Summary. A method is described for dispersing C^{14}-labeled triglyceride in plasma to form low density fat particles which are similar in size, flotation and electrophoretic mobility to the particulate fat present in plasma during fat absorption. The removal rate in man of a trace dose (less than 1 mg) of this preparation of radioactive particulate fat is rapid. Small loads (2 g) of unlabeled lymph or emulsified fat given simultaneously significantly prolong the removal rate. This rate is comparable to that observed after administration of a similar load of labeled human chylomicrons.

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Nephelometric Determination of Glycogen in the Rat Liver Glycogen Deposition Assay of Adrenal Steroids. (27327)

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In liver glycogen deposition assays for endocrine principles of the adrenal cortex and synthetic adrenal steroids, glycogen is precipitated from alkaline liver digests with ethanol and subsequently determined by somewhat lengthy, indirect, quantitative procedures(1-7). Previously reported nephelometric methods require preliminary precipitation of glycogen with an ethanol-sodium chloride solution(8) or preparation of a protein-free filtrate(9). During routine use of the NF XI method(3), one that does not involve deproteination, it was observed that glycogen formed a mildly stable suspension before its precipitation and hence suggested the possibility of a nephelometric procedure. The studies done to determine the proper experimental conditions and practicability of such an analysis are the subject of this report.

Procedure. Experimental rat liver digests (diluted digests) are prepared as follows: Each liver is freed of surface blood with 0.9% NaCl and digested in 12 ml of hot 30% KOH. The digest is diluted to 100 ml with water after storage at room temperature overnight. Simultaneously, livers of several adrenalectomized, starved, uninjected rats are also obtained for each series of glycogen determinations. They are treated as above, then pooled (blank pool) for use in preparation of an instrument blank and as a diluent for the glycogen standard used in positioning the galvanometer index.

In separate stoppered glass vessels, 2 ml of blank pool and 2 ml of standard glycogen solution consisting of 0.5 mg of rat liver glycogen per ml of blank pool are added to 42 ml of 57.5% alcohol. Two ml of each diluted digest are treated in the same manner as the blank pool and glycogen standard. The vessels are closed, shaken, and allowed to stand 35 min. The samples are thoroughly mixed again and transferred to 20 $\times$ 40 mm cuvettes. Nephelometric readings are obtained against the blank using a Coleman Model 14 Universal Spectrophotometer at a wave length of 575 mμ and at a galvanometer sensitivity determined by the glycogen standard.

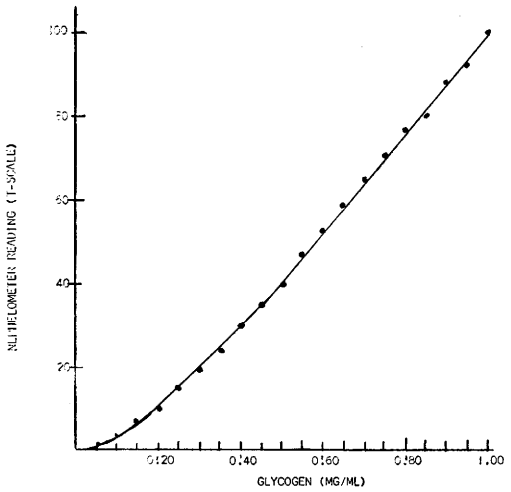

FIG. 1. Standard curve for rat liver glycogen. Readings were made at 575 μ with the galvanometer index maintained at a setting so that 0.50 mg of glycogen per ml gave a reading of 40 against the blank pool.

The desired sensitivity is obtained using the "T" scale as an arbitrary intensity scale and positioning the galvanometer index so that it reads "40" with a cuvette containing the glycogen standard in place and "0" with the blank. The sensitivity used for the 0.5 mg/ml glycogen sample is the same as that of the standard curve constructed as follows:

Purified rat liver glycogen, prepared by method of van der Vies(10), is dissolved in the blank pool at a concentration of 1.0 mg/ml. Aliquots of this sample are further diluted with the blank pool to give glycogen concentrations ranging from 0.05-1.0 mg/ml at increments of 0.05 mg. Two ml of each solution are then added to 42 ml of 57.5% alcohol and thoroughly mixed. After standing for 35 min, the samples are remixed and transferred to 20 $\times$ 40 mm cuvettes. Instrument readings are made with the galvanometer sensitivity positioned at 40 for the 0.50 mg/ml sample against the blank pool treated in the same manner as the standard glycogen samples. Observed nephelometric readings are shown for the standard curve of purified rat liver glycogen (Fig. 1).

In determining the amount of glycogen in each liver, nephelometric readings are converted to mg of glycogen per liver from the standard curve.

Results and discussion. To determine a suitable alcohol-glycogen relationship, glycogen was dissolved in the blank pool at concentrations of 0.1, 0.5, and 1.0 mg/ml, and aliquots were added to alcohol of varying strengths at 25°C so that final alcohol concentrations ranged from 40 to 95%. Aliquots of the blank pool were also treated with varying concentrations of alcohol in the same manner as the glycogen solutions. Instrument readings indicated that a final concentration of 55% alcohol was satisfactory.

To determine the stability of the glycogen suspension, various concentrations of glycogen were prepared using a blank pool as the diluent. Two ml aliquots were added to 42 ml of 57.5% alcohol and mixed. Readings at 25°C against the same blank pool prepared in an identical manner were obtained at 5-min intervals for 3 hrs. Four additional readings were made at 15-min intervals thereafter (Fig. 2).

Readings of glycogen samples in the range of 0.1-1.0 mg/ml reached a peak within 35 min and remained stable for approximately 135 min. Therefore, it should be relatively simple for a single operator to determine the glycogen content of a large number of diluted digests during this period.

To determine whether glycogen added to diluted digests containing varying amounts of glycogen could be accurately measured by the nephelometer procedure, aliquots of the blank pool containing various concentrations of glycogen were combined with those of diluted digests from rats previously injected with adrenal steroids. One ml of diluted digest and 1.0 ml of blank pool containing various

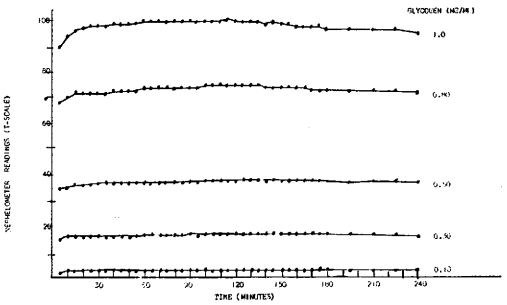

FIG. 2. Curves showing stability of 55% alcoholic suspensions of rat liver glycogen. Readings were made at 575 μ against a "glycogen-free" liver digest. Points on the curve indicate nephelometric readings.

TABLE I. Recovery of Glycogen from Diluted Alkaline Liver Digests Using Nephelometry as Described in This Report.

Glycogen present in digest (mg/ml)	Glycogen added (mg/ml)	Glycogen found (mg/ml)	Recovery (%)
.320	.100	.418	99.52
"	.200	.513	98.65
"	"	"	"
"	.300	.598	96.45
"	"	.603	97.26
"	.400	.703	97.64
"	"	.698	96.94
"	.500	.803	97.93
"	"	"	"
.326	.250	.555	96.35
.350	"	.570	95.00
.269	"	.505	97.30
.419	"	.645	96.41
.196	"	.465	104.26
.223	"	.485	102.54
	Avg		98.19
	Mean stand. dev.		± .62

amounts of added glycogen were treated with alcohol and analyzed. An average recovery of 98.19% was obtained. The mean std. dev. for the 15 determinations (Table I) was ± 0.62%.

The reproducibility of the method and the effect that the blank pool might have were investigated. Twelve solutions of glycogen at a concentration of 0.50 mg/ml were prepared using a different "glycogen free" diluent for each sample. Only one of the digest diluents, selected at random, was used in preparation of the instrument blank. An average glycogen concentration of 0.495 mg/ml ± 0.009 mg was found. The results show that the method offers good reproducibility and that "glycogen-free" liver digests prepared as described in this study are satisfactory glycogen diluents. The mean std. dev. found for this study is indicative of the precision that can be expected for glycogen determinations using this method.

Recovery studies indicate that the utility of the standard curve may be increased by using the blank pool as a diluent for experimental liver digests yielding nephelometer readings in excess of 100. Also, that "glycogen-free" liver digests serving as diluents contribute nothing of significance toward increasing actual glycogen content. Were this not the case, average recovery value should have exceeded 100%.

Biological assays of adrenal steroids were conducted using the method essentially as described in NF XI for Adrenal Cortex Injection. Glycogen determinations on parallel samples of diluted digests were also made using nephelometry. Table II shows glycogen values and statistical analyses of a representative assay on a sample of adrenal cortex extract used in manufacture of Adrenal Cortex Injection.

Some variation in liver glycogen occurred between the two methods, but, on the whole, there was good agreement. It is possible that the nephelometric method gave a better estimate of glycogen because of more numerous manipulations required for the NF XI procedure. Using calculations for a 2×2 balanced assay as described in USP XVI(11), valid assays with comparable potencies were obtained with both glycogen procedures.

A comparison of statistical results of 8 additional assays, using methods previously described, is shown in Table III. Calculations for assays No. 1, 2, 4, and 5 shown in Table III were performed according to Bliss(12) where joint assays were calculated using a common standard. Calculations of a single unknown against a standard were used for assays No. 3, 6, 7, and 8(11). In all cases, comparable potencies were obtained by both methods of glycogen determination. Valid assays resulted in all instances except assay No. 3 which exceeded the "F" test at $P_{.05}$, for both methods, thereby failing the test for parallelism. Analysis of statistical data of Tables II and III indicates that the nephelometric method yields results comparable to those of NF XI. Due to the similarity in results of both methods, the ease and rapidity offered by the nephelometric method should make it advantageous for glycogen determinations in the glycogen deposition assay.

Modification of the procedure should make it possible to determine glycogen from other sources than rat liver.

Summary. A method is described for rapid and precise nephelometric determination of rat liver glycogen. Instrument readings are made following addition of diluted alkaline liver digests to 57.5% alcohol. Results indicate good recovery and reproducibility, with adherence to conditions used for the std.

TABLE II. Protocol of Rat Liver Glycogen Deposition Assay for Adrenal Cortex Injection, NF XI. Glycogen concentration of each rat liver was determined by both the NF XI method and the nephelometric procedure described here. Potency and validity tests were calculated according to USP XVI statistical procedures for a 2 × 2 balance assay with 8 rats/dose for a total of 32 rats.

	mg glycogen per individual rat liver							
	USP hydrocortisone standard				Unknown			
	.288 mg		.48 mg		1.8 ml		3.0 ml	
	NF XI	Neph.	NF XI	Neph.	NF XI	Neph.	NF XI	Neph.
	40	62	70	77	30	35	74	77
	45	51	57	58	30	29	54	51
	45	49	74	72	25	24	73	75
	49	55	37	33	43	40	69	71
	43	42	78	81	42	45	67	67
	55	54	74	78	46	49	57	58
	23	20	64	62	51	49	66	59
	38	39	69	70	36	35	76	79
Totals	338	372	523	531	303	306	536	537

	Statistical values	
	NF XI	Neph.
Potency (mg F ≅ ml)	.16	.15
Log-confidence interval	.13	.18
95% confidence limits (mg F ≅ ml)	.13–.18	.12–.18

TABLE III. Statistical Results of Biological Assays with Liver Glycogen Determined by NF XI Method and Nephelometric Procedure of This Report.

	NF XI		Neph.	
Assay No.	Potency (% of theory)	Log-confidence interval (L)	Potency (% of theory)	Log-confidence interval (L)
1	116	.22	119	.24
2	120	"	120	"
3*	89	.16	85	.15
4	105	.21	106	.18
5	98	"	101	"
6	123	.15	115	.16
7	100	.16	98	.18
8	77	.14	71	.19

* Failed test for parallelism.

curve. Fifteen solutions containing varying amounts of glycogen showed an average recovery of 98.19% ± 0.62%. The procedure was used in bio-assay of adrenal steriods and found to compare favorably to that recommended by NF XI.

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