with the identity test based upon the evolution of iodine vapor and comparison of the infrared spectrum provide a satisfactory identification of idoxuridine.

Purity Tests.—Thin-layer or paper chromatographic procedures may be included for testing the purity of bulk idoxuridine. Comparison of idoxuridine to a reference standard is made by examination of developed chromatograms using an ultraviolet light and/or color producing reagents, *i.e.*, cysteine–sulfuric acid. The idoxuridine spot should be equivalent in position to the reference standard spot for idoxuridine, and no other spots at other positions should be visible. The spotting of control solutions containing the degradation products of idoxuridine (5-iodouracil, uracil, and deoxyuridine) will aid in detecting the position of extraneous spots on the chromatograms.

Quantitative Methods.—The quantitative determination of the iodine content of idoxuridine is similar to the official assay for sodium liothyronine (3) and gave an average value equivalent to 36.1 ± 0.1%[3] iodine. A rapid, precise measure of the iodine content may also be determined by the oxygen flask method (4–6). Nonaqueous titration of idoxuridine with sodium methoxide gave an average value of 99.8 ± 0.6%.[3] Azo violet indicator may also be used for the end point detection of the titration. Analysis of the sterile ophthalmic solutions by column partition chromatography was based on the procedure of Simpson and Zappala (7). Acid washed Celite 545 was used as the supporting phase without prior treatment. Celite 545 may be used as the adsorbant by prewashing an acidified column with organic solvents to remove extractable impurities. The organic solvents are then removed by oven drying. Incorporation of water or 0.1 *N* hydrochloric acid in the preparation of the Celite columns gave comparable results representing an average recovery of 97.4 ± 5.7%[3] of the theoretical amount of idoxuridine in the ophthalmic solutions.

The volume of eluting solvent included for the assay of idoxuridine ophthalmic solution gave quantitative recoveries with the Celite used. However, it should be noted that the elution rate for idoxuridine may vary from lot to lot of Celite which then necessitates a minor change in the volume of eluate collected. This should be demonstrated for each batch of Celite by a satisfactory recovery of a standard aqueous idoxuridine solution subjected to the column procedure.

[3] Maximum deviation from the mean value.

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Technical Articles

Automated Nephelometric Determination of Rat Liver Glycogen in Adrenal Steroid Bioassays

By WILLIAM F. BEYER

An automated procedure for the nephelometric determination of rat liver glycogen is described. Alkaline liver digests are mixed with 57.5 per cent alcohol and heated at 45°. Samples are analyzed at a rate of 60/hr. using an automatic sampler and analyzer (Technicon AutoAnalyzer) in conjunction with a commercially available fluorometer. The coefficient of variation for the automated procedure is approximately 1 per cent.

RAT LIVER glycogen is used as the criterion of response in the bioassay for endocrine principles of the adrenal cortex and also for synthetic steroids. The assay is based on the method of Pabst *et al.* (1) and is currently an official procedure for adrenal cortex injection as directed by N.F. XII (2). For the assay, livers of adrenalectomized rats, previously injected with test samples, are digested in hot 30% potassium hydroxide. After standing overnight at room temperature, the alkaline liver digests are diluted with water and glycogen is determined.

An automated procedure for glycogen has been described by Singer *et al.* (3) requiring manual deproteination with trichloroacetic acid. Glyco-

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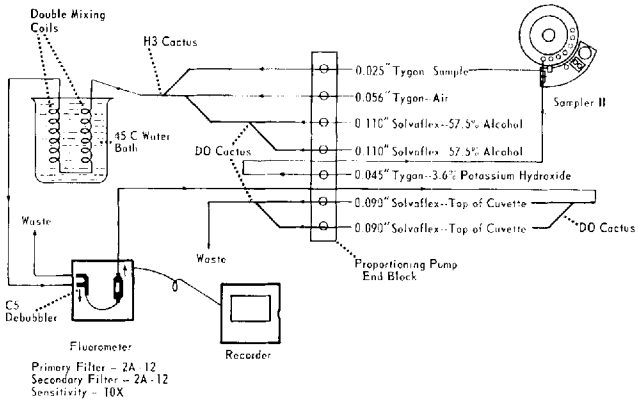

Fig. 1.—Manifold flow diagram for the automated determination of rat liver glycogen using an automatic sampler, proportioning pump, and recorder in conjunction with a commercially available fluorometer adapted for nephelometry.

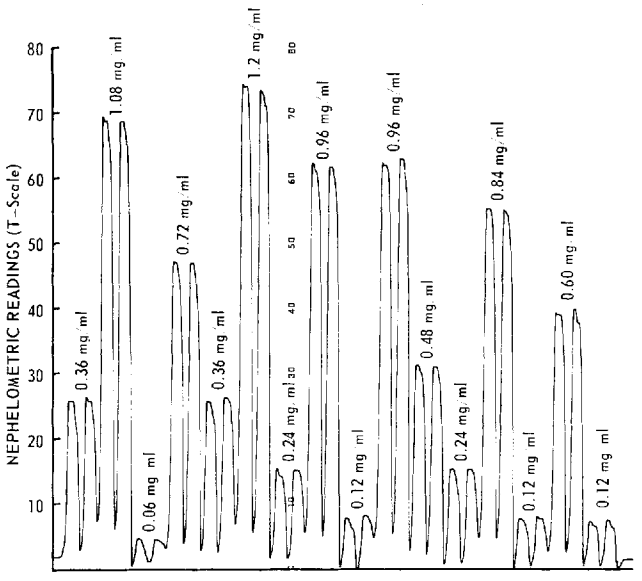

Fig. 2.—Recordings of various quantities of rat liver glycogen using automated nephelometry at a rate of 60 samples/hr.

gen determinations are then made automatically by measuring the intensity of the glycogen–iodine complex. This report gives details for an automated assay of rat liver glycogen using a fluorometer adapted for nephelometry. The method, based on a previously described procedure (4), depends upon the cloud produced by glycogen from alkaline liver digests in 55% alcohol. Technicon instruments and a commercially available fluorometer equipped with a square quartz flow cell are used in the analyses, permitting glycogen determinations at a rate of 60 samples/hr.

MATERIALS AND METHODS

Instrument and Equipment.—(*a*) Automatic sampler,[1] proportioning pump, flow through door for fluorometer, recorder, chart reader, double mixing coils, and assorted glass fittings and tubing. (*b*) Fluorometer[2] with Wratten 2A-12 primary and secondary filters and blue lamp, permitting measurements at approximately 510 mμ. (*c*) Square quartz flow cell, 3 mm. i.d. × 5-mm. o.d. with 12-mm. masked adapter insert.[3] (*d*) Water bath operated at 45°.[4]

Reagents.—(*a*) Potassium hydroxide, 3.6% and 30%. (*b*) Alcohol, 57.5%, (v/v). (*c*) Test rat liver digests. Adrenalectomized rats are injected with adrenal steroid preparations as directed by N.F. XII. Livers are removed, digested in 12 ml. of hot 30% potassium hydroxide, and diluted to 100 ml. with water after standing overnight at room temperature. (*d*) Pooled livers of adrenalectomized uninjected rats (blank pool). Prepared in the same manner as liver digests of test rats for each series of glycogen determinations. (*e*) Standard rat liver glycogen. Prepared according to van der Vies (5). (*f*) Standard solutions of rat liver glycogen. Fresh solutions of glycogen standard are prepared in the blank pool. For a standard curve, solutions in the range of 0.06 to 1.2 mg./ml. are adequate. To check instrumental and reagent changes, a 0.5-mg./ml. standard is used.

Method.—The sample line is placed in the blank

[1] AutoAnalyzer Sampler II, Technicon Controls, Chauncey, N. Y.
[2] Turner Fluorometer model III, Arthur H. Thomas, Philadelphia, Pa.
[3] Catalog numbers B16-63019 and A363-62140, American Instrument Co., Barrington, Ill.
[4] Tamson water bath, Witt Sales, Cincinnati, Ohio.

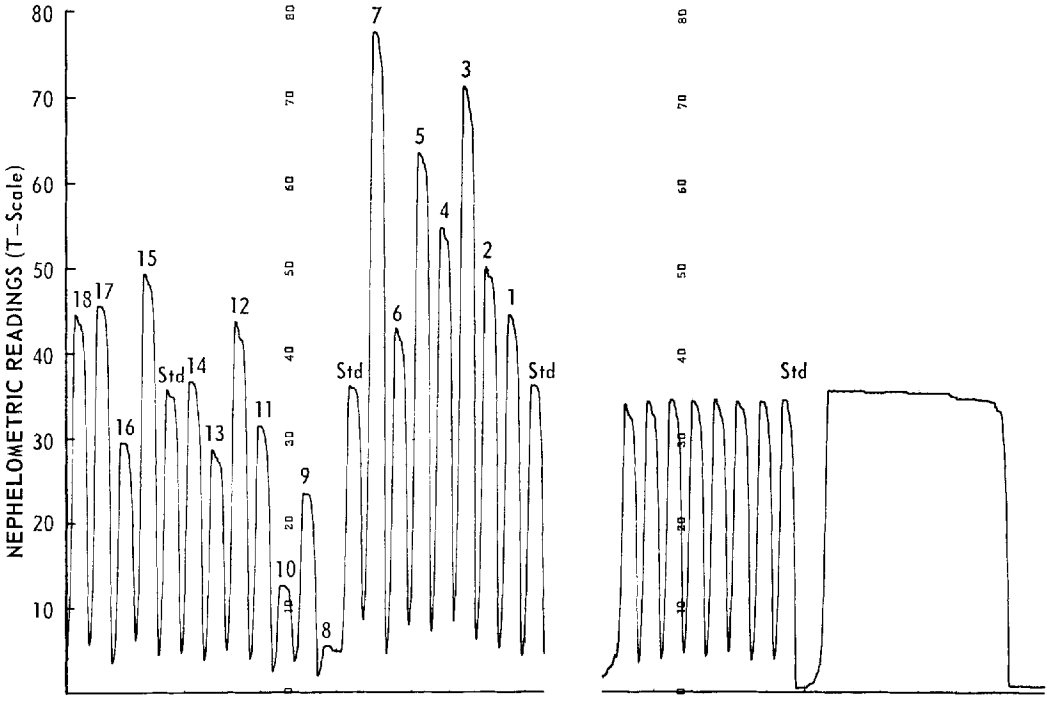

Fig. 3.—Automated nephelometric recordings of rat liver glycogen standard and representative recordings of unknown test samples. Key: numbered peaks, recordings of unknown test samples; Std, 0.5, mg./ml. standard glycogen dissolved in the blank liver pool.

TABLE I.—RECOVERY OF GLYCOGEN FROM RAT LIVER DIGEST USING THE AUTOMATED NEPHELOMETRIC PROCEDURE

Glycogen Present in Digest, mg./ml.	Glycogen Std. Added, mg./ml.	Theoretical Amt. Glycogen Present, mg./ml.	Glycogen Found, mg./ml.	Recovery, %[a]
0.055	0.430	0.485	0.480	99.0
0.111	0.430	0.541	0.547	101.1
0.221	0.430	0.651	0.648	99.5
0.332	0.430	0.762	0.761	99.9
0.443	0.430	0.873	0.873	100.0
0.553	0.430	0.983	0.972	98.9

[a] Average recovery = 99.7%.

pool and all other lines in proper reagents as shown in the manifold flow diagram of Fig. 1. Sensitivity of the fluorometer is positioned at 10× and 2A-12 filters are used for both primary and secondary filters. With all instruments operating, a zero base line is established. The 0.5-mg./ml. glycogen standard is placed in duplicate cups initially and singly thereafter at regular intervals following a group of test samples. The analyses are made at a rate of 60/hr. using 3.6% potassium hydroxide in the rinsing system of the automatic sampler.

Calculations.—The quantity of glycogen in each rat liver is calculated using the formula:

$$Gu = \frac{Nu}{Ns} \times Gs \times V$$

where, Gu = milligrams glycogen per liver; Gs = milligrams glycogen standard per milliliter of blank pool; Ns = nephelometric reading of glycogen standard on transmission scale; Nu = nephelometric reading of test sample on transmission scale; and V = milliliters of liver digest.

To correct for changes in the automated system average readings of standards on either side of a group of test samples are used with appropriate liver digests.

TABLE II.—PRECISION OF REPLICATE RAT LIVER GLYCOGEN DETERMINATIONS

Rat Liver No. 11 mg. Glycogen/Liver		Rat Liver No. 25 mg. Glycogen/Liver	
52	52	65	66
52	52.5	66.5	65.5
52.5	51.5	65.5	64.5
52	51.5	65	65.5
51.5	51	65.5	66.5
52	51.5	64	65.5
52	51.5	65	65
		65.5	66.5
Av. = 51.8		Av. = 65.4	
Coefficient of variation = 0.81%		Coefficient of variation = 1.07%	

TABLE III.—PROTOCOL OF A RAT LIVER GLYCOGEN DEPOSITION ASSAY FOR ADRENAL CORTEX EXTRACT[a]

mg. Glycogen/Individual Rat Liver							
U.S.P. Hydrocortisone Std.(F)				Adrenal Cortex Extract			
0.288 mg./Rat		0.48 mg./Rat		0.288 mg./Rat		0.48 mg./Rat	
A	M	A	M	A	M	A	M
32	32	55	54	16	13	53	54
27	29	60	61	46	46	53	54
26	25	66	63	31	31	52	55
29	30	61	60	38	40	53	52
56	57	55	57	33	32	53	52
58	57	59	58	46	46	52	52
50	51	44	43	36	38	54	54

	A	M
Potency, mg. F ≅ ml.	0.141	0.142
Log-confidence interval	0.2253	0.2338
95% Confidence limits, mg. F ≅ ml.	0.105–0.177	0.105–0.181
Test for parallelism of slopes (F)	0.0015	0.0710

[a] Liver glycogen of each rat was determined by both automated (A) and manual (M) procedures. Potency and validity tests were calculated as directed by N.F. XII.

TABLE IV.—STATISTICAL RESULTS OF RAT LIVER GLYCOGEN DEPOSITION ASSAYS OF ADRENAL CORTEX EXTRACTS

	Automated		Manual	
Prepn.	Potency, % of Theory	Log-Confidence Interval (L)	Potency, % of Theory	Log-Confidence Interval (L)
1	126	0.1757	122	0.1744
2	141	0.2253	142	0.2388
3	97	0.2758	94	0.3135
4	147	0.3281	152	0.3830
5	101	0.3005	106	0.2479
6	73	0.2236	73	0.2105
7	125	0.2117	125	0.2127

Alternatively, the chart reader and a standard curve for rat liver glycogen may be used. Liver glycogen values of test samples are adjusted in accordance with nephelometric readings of the 0.5-mg./ml. standard placed at regular intervals.

RESULTS AND DISCUSSION

Following preliminary experiments with various manifold systems and temperatures, optimum operating conditions and manifold design were determined. The 0.025-in. sample line (Fig. 1), delivering 0.23 ml./min., dilutes the 57.5% alcohol carried by two 0.110-in. lines at a rate of 6.78 ml./min., to 55%. This concentration of alcohol was found to effectively form the glycogen cloud.

Nephelometric responses to liver glycogen concentrations when plotted were linear over the range of 0.06 to 1.2 mg./ml. (6 to 120 mg./liver) and had a zero intercept. An upper limit was not established; however, the concentration of liver glycogen encountered in the bioassay of adrenal steroids normally does not exceed 120 mg./liver. Although glycogen values may vary by a factor of 10 in glycogen deposition assays, sample carry over with the automated procedure is minimal. Figure 2 shows that very similar readings are recorded for duplicate samples of glycogen standard even though preceded by a standard having either high or low nephelometric readings.

Figure 3 gives a typical recorder tracing of unknown test liver digests and glycogen standard showing the variation in liver glycogen that is encountered normally. A portion of the recording, extreme right, shows continuous sampling of the 0.5-mg./ml. glycogen standard and duplicate sampling of the same standard.

Recovery studies were carried out using the automated procedure on samples of glycogen standard added to varying quantities of test liver digest. Table I shows that satisfactory recovery was obtained, with results varying from 98.9–101.1% for the six samples tested.

The reproducibility of the procedure was determined by sampling repeatedly two test liver digests. Table II gives the results: a coefficient of variation of 0.81% was obtained for 14 analyses and 1.07% for 16 subsequent analyses carried out at a later date.

No significant differences in liver glycogen determinations occurred for the manual procedure (4) and the automated method. Table III gives the results of a representative adrenal cortex assay with glycogen determined by both methods on parallel samples of liver digests. It can be noted that only small differences occurred in individual glycogen values; consequently, statistical results are very similar. A comparison of seven additional liver glycogen deposition assays is shown in Table IV. In all cases, comparable potencies and log-confidence intervals were obtained, indicating that the automated procedure is suitable for rat liver glycogen determinations in the glycogen deposition assay of adrenal steroids.

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