ram spermatozoa by 50%; 0·0008–0·0012M was required for complete inhibition of fructolysis and motility. Among similarly active substances were dodecyl sulphate, 2-phenoxyethanol, dodecylamine hydrochloride, sodium taurocholate, 'Lubrol' and 'Teepol'.

3. The surface-active compounds induced a change in the permeability of spermatozoa as indicated by the diffusion of cytochrome *c* from the cells into the surrounding fluid.

4. When succinate was added to detergent-treated immotile spermatozoa, the percentage increase of the oxygen uptake was much greater than in intact motile sperm; thus, the stimulating effect of succinate on the respiration of spermatozoa was an indication of a moribund cell population.

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Two Methods for the Determination of Glycogen in Liver

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Several methods for the determination of glycogen in tissues have been described (Good, Kramer & Somogyi, 1933; Morris, 1948; Boettiger, 1946; Seifter, Dayton, Novic & Muntwyler, 1950; van Wagtendonk, Simonsen & Hackett, 1946; van der Kleij, 1951). As we needed a simple technique for the study of the glycogenetic properties of adrenal steroids a survey was made of several techniques often used (see Table 1). For serial determinations the methods of Seifter *et al.* and van der Kleij are attractive, in part because the time-consuming separation of the glycogen by precipitation with alcohol is avoided. However, in Seifter's method this separation can only be omitted if the glycogen content of the liver exceeds 1%, while van der Kleij's technique is not specific for glycogen, since substances like glucose and glucose phosphates, which are known to occur in liver, are also estimated.

The results submitted in this paper show that the process of extracting the glycogen with trichloroacetic acid as proposed by van der Kleij can be used in combination with colorimetric techniques for the estimation of glycogen as described by van Wagtendonk *et al.* (with iodine) and Morris (with anthrone). In this way two methods were developed, which can be used for the serial determination of glycogen in liver. In particular the application of an iodine reagent proved to be very convenient for routine determinations of glycogen. In our hands, the use of this method instead of the more elaborate techniques of Pflüger (1905) and others led to a considerable saving of time and materials. Satisfactory results have been obtained, despite some limitations of the method, which will be discussed in detail.

REAGENTS AND METHODS

Extraction of glycogen from liver with trichloroacetic acid

If the iodine reagent is used for the determination of glycogen in the extracts the concentration of trichloroacetic acid (TCA) should not exceed the limits 4·9–5·1% (w/v). This is checked by titration with 0·1N alkali, and the solution adjusted if necessary by adding water or a concentrated solution of TCA.

Procedure. A 200 mg. sample of liver is weighed on a torsion balance and finely ground with 20 ml. of 5% TCA

in a mortar or preferably in a homogenizer (Potter & Elvehjem, 1936). The precipitate of proteins is filtered off and the clear filtrate submitted to analysis.

Estimation of glycogen in the extracts

(*a*) *With iodine*

Iodine reagent: 16·5 ml. of Lugol's solution, prepared by dissolving 1 g. of iodine and 2 g. of KI in 20 ml. of water, is added to 990 ml. of an aqueous solution, containing 25% (w/v) of KCl.

Procedure. In a colorimeter tube (1·2 cm. diameter) 2 ml. of a liver extract is added to 3 ml. of iodine reagent. After mixing, the optical density is read in a photometer at 650 mμ. against a blank, obtained by adding 2 ml. of 5% TCA to 3 ml. of reagent in the same way. The amount of glycogen is read from a calibration curve, which must be prepared using glycogen of the same origin as the sample to be estimated.

(*b*) *With anthrone*

Anthrone reagent: 0·5 g. of anthrone is dissolved in 250 ml. of 95% (w/v) H_2SO_4. As this reagent deteriorates rapidly it should be freshly prepared before each series of determinations.

Procedure. 2 ml. of the liver extract are pipetted into a test tube calibrated at 20 ml., 2 ml. of 10N-KOH are added and the tube placed in boiling water for 1 hr. After cooling, 1 ml. of glacial acetic acid is added to neutralize the excess of alkali, and the fluid brought up to the mark with water: 2 ml. of the solution are added slowly to 4 ml. of anthrone reagent in a test tube, which is placed in cold water to prevent excessive heating. After mixing by lateral shaking, the tube is placed in boiling water for exactly 10 min. for colour development and cooled with tap water. The optical density is read within 2 hr. in a photometer at 650 mμ. against a blank, which is prepared by submitting 2 ml. of 5% TCA to the same procedure. For the calculation of the amount of glycogen, glucose standards are prepared in each series as follows: 2 ml. of TCA are heated to 100° with 2 ml. of 10N-KOH and the solution is neutralized with 1 ml. of glacial acetic acid as described above. Then 4 ml. of a solution containing glucose (0·15 and 0·60 mg.) are added. After dilution to 20 ml. with water the procedure is continued in the usual way.

Preparation of glycogen

The livers (or muscles) of 10–20 rats were quickly removed and transferred as rapidly as possible to 300 ml. of 30% (w/v) NaOH at 100°. Heating was continued for 2 hr. and the crude glycogen precipitated by adding twice the volume of 96% (v/v) ethanol. After separation, the precipitate was dissolved in a small volume of water and the solution adjusted to pH 3 with dilute HCl. The glycogen was re-precipitated by the addition of an equal volume of ethanol. This process of precipitation was repeated once more, and the resulting substance washed with ethanol and ether and finally dried *in vacuo*. The purity of the glycogen determined after conversion into glucose usually amounted to 90–95%. For these estimations glucose was determined with the Hagedorn & Jensen method. In all experiments optical densities were measured in a Lumetron photometer (model 402 E) provided with monochromatic filters.

Table 1. *Survey of some methods for the determination of glycogen*

	Pflüger (1905)	Good *et al.* (1933)	Morris (1948)	Boettiger (1946)	Seifter *et al.* (1950)	van Wagtendonk *et al.* (1946)	van der Kleij (1951)
Separation of glycogen from the tissues	Destruction by alkali, 2 hr.; precipitation by ethanol	Destruction by alkali, 20 min.; precipitation by ethanol	Destruction by alkali, 20 min.; precipitation by ethanol	Destruction by alkali, 2 hr.; precipitation by ethanol	Destruction by alkali, 20 min.	Destruction by alkali, 2 hr.; precipitation by ethanol	Grinding with trichloroacetic acid
Purification of glycogen	Washings with 66, 85 and 96% ethanol and ether	None	None	Washings with 66, 85 and 96% ethanol and ether	None	None	None
State at estimation	Glucose	Glucose	Glucose	Glucose	Glucose	Glycogen	Glucose
Conversion into glucose	By hydrolysis (3 hr.)	By hydrolysis (2 hr.)	During colour development	During colour development	During colour development	None	During colour development
Final titration	Any glucose determination	Folin & Wu's colorimetric method	Colorimetric method with anthrone	Colorimetric method with diphenylamine	Colorimetric method with anthrone	Colorimetric method with iodine	Colorimetric method with sulphuric acid

RESULTS

Estimation with iodine reagent

Fig. 1 shows the relationship between the optical density of a glycogen-iodine complex and the glucose equivalent of glycogen at various wavelengths. This diagram was constructed with a glycogen sample obtained from rat liver. As these relationships can be represented by straight lines the optical density can be read at different wavelengths. In practice we adopted 650 mμ. as the most suitable one for estimation of glycogen over a wide range of concentrations without necessitating dilution of glycogen-rich liver extracts. The colour intensity of the glycogen-iodine complex decreases with increase in the concentration of TCA in the extract, but within the limits of 4·9–5·1 % TCA, variations in optical density due to different acidity can be neglected.

Glycogen is very stable in the extracts. The differences between estimations of thirty-seven samples from various livers, performed immediately after extraction of the tissues and 18 hr. later, respectively, averaged $+0{\cdot}01 \% \pm 0{\cdot}03$, the greatest deviation being 0·08 %. The extracts can therefore be left overnight, which adds to the usefulness of the TCA method.

The reproducibility of the technique was investigated by performing duplicate determinations on the same liver. In view of the irregular distribution of glycogen in liver found by Nijs, Aubert & de Duve (1949), adjacent samples of tissue were taken for analysis. In eighteen experiments a mean difference of $0{\cdot}06 \pm 0{\cdot}068$ in percentage liver glycogen was found between duplicate samples. Similar liver samples were analysed using the more elaborate Pflüger method; in eleven experiments a mean difference of $0{\cdot}33 \pm 0{\cdot}225$ was found, so with the iodine method more reproducible results are obtained than with Pflüger's technique.

The calibration curves relating optical density to glucose equivalent have a different slope with glycogen samples of different origin (Fig. 2); this emphasizes the necessity for a standard curve, obtained with glycogen from the same source as the glycogen samples which are estimated. The magnitude of this slope may be used to characterize glycogen for comparative purposes and is termed here the 'colour intensity', being related to the extinction coefficient. Using the data of Fig. 2, values of colour intensity for samples of glycogen from mouse liver, rat liver and rat muscle were calculated to be 1·51, 1·00 and 0·81, respectively. In subsequent experiments the colour intensity of a glycogen was determined by submitting a solution to two determinations, one by iodine-staining and the other by anthrone.

It was found that differences in colour intensity exist between samples of glycogen prepared from the livers of different individuals of one species (rats). The colour intensities were estimated at various wavelengths for five samples of glycogen prepared from different livers (Table 2). Determinations in triplicate were performed on one

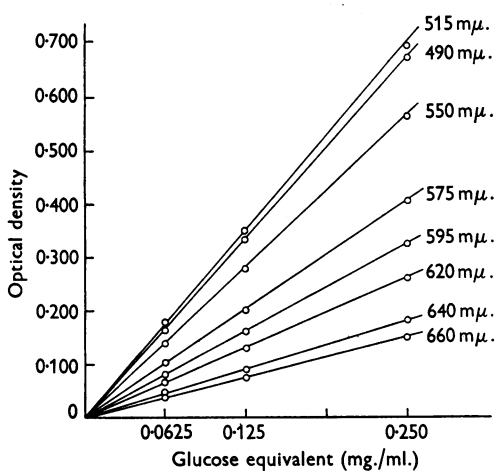

Fig. 1. Relations between optical density of a glycogen-iodine complex and glucose equivalent of glycogen at various wavelengths. A homogenous sample of glycogen from the livers of rats was used. The glucose equivalents indicated refer to 1 ml. of iodine-stained solution.

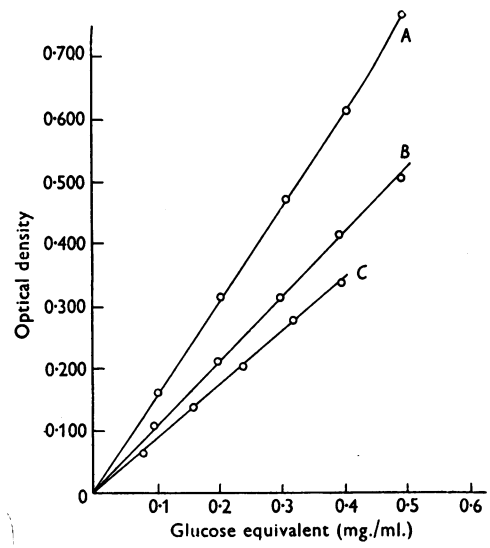

Fig. 2. Calibration curves for glycogen from different sources. Optical densities were read at 650 mμ. The glucose equivalents indicated on the abscissa refer to 1 ml. of iodine-stained solution and were calculated from the estimated purities of the glycogen preparations. *A*, mouse liver; regression coefficient of the line, 1·51. *B*, rat liver; regression coefficient, 1·00. *C*, rat muscle; regression coefficient, 0·81.

homogeneous sample of glycogen as well. The variations in the values for glycogen from different livers (standard deviations in upper part of the table) exceed significantly those solely due to technical errors (standard deviations in lower part of the table). This was proved statistically by computing a mean square for each part of the table by pooling the standard deviations according to the formula $(\Sigma s^2)/n$. The ratio of these mean squares was found to be 4·36 and indicates a significant difference with $P < 0{\cdot}001$. Hence, colour intensities of glycogen differ from one liver to another.

As the differences between the samples of glycogen existed at all wavelengths, only colour intensities at 650 mμ. were compared in the following experiment. After preliminary starvation, rats were dosed with glucose and the colour intensities of liver glycogen compared with those of normally fed rats (Table 3). The results suggest that following a treatment which effects a rapid deposition of glycogen in the liver the colour intensity of the glycogen increases, but independently of the actual amount of glycogen in the liver. This suggests that newly formed glycogen has the greater colour intensity.

Estimation with anthrone reagent

The estimation of glycogen in liver extracts by means of the anthrone reagent includes a simultaneous hydrolysis of glycogen to glucose, so this estimation is not influenced by the origin of the glycogen. Treatment of liver extracts with hot alkali resulted in an elimination of interfering glucose, without affecting the glycogen (Table 4); this enhances the specifiaty of the method.

A liver incubated at 37° in Krebs-Ringer phosphate buffer is a suitable object to demonstrate the estimation of glycogen in the presence of increased amounts of physiological degradation products, such as can occur in the system to be studied. Experiments were performed with eight livers, incubated at 37° for 15 min. The livers were extracted by grinding 500 mg. of tissue with 30 ml. of TCA. In order to hydrolyse the glycogen, 10 ml. of extract were heated with 0·5 ml. of conc. HCl for 3 hr. at 100°. After neutralization of the acid with alkali and readjusting the quantity of fluid to 10 ml. with water, 10 ml. of 10% TCA were added, giving a final concentration of 5%, as the TCA originally present was decomposed by boiling with hydrochloric acid. A comparable solution was obtained by mixing the components in a sequence which prevented the hydrolysis of the glycogen. In the solutions, glycogen was estimated both including and excluding a treatment with alkali

Table 2. *Colour intensities at various wavelengths for glycogen from the livers of individual rats*

	Wavelength (mμ.)								
	465	490	515	550	575	595	620	640	660
	Colour intensities								
Mean ± S.D. of glycogen preparations from the livers of five different rats	2·12 ±0·23	2·53 ±0·32	2·46 ±0·24	2·24 ±0·24	1·61 ±0·21	1·32 ±0·25	1·06 ±0·15	0·74 ±0·11	0·68 ±0·09
Mean ± S.D. triplicate determinations on one glycogen preparation	2·63 ±0·29	2·78 ±0·07	2·85 ±0·06	2·30 ±0·02	1·64 ±0·05	1·30 ±0·01	1·07 ±0·01	0·73 ±0·02	0·63 ±0·02

Table 3. *Colour intensities at 650 mμ. for glycogen from the livers of individual rats*

Normally fed rats		Glucose-treated rats*	
Liver glycogen (% of fresh wt.)	Colour intensity	Liver glycogen (% of fresh wt.)	Colour intensity
1·76	0·59	3·50	1·38
2·42	0·89	1·36	1·52
5·76	0·95	1·25	1·80
1·34	0·80	1·68	1·85
5·05	0·87		
Av. 3·27	0·82	1·95	1·64

* After fasting for one night each animal received 7 hourly doses of 0·5 g. of glucose subcutaneously. The mean difference between the colour intensities is statistically significant ($t_7 = 5{\cdot}21$, $P < 0{\cdot}01$).

Table 4. *Influence of treatment with alkali on glucose and glycogen estimation with anthrone*

		Time of treatment with alkali at 100° (min.)					
		0	15	30	45	60	90
	No. of observations	Recovery of glucose or glycogen (%)					
Glucose (0·60 mg./ml.)	4	100	4	3	4	3	3
Glycogen (glucose equivalent 0·32 mg./ml.)	2	100	104	104	103	99	101

The experiments were performed as indicated in the Methods section.

for 1 hr. at 100° (Table 5). By omitting the treatment with alkali both glycogen and glucose are estimated. After previous hydrolysis of the glycogen the same results are obtained. Hydrolysis of glycogen with subsequent alkali treatment eliminates both glycogen and glucose and consequently no carbohydrate is found. Finally, treatment with alkali only destroys glucose, leaving the glycogen intact, which results in lower figures than without alkali treatment.

For routine determinations treatment with alkali for 1 hr. at 100° was adopted. After adding known amounts of glycogen to liver extracts the glycogen was fully recovered (Table 6).

Comparison of methods for estimation of glycogen in liver and muscle. The results of the method were compared with those of the technique described by Good *et al.* (1933). Two adjacent samples were taken from several livers, one sample extracted with TCA and the other one submitted to analysis according to Good *et al.* In the extracts glycogen was estimated with the anthrone reagent. The same experiments were performed with muscles of rats. To extract the glycogen from muscle, a 500 mg. sample was ground finely in a mortar with about 1 g. of purified sand and 10 ml. of 5% TCA. The sand and proteins were filtered off and the clear filtrate was used for analysis.

The data in Table 7 indicate a satisfactory agreement for liver but not for muscle, suggesting an incomplete extraction of glycogen from the latter. This assumption was tested as follows:

Two samples were taken from the same muscle; one extracted with TCA and the other one heated with alkali until a homogenous solution was obtained. From this solution the glycogen was precipitated by adding twice the volume of ethanol and the precipitate separated. The glycogen was dissolved in 10 ml. of TCA, yielding a solution comparable with that obtained by direct extraction of muscle. In the two extracts glycogen was estimated with the anthrone reagent (Table 8). Lower figures were obtained when the muscle was directly extracted with TCA than after liberation of the glycogen by destruction of the tissue with alkali. So apparently a considerable part of the glycogen in muscle is not extracted with TCA. Subsequent extractions of the protein residue with fresh TCA did not increase the yield of glycogen. However, the unextracted glycogen could be recovered after destruction of the protein residue with alkali.

In a similar series of experiments both the extractable and non-extractable part of the glycogen were estimated in the livers of rats, submitted to various treatments. The non-extractable glycogen left in the protein residue of the tissue after extraction with TCA was liberated by alkali destruction and separated by ethanol precipitation. In some cases the colour intensities of the glycogen-iodine complexes were determined as well. All results are

Table 5. *Influence of treatment with alkali on glucose and glycogen in extracts of incubated livers*

Results* (as % glucose equivalent of fresh wt. of liver)			
Acid hydrolysis previously		No acid hydrolysis	
Including KOH treatment	Without KOH treatment	Including KOH treatment	Without KOH treatment
0·01±0·022	1·01±0·424	0·33±0·328	0·99±0·471

* Each result is the average of eight experiments.

Table 6. *Recovery of glycogen added to liver extracts*

Estimation with anthrone reagent.

Added glycogen (mg./ml.)	Recovered glycogen (mg./ml.)	Recovery (%)
0·159	0·157	98·8
0·220	0·221	100·3
0·272	0·280	102·9
0·316	0·313	99·1
0·356	0·366	102·9
0·389	0·382	98·4
		Av. 100·4

Table 7. *Agreement between estimations with the anthrone reagent and the method of Good* et al. (1933)

	Glycogen (Mean % fresh wt. tissue)		
	With anthrone reagent	With Good's method	Mean difference
Liver	(10) 2·72	(10) 2·51	+0·21±0·227
Muscle	(12) 0·34	(12) 0·52	−0·18±0·050

The figures in parentheses give the numbers of observations. For the mean values of the glycogen amounts in liver and muscle no standard deviations have been calculated as the latter are only indicative of the variability between individual animals. For the comparison of the two methods only the differences between the data obtained with both methods in the same organ are relevant. The relatively greatest mean difference between the estimations in muscle is highly significant ($t_{11} = 12{\cdot}5$, $P < 0{\cdot}001$).

Table 8. *Determinations of glycogen in muscle of rats using trichloroacetic acid extraction or alkali-destruction*

	Glycogen found (Mean % fresh wt.)		
No. of observations	Glycogen extracted with TCA	Glycogen liberated by destruction of the tissue with alkali	Mean difference
18	0·42	0·61	0·19±0·126

Statistical significance of mean difference $t_{17} = 6{\cdot}37$, $P < 0{\cdot}001$.

Table 9. *Extractable and non-extractable glycogen in the livers of rats and mice after various treatments*

	Expt.	Glycogen fraction	No. of observations	Glycogen (% of fresh wt.)	Colour intensity*
1	Normal rats, not starved	Extractable	10	4·71±2·22	0·93±0·085
		Non-extractable	—	0·16±0·021	0·58±0·121
2	Normal rats, starved for 24 hr.	Extractable	10	0·04±0·024	—
		Non-extractable	—	0·03±0·016	—
3	Adrenalectomized rats, starved for 24 hr.	Extractable	5	0·06±0·045	—
		Non-extractable	—	0·01±0·007	—
4	Adrenalectomized rats, starved for 24 hr. and treated with cortisone†	Extractable	5	0·63±0·240	—
		Non-extractable	—	0·08±0·050	—
5	Adrenalectomized mice, starved for 24 hr. and treated with glucose‡	Extractable	5	0·46±0·274	0·87±0·224
		Non-extractable	—	0·10±0·049	0·50±0·174
6	Adrenalectomized mice, starved for 24 hr. and treated with cortisone and glucose‡	Extractable	5	1·98±0·541	1·26±0·263
		Non-extractable	—	0·13±0·32	0·64±0·131

* Statistical significance of differences between colour intensities of extractable and non-extractable glycogen fractions: Expt. 1: $t_9 = 7{\cdot}72$, $P < 0{\cdot}001$; Expt. 2: $t_7 = 3{\cdot}26$, $P < 0{\cdot}05$; Expt. 3: $t_7 = 9{\cdot}50$, $P < 0{\cdot}001$.

† After fasting for 16 hr. each rat received 5 doses of 0·18 mg. cortisone acetate in peanut oil subcutaneously.

‡ Treatment with glucose and cortisone as described by Venning, Kazmin & Bell (1946). Total doses for each animal: cortisone acetate 0·08 mg., glucose 35 mg.

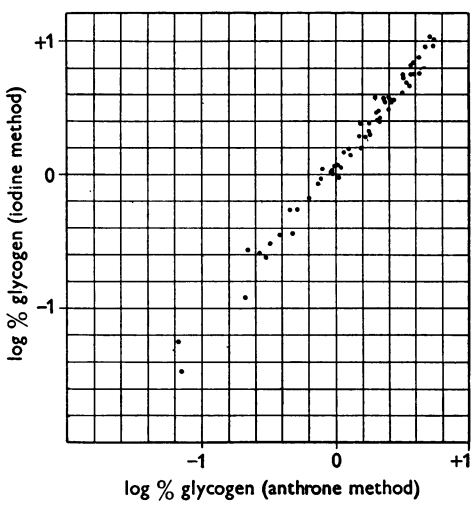

Fig. 3. Parallelism between the results of the anthrone and the iodine method. The logarithms of the percentage glycogen found with the two methods are plotted against each other.

summarized in Table 9. In the livers of normal animals the non-extractable fraction is only a small part of the total glycogen. The colour intensities with iodine at 650 mμ. of the non-extractable glycogen fractions are less than those of the extractable parts. Finally it can be seen that alterations in the glycogen content of liver, caused by starvation or treatment with cortisone acetate, are mainly present in the extractable fraction of the glycogen.

Comparison of anthrone and iodine methods. In many cases, the determination of the extractable glycogen will furnish sufficient information about the glycogen contents of the liver, and then the TCA extraction procedure may be adopted, with subsequent estimation by the iodine or anthrone method. A comparison between the anthrone and the iodine method was made using extracts obtained during a glycogenetic test. A close parallelism was found between both series of results as can be seen from Fig. 3 and the final results of the test proved to be nearly the same, irrespective of the method used for the estimation of glycogen. It may be noted that in the graph logarithms of the percentage are plotted. It could be shown that the logarithms of liver glycogen values in rats have a normal frequency distribution. So the logarithm rather than the actual percentage should be used for the statistical calculation of a test.

DISCUSSION

In the foregoing the usefulness of the iodine and anthrone methods has been demonstrated. Both methods are simple and much more appropriate for serial determinations than all other methods previously described. Nevertheless they have their limitations. The TCA extraction, which is used for both methods, is very convenient but a considerable part of the glycogen in the muscles of rats is not extracted with TCA. Consequently the TCA extraction cannot be recommended for the estimation of glycogen in muscle.

In liver the non-extractable glycogen fraction proved to be only a minor part of the total glycogen and alterations in the amount of glycogen in this tissue were found to be mainly associated with the extractable glycogen. For many purposes the determination of the extractable glycogen will give sufficient information about the amount of glycogen in a liver. The suitability of the extraction

procedure should be determined for other organs. If one wants to be sure that 100% of the glycogen present in any organ is determined, the usual hydrolysis of the tissue with strong alkali followed by precipitation of the glycogen remains the method of choice. Using the iodine reagent only approximate figures are obtained for the glycogen content of a liver extract. Nevertheless, this very convenient method may be useful for investigations where some accuracy may be sacrificed in order to make possible experiments with a large series of rats. For instance this was the case in our studies of the glycogenetic properties of adrenal steroids by estimation of liver glycogen, in which the experimental animals are treated following a standard procedure, and the results are always mutually compared. The results of these tests were hardly influenced by the variable colour intensities of the glycogen from different livers. However, if approximate values cannot be accepted the use of the anthrone method is indicated. Providing the lower saccharides are previously destroyed with alkali, more correct results are obtained, but the method is more elaborate than the estimation with iodine.

SUMMARY

1. Two methods are described for the determination of glycogen. In both procedures the glycogen is extracted from the tissue with trichloroacetic acid.

2. In these extracts glycogen can be estimated approximately by means of an iodine reagent. The colour intensity of the glycogen-iodine complex varies from one liver to another and is further influenced by the treatment of the animals.

3. More correct results are obtained if the glycogen in the extract is determined using an anthrone reagent, after treating the liver extracts with alkali which eliminates interfering glucose.

4. The application of the methods is briefly discussed.

I wish to express appreciation and thanks to Dr G. A. Overbeek for his advice and encouragement given during the course of this work and to Miss C. W. N. N. Schellekens and Mr J. J. de Koning for valuable technical assistance.

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The Use of Substances Depressing Antithrombin Activity in the Assay of Prothrombin

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Determination of the prothrombin activity of body fluids has become of clinical interest in connexion with the diagnosis of haemorrhagic disorders, and during treatment with anticoagulants. Most techniques for the determination of prothrombin are based on one or other of two principles. In both, prothrombin is converted into thrombin in the presence of optimum amounts of the other clotting factors. In one, the time interval required for clot formation in the specimen is taken as a measure of prothrombin activity; this is a direct method, and is referred to in the literature as the one-stage technique (Quick, 1942). In the other, the thrombin potency developed is estimated with an added substrate containing fibrinogen; this is an indirect method often called the two-stage technique (Warner, Brinkhous & Smith, 1936).

In the direct method a fibrin clot appears at a certain time corresponding to the formation of a certain concentration of thrombin. The rate of thrombin formation depends upon the concentration of prothrombin and the activity of accelerating and inhibiting factors. The direct method is useful in determining the haemostatic condition of a patient, but it does not indicate the actual prothrombin concentration of the specimen.

In the direct method, fibrin formation takes place at a very low thrombin concentration, which is