which cover the accurate range of the spectrophotometer, are given by 20-100 μg glycogen per tube. The columns headed "unprecipitated" in Table 1 contain examples of calibration curves. Log transmission is linearly related to the concentration of glycogen. However, secular shifts in the position of the calibration curve are a characteristic finding, and thus it is essential to determine a calibration curve for each experiment.

In tissue samples where the glycogen content is less than 20 μg it is necessary to boost the solutions of extracted glycogen by the addition of a known quantity of glycogen. When low levels of glycogen are expected, either 20, 30, or 40 μg of glycogen in 0·4 ml of water are added to the dissolved extracted glycogen, and at the same time the total volume of the glycogen solution is adjusted to 2·5 ml.

(*c*) *Recovery of Glycogen from Aqueous Solution* (*Table* 1)

An experiment was designed to study the precipitation with 60 per cent. ethanol of amounts of glycogen of less than 100 μg. Preliminary experiments had shown that the volume (up to 0·5 ml) of KOH in which the glycogen is dissolved is not critical for the recovery of glycogen. It had also been found that the speed of centrifugation is important for the collection of the precipitated glycogen, appreciable losses occuring with speeds less than 2500 r.p.m. The details of the experiment are shown in Table 1.

The sets of four tubes corresponding to each glycogen level on each day are prepared from a common glycogen solution, and the results are therefore correlated. The experiment is thus of the split-plot type and calls for a special form of analysis (Cochran and Cox 1950). The analysis of variance is divided into two parts, each having a separate error term. The day and glycogen effects are examined from the plot totals and an inter-plot error, while the treatment effects are tested against an intra-plot error. The analysis shows that a large difference exists between the mean results on each day. The calibration curve is parallel on the 2 days and is linear over the range of glycogen levels studied. No difference is observed between the unprecipitated and the precipitated material, and thus the addition of 60 per cent. ethanol leads to quantitative precipitation of glycogen from aqueous solution. This differs from the results of Walaas (1950*a*, 1950*b*) who found that losses of glycogen occurred in the precipitation process.

The experiment has not been repeated for the glycogen range of 5-20 μg since a final check of the method includes this range (see below). The highly significant day difference indicates that it is essential to determine a separate calibration curve on each day.

TABLE 1

RECOVERY OF GLYCOGEN FROM AQUEOUS SOLUTIONS

Glycogen (μg)	Optical Density							
	Day 1				Day 2			
	Unprecipitated		Precipitated		Unprecipitated		Precipitated	
20	1·79	1·79	1·81	1·83	1·83	1·84	1·83	1·84
30	1·70	1·67	1·71	1·68	1·73	1·68	1·76	1·75
40	1·58	1·56	1·62	1·62	1·65	1·68	1·60	1·65
50	1·50	1·46	1·50	1·47	1·54	1·58	1·54	1·60
60	1·40	1·41	1·45	1·36	1·46	1·49	1·44	1·48
70	1·34	1·32	1·32	1·30	1·37	1·37	1·38	1·39
80	1·23	1·32	1·25	1·20	1·26	1·32	1·29	1·28
90	1·11	1·28	1·15	1·15	1·20	1·11	1·20	1·24
100	1·04	1·00	1·04	1·04	1·08	1·13	1·15	1·16

Analysis of Variance

Source of Variation	D.F.	Mean Square†	F
Between days	1	50·1	35·8***
Between glycogen levels:	(8)		
Linear	1	392·8	2800***
Remainder	7	0·6	< 1
Inter-plot error	8	1·4	
Between treatments	1	0·9	< 1
Days $\times$ treatments	1	1·0	< 1
Glycogen levels $\times$ treatments	8	0·7	< 1
Intra-plot error	44	1·25	

*** $P < 0{\cdot}001$. † Mean squares have been multiplied by 1000.

(*d*) *Substances which Interfere with the Estimation of Tissue Glycogen*

Seifter *et al.* (1950) pointed out that proteins which are precipitated along with glycogen in 60 per cent. ethanol are likely to cause an over-estimation of glycogen when reducing sugar methods are used. The amount of interference is smaller with the anthrone reagent than with other methods of sugar estimation, as tryptophan is the only amino acid giving a colour with anthrone at 620 mμ (Seifter *et al.* 1950; Sheltar 1952). It is also possible that other substances such as hexose sugars and related compounds may interfere, as these give a colour reaction with the anthrone

reagent (Sattler and Zerban 1950; Sheltar 1952; Hill, Hawkins, and Strasser 1953). However, since Little (1949) and Young and Raisz (1952) have shown that free reducing sugar is completely destroyed by heating with alkali, protein material seems likely to be the most serious contaminant in the present method.

TABLE 2
THE REDUCTION OF THE BLANK BY TCA EXTRACTION

Test No.	Glycogen Equivalent of Blank (μg)			
	No TCA Extraction		TCA Extraction	
1	49	44	38·5	35
2	18	21	12	16
3	9·5	7	6	5·5
4	8	14·5	4	5·5
5	12	12	9·5	8·5
6	17	13	12·5	12
7	12·5	18	12	13·5
8	9	5	7	9·5
9	9	13	9	6·5
10	17	7	7	6
11	8	6·5	6	7
12	7	11	9	8
13	10	11·5	9	8

Analysis of Variance

Source of Variation	D.F.	Mean Square	F
Between tissue samples	12	186·6	19·2***
Between treatments	1	171·9	17·7***
Samples × treatments	12	11·4	1·17
Error	26	9·72	

*** $P < 0{\cdot}001$.

Several workers have attempted to purify glycogen by dissolving it again and reprecipitating it with 60 per cent. ethanol (Seifter *et al.* 1950; Walaas 1952*a*, 1952*b*; Bowman 1953). In our experience this procedure was found to reduce the "blank" by only a small amount. Increasing the concentration of KOH beyond 30 per cent. lowered the blank but also affected the recovery of glycogen. However, increasing the volume of KOH solution from 0·2 ml as used by Walaas (1952*a*, 1952*b*), to 0·4 ml significantly reduced the blank while it did not affect the recovery of glycogen. A volume of 0·4 ml has thus been used throughout this work.

(*e*) *Reduction of Tissue Blank by Trichloroacetic Acid Extraction* (*Table* 2)

If tissues are incubated at 37°C in phosphate-Ringer solution (pH 7·4), the glycogen breaks down due to enzyme action. When the tissue is then extracted for

glycogen, and the anthrone reaction is carried out, a considerable blank is obtained, presumably due to non-specific protein substances (see below).

Since TCA dissolves glycogen and precipitates proteins, it was introduced into the method to see if it reduced the blank. The experiment described in Table 2 demonstrates the effect of TCA extraction of the ethanol precipitate. The analysis of variance shows that the difference between the tubes treated and those not treated with TCA is highly significant. The use of TCA leads to a 23·5 per cent. reduction of the blank, and since the samples × treatments mean square is not significant, the reduction of the blank may be considered uniform for all samples.

(*f*) *Preparation of Tissue for the Study of the Blank* (*Fig.* 1)

The TCA extraction technique was used to follow the breakdown of glycogen in the vagina of the mouse during incubation at 37°C in phosphate-Ringer (pH 7·4).

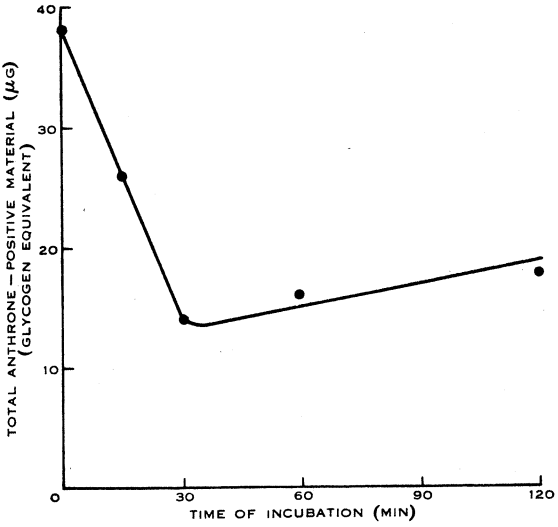

Fig. 1.—The breakdown of glycogen in mouse vaginae during incubation in phosphate-Ringer at 37°C.

The design and results of the experiment are shown in Figure 1. There is a rapid reduction in the quantity of anthrone-positive material during the first 30 min, after which no further reduction takes place. The initial fall is due to glycogen breakdown, and the residual material may be attributed to interfering substances. The slight rise occurring after 30 min is statistically significant ($0{\cdot}01 > P > 0{\cdot}001$) but its cause is unknown. Its presence stresses the necessity of incubating the tissue for a constant time in the determination of non-glycogen substances.

In a further experiment, the blanks of vaginae incubated at 37°C in phosphate-Ringer and in phosphate-Ringer containing 0·2 ml saliva were compared. No significant difference was found between them ($t_{(8)} = 1{\cdot}08$, $P > 0{\cdot}05$), which confirms the non-glycogen nature of the blank.

(g) Quantitative Recovery of Glycogen in the Presence of Tissue (Table 3)

The experiment shown in Table 3 was designed to examine the quantitative recovery of glycogen in the presence of tissue with the TCA extraction procedure. The linear regression of log transmission on concentration of glycogen is highly significant when compared with the deviations from linear regression (remainder).

TABLE 3

RECOVERY OF GLYCOGEN IN THE PRESENCE OF HYDROLYSED GLYCOGEN-FREE TISSUE

Glycogen (μg)	Optical Density			
	Unprecipitated Glycogen plus Precipitated Tissue		Precipitated Glycogen plus Precipitated Tissue	
5	1·71	1·69	1·71	1·69
10	1·66	1·66	1·59	1·63
15	1·53	1·62	1·56	1·57
20	1·58	1·57	1·59	1·56
25	1·48	1·52	1·52	1·52
30	1·45	1·44	1·40	1·47
35	1·44	1·45	1·33	1·40
40	1·39	1·39	1·27	1·34
45	1·30	1·32	1·32	1·31
50	1·20	1·21	1·28	1·26

Analaysis of Variance

Source of Variation	D.F.	Mean Square	F
Between glycogen levels	(9)		
Linear	1	805·0	1103***
Remainder	8	1·0	1·4
Between treatments	1	2·4	3·3
Glycogen levels × treatments	(9)		
Linear	1	0·2	< 1
Remainder	8	2·3	3·2*
Error	20	0·73	

* $0{\cdot}05 > P > 0{\cdot}01$; *** $P < 0{\cdot}001$.

However the glycogen × treatment mean square is also just significant. Further analysis shows this to be due to high-order, opposed, curvature effects of unknown origin. The presence of this effect, however, does not invalidate the conclusion that there is no significant difference between the treatments at the $P = 0{\cdot}05$ level. Thus there is adequate recovery of glycogen in the presence of hydrolysed tissue, and the method may be considered satisfactory for the microestimation of glycogen.

III. Proposed Method for the Microestimation of Glycogen

(*a*) *Reagents*

Potassium hydroxide: 30 per cent. aqueous solution.
Absolute ethanol.
Trichloroacetic acid: 5 per cent. aqueous solution.
Sulphuric acid: 95 per cent. aqueous solution, prepared by adding 1 l. A.R. sulphuric acid (S.G. 1·84) to 50 ml distilled water.
Anthrone reagent: 0·2 per cent. solution of anthrone (B.D.H.) in 95 per cent. sulphuric acid, prepared each day.

(*b*) *Procedure*

The vagina is bisected so that one-half may be used for total glycogen, and the other half for a blank or other determination. Similarly each uterine horn can be used for separate determinations. The piece of tissue is mopped roughly dry on blotting-paper, weighed quickly on a torsion balance (capacity 200 mg), and placed in a small tube (capacity 2 ml) containing 0·4 ml 30 per cent. KOH. The tube is then heated at 100°C for 30 min. The time taken from the death of the animal to placing the tissue in KOH is less than 2 min.

After digestion of the tissue with KOH, the glycogen is precipitated by the addition of 0·8 ml absolute ethanol giving a final concentration of about 60 per cent. Thorough mixture of the solutions is ensured by means of a thin glass rod, which is reserved for later steps. The ethanol mixture is heated to boiling in a water-bath and cooled for 5-10 min in a refrigerator. Glycogen is removed from this suspension by centrifugation at 3500 r.p.m. for 30 min. The supernatant is then decanted off and the tubes drained for at least 15 min on blotting-paper. When large numbers of estimations are being made it is convenient to store the precipitated glycogen at this stage by keeping the tubes in a refrigerator overnight.

Glycogen is obtained quantitatively from the microtubes by two successive extractions with 5 per cent. TCA, using 1·0 ml for the first washing and 1·5 ml for the second (total 2·5 ml). For each washing the TCA is added to the tubes, the mixture being thoroughly agitated by means of the stirring-rod used previously. The tubes are then centrifuged at 3500 r.p.m. for 20 min, and the supernatants are decanted carefully into 6 × 1 in. boiling tubes, each microtube being well drained.

The solutions collected in the boiling tubes are then subjected to the anthrone reaction as described earlier. The values obtained give an estimate of the "total glycogen", which will consist of "true glycogen" together with ethanol-precipitated interfering substances (i.e. the blank).

If the true glycogen content of the tissue is required, then a similar procedure is carried out on the other half of the tissue after incubation at 37°C in phosphate-Ringer (pH 7·4) for 60 min, in order to obtain an estimate of the blank. The difference between the total glycogen and the blank gives an estimate of the true glycogen content of the tissue. By the adoption of suitable randomization procedures an estimate of the true glycogen content of an organ may be made without taking exactly equal weights of tissue for the analysis of total and blank glycogen (see Balmain *et al.* 1956).

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