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Histochemistry LXVII. Microscopic Microbiological Assay. Determination of Biotin to 10^{-15} Gram.* (27343)

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Previously, a system for microbiological assay depending on measurement of light absorbance of bacterial suspensions in microcuvettes was described in which 500 to 1000 times less sample than the commonly employed ml-range was used(1). Subsequent developments included elaboration of methods for determination of coenzyme A(2) and biotin(3) in μg-amounts of tissue and their applications to analyses of microtome sections of adrenal(4,5).

The technic has now been extended to determinations with submicroliter volumes of total reaction mixture, as droplets under oil, by measurement of reflectance from suspended bacteria; and, to illustrate its use, analysis of biotin with *Lactobacillus arabinosis* 17-5 (*L. plantarum*, ATCC 8014) in volumes of reaction mixture from 0.6 to 0.01 μl with a sensitivity for the smaller droplets of 10^{-15} g was developed.

Methods. The assembly used for reflectance measurement (Fig. 1) consists of a microscope (Zeiss, standard GFL, with phase contrast condenser, 111Z, having annular diaphragm in position 2 to furnish dark-field illumination—superior to usual dark-field condenser in providing greater intensity of illumination and finer adjustment of area illuminated) with a 2.5 × objective and a microphotographic adapter (Leitz, No. 72.005) containing a shutter, an observation eyepiece, a 12.5 × ocular, and individual cable releases to operate the shutter and a prism which directs the light either to the eyepiece or the photomultiplier tube (Photovolt, Type E) mounted above the adapter. A Photovolt photometer, Model 512, is used. The arrangement of the sample chamber for measurement is shown in Fig. 2.

For each use a clean glass slide (38 × 75 mm) is placed in hot half-conc. nitric acid for 10 min, rinsed well with distilled water, dried in an air stream filtered through cotton, and siliconed on one side by rubbing with clean gauze saturated with 5% Drifilm (General Electric) in chloroform, air-drying, and rinsing with distilled water and draining dry. The surface may be improved by polishing with gauze. The aqueous droplets will rest on the slide with a constant interface diameter if the siliconing is proper.

The sample chamber is made by sealing a Lucite frame (32 × 70 × 8 mm deep) to the siliconed slide by interposing a 2-layer frame of Parafilm M between the Lucite and the slide and applying gentle pressure while heating on a hot plate until the Parafilm loses its milky appearance and becomes transparent. The chamber is filled with about 6 ml of oil (3 vol. *n*-tetradecane, Eastman Kodak No. P2221, plus 1 vol. light liquid petrolatum,

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U.S.P.) previously saturated with single-strength assay medium by shaking. It is essential that no water be lost from droplets and the efficacy of saturating the oil with medium to accomplish this is illustrated in Table I.

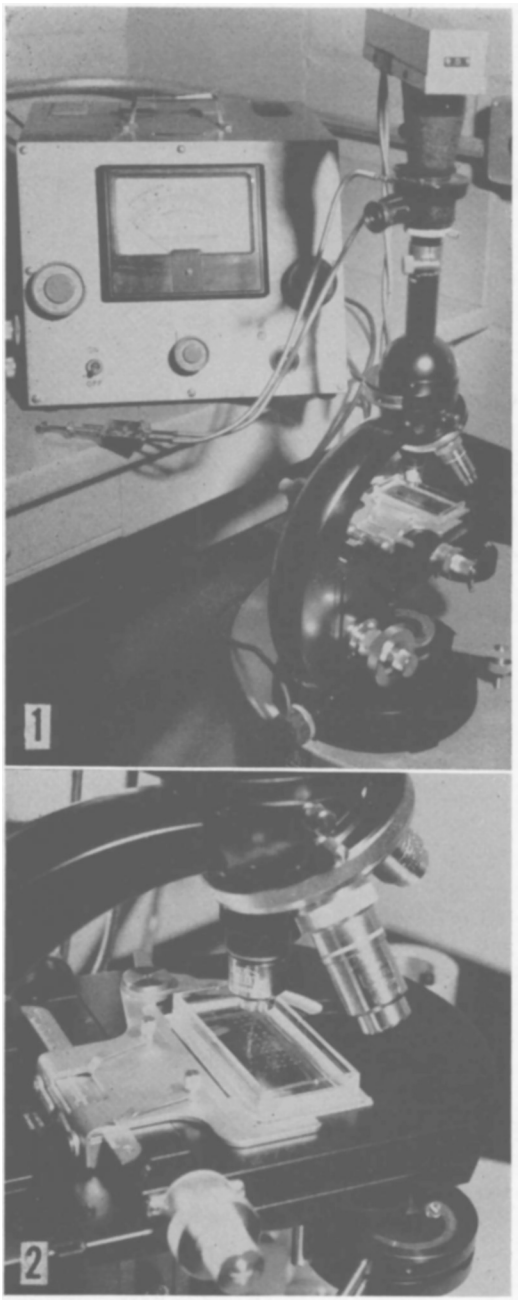

FIG. 1. Microscope assembly for measuring reflectance from aqueous droplets of bacterial suspension under oil in sample chamber on the stage.

FIG. 2. Sample chamber in position for measurement. Three rows (triplicate analyses) of droplets for measurement of control, standards, and unknowns are used.

Siliconed micropipettes with straight, finely drawn-out tips are used, the larger ones (1.0, 0.6 μl) being graduated with a line etched on the stem, while for those smaller a scale in the ocular of a small table cathetometer (O. Dich, Brøndby Strand, Copenhagen, Denmark) is employed to control the volume delivered. The pipettes are rigidly clamped in a vertical position, filled by suction with a rubber or plastic mouth-tube, and emptied by constant air pressure to assure reproducible delivery.

The oil-filled sample chamber is positioned on a vertically adjustable rack and pinion stand under the pipettes. For delivery, the tip of the pipette is placed just below the oil surface, the liquid is discharged, and the pipette is withdrawn slowly while maintaining the constant air pressure to prevent the droplet which adheres to the tip from being drawn up into the pipette by capillarity. As the tip passes through the oil surface the droplet is pulled off by surface tension and sinks to the slide.

Incubation is carried out by placing the slide with the charged sample chamber in a forced-convection incubator containing a pan of water and covering the slide with a glass specimen jar. A damp sponge is set next to the slide under the jar to assure no significant loss of water from the saturated oil.

For biotin assay an inoculum of *Lactobacillus arabinosis* 17-5 is prepared the day before assay by seeding 5 ml of inoculum broth (1% each of glucose, Casitone-Difco, and yeast extract-Difco, and 0.5% K_2HPO_4) with 1-3 loopsful of stock culture (culture medium: 1.5% APT agar, Case) and incubating at 30°C for 6-7 hr. The inoculum, first washed 3 times by centrifugation and resuspension in 5 ml of physiological saline solution, is diluted 10,000 to 50,000 times with the saline solution. One drop, about 0.05 ml, of this is added to 6 ml of recently sterilized (autoclaved 15 min at 121°C) double-strength Wright-Skeggs assay medium(6) in which the cystine was replaced by cysteine

TABLE I. Effect of 24-Hour Incubation on Diameter of Pairs of Droplets of Uninoculated Medium.

Vol (μl) of droplet	Diameter (mm, ocular scale) Before A	Before B	After A	After B	Change A	Change B	Light beam
.6	1.081	1.258	1.084	1.258	+.003	0	.309
.035	.458	.408	.458	.406	0	−.002	.097
.010	.269	.280	.265	.280	−.004	0	.058

and 1 mg of folic acid per liter of medium was added(1). The tube of inoculated medium is incubated at 30°C until used the following day, to use up any residual biotin and to obtain disperse growth with minimum clustering.

Sterile buffered (0.015 M potassium phosphate, pH 6.8) standard biotin solutions (0.1, 0.2, 0.4, 0.6, 1.0, 2.0 mμg/ml) and unknowns are mixed individually with equal volumes of inoculated medium and 3 droplets of each solution are placed under oil in the same chamber for incubation at 30° for 24 hr. Starting with a droplet containing the greatest concentration of biotin, the light beam is centered in the droplet, and the area illuminated is adjusted with the radiant field diaphragm to read 75-85% transmission on the photometer scale (diameter of beam is 22-30% of maximum diameter of droplet). For the smallest droplets (0.01 μl) a black disc with a 0.48 mm hole is used for the diaphragm. Without changing the optical system, each droplet in turn is centered and the reflectance is recorded. The data are used to construct a standard curve for the assay from which values of the unknowns are obtained by interpolation.

Results. The accuracy and precision of the droplet technic is shown by Table II in which the degree of agreement with analysis by the previously employed absorption method, and the reproducibility of analyses by each method, are evident. The greater sensitivity of reflectance measurement over absorption is apparent from Table III in which each increment in biotin concentration effected an increment in the reflectance measured of about twice the corresponding change in absorption. Application of the droplet technic to biotin assay of liver is illustrated by Table IV. Different amounts of each of 2 different livers gave analyses that had maximum variations of 6 and 3%, respectively. Recovery of biotin added to liver hydrolysates was found to be within ± 10% (Table V).

TABLE III. Comparison of Biotin Measurements by Light Scatter and Absorption with 1 μl Droplets of Assay Mixture.

Biotin (10^{-14}g) /droplet	% transmission* Scatter	Absorption†
0	6	96
5	15	91
20	37	82
100	73	65

* Each value mean of 3 droplets, 0.2 mm diam. light beam, sensitivity setting 1000×.
† Neutral filter used to reduce light intensity.

Response of *Lactobacillus arabinosis* 17-5

TABLE II. Comparison of Biotin Analyses of Fat-Free Dry Rat Liver by Light Scatter with Droplets and Absorption in Cuvettes.

Sample	Vol (μl) of reaction mixture Cuvette*	Droplet†	No. assays	Liver (10^{-9}g) /assay	Biotin (10^{-12}g)/μg liver Mean	S.D.	Coeff. of variation (%)
A	120		15	4800	4.75	.18	3.8
B	120		15	4800	4.90	.17	3.5
A		.6	9	24	4.73	.26	5.5
A		.035	12	1.4	4.78	.23	4.8
B		.035	12	1.4	4.91	.29	5.9
A		.010	6	.4	4.74	.23	4.9

* Single analysis/assay. † Triplicate analyses/assay.

to biotin in both ml and sub-μl volumes of total incubation mixture is represented in Fig. 3. It is apparent that, with the 0.010 μl droplets, a change of over 10% transmission per 1×10^{-15} g of biotin was obtained up to 50% transmission.

Summary. A technic for microscopic microbiological assay was elaborated in which droplets of total incubation mixture down to 0.010 μl volume were employed under oil. Measurement of bacterial growth by light scatter was used. The technic was applied to determination of biotin to 10^{-15} g with approximately the same percent accuracy and precision as the macrotechnic with ml-volumes.

TABLE IV. Biotin Assay of Fat-Free Dry Rat Liver by Light Scatter with 0.6 μl Droplets of Assay Mixture.*

Liver (10^{-10}g) /assay	Biotin (10^{-15}g) /droplet		Biotin (10^{-14}g) /μg liver	
	A	B	A	B
127	55	60	433	472
255	117	120	459	470
510	232	247	455	484

* Assay values mean of triplicate analyses of liver from two rats, A, B, respectively.

TABLE V. Recovery of Biotin Added to Hydrolyzed Rat Liver Measured by Light Scatter with 0.6 μl Droplets of Assay Mixture.*

Biotin (10^{-15}g)			
Hydrolysate	Added	Total found	% recovery
37	80	110	91
40	80	126	107
55	60	113	97
55	60	110	92
60	60	120	100
66	60	129	105
68	60	129	102
73	40	117	110

* Assay values mean of triplicate analyses.

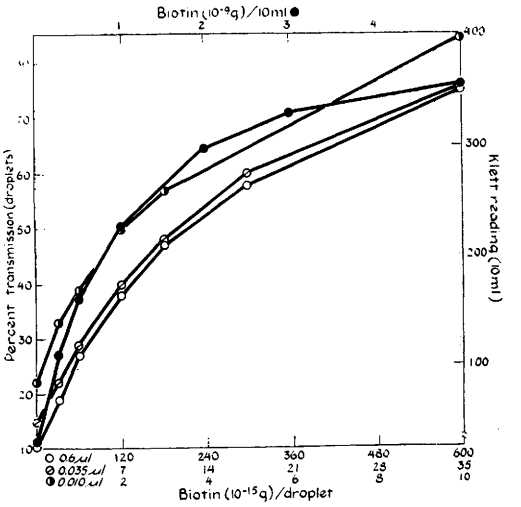

FIG. 3. Response of *Lactobacillus arabinosis* (17-5) to biotin in 10 ml and droplet assays. Sensitivity settings on the Photovolt photometer for droplets 0.60, 0.035, and 0.010 μl are 1000, 100, and 10×, respectively.

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Effect of Sulfated Mucopolysaccharides and of Endogenous Plasma Heparin(s) on Thromboplastin Generation. (27344)

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The first stage of clotting accomplishes the formation of plasma thromboplastin. Heparin delays the rate of thromboplastin production at a concentration as low as 1/80 unit/ml(1) and prevents its formation at 1/20-1/30 unit/ml(1,2). It therefore seemed desirable to evaluate the effect on thromboplastin generation of an extract of normal human plasma which we believe contains heparin(3). Since other acid mucopolysac-