and differential leucocyte counts. Sulfobromophthalein retention, serum urea nitrogen, total serum bilirubin, and serum alkaline phosphatase never deviated significantly. At each of the 20 periods when cholinesterase was determined the values were calculated as percentages of their predose values. The mean percentages for the Sevin-dosed dogs then were compared to those of the control dogs similarly handled. None of these was significantly different.

The weights of livers and kidneys of the Sevin-dosed dogs, individually or collectively by group, were not significantly higher than those of their controls. The obese female had organs of normal weight but on the basis of percentage of body weight they were about one half the usual value.

Microscopic examination of sections of the kidney revealed diffuse cloudy swelling of the proximal convoluted and loop tubules and focal Sudanophilic dust in the glomeruli of dogs which received 400 p.p.m. of Sevin. These are interpreted as transitory conditions and not as early stages of toxic degeneration, because the same conditions were present in the control dogs but to a lesser extent. Considerable intracellular fat was observed in the proximal kidney tubules of females. Its presence apparently does not represent a significant pathological lesion resulting from specific action of the compound but rather variability within the normal range.

One of the two female dogs that received 25 p.p.m. of Sevin had a transient hind leg weakness after the 189th dose of Sevin. Dosing was continued unremittingly and before 21 days elapsed the dog appeared normal. At the end of the study there was no microscopically detectable difference between the tissues of this dog and those of the other animals which had demonstrated no such weakness even though many of them received 16 times as much insecticide.

The tissues from the 14 dogs killed after 1 year of oral doses of 400 p.p.m. or less of Sevin showed no permanent degenerative changes. Neither was there any statistically significant deviation from the controls in the other criteria of effect studied—namely, body weight change, organ weights, hematological studies, biochemical tests, cholinesterase levels, and mortality.

Acknowledgment

The authors thank Mary Ann Babyak for biochemical assistance, Jean A. Striegel and Naomi Condra for animal treatment, and Frances Mocker for preparation of tissues for micropathological interpretation.

Literature Cited

(1) Barnes, J. M., Denz, F. A., *J. Pathol. Bacteriol.* **65,** 597–605 (1953).
(2) Berenbom, M., Young, L., *Biochem. J.* **49,** 165–9 (1951).
(3) Boyland, E., Wiltshire, G. H., *Ibid.*, **53,** 636–41 (1953).
(4) Burgen, A. S. V., *Brit. J. Pharmacol.* **4,** 214–28 (1949).
(5) Corner, E. D. S., Billett, F. S., Young, L., *Biochem. J.* **56,** 270–4 (1954).
(6) Fisher, R. A., "Statistical Methods for Research Workers," 11th ed., pp. 92–5, Oliver and Boyd, Edinburgh, 1950.
(7) Fitzhugh, O. G., Buschke, W., *Arch. Ophthalmol. (Chicago)* **41,** 572–82 (1949).
(8) Frawley, J. P., Fuyat, H. N., J. AGR. FOOD CHEM. **5,** 346–8 (1957).
(9) Frawley, J. P., Hagan, E. C., Fitzhugh, O. G., *J. Pharmacol. Exptl. Therap.* **105,** 156–65 (1952).
(10) Haynes, H. L., Lambrech, J. A., Moorefield, H. H., *Contrib. Boyce Thompson Inst.* **18,** 507–13 (1957).
(11) Kolbezen, M. J., Metcalf, R. L., Fukuto, T. R., J. AGR. FOOD CHEM. **2,** 864–70 (1954).
(12) Merrell, M., Shulman, L. E., *J. Chronic Diseases* **1,** 12–32 (1955).
(13) Michel, H. O., *J. Lab. Clin. Med.* **34,** 1564–8 (1949).
(14) Moorefield, H. H., Tefft, E. R., *Contrib. Boyce Thompson Inst.* **19,** 295–301 (1958).
(15) Rao, C. R., *Biometrics* **14,** 1–17 (1958).
(16) Weil, C. S., *Ibid.*, **8,** 249–63 (1952).

Received for review March 16, 1960. Accepted September 9, 1960.

ANIMAL METABOLISM OF INSECTICIDES

The Metabolism of Orally Administered Malathion by a Lactating Cow

R. D. O'BRIEN,[1] W. C. DAUTERMAN, and R. P. NIEDERMEIER

Departments of Entomology and Dairy Husbandry, University of Wisconsin, Madison, Wis.

Malathion was rapidly excreted by a lactating cow, principally via the urine, which accounted for 90% of the excreted material. About 23% of the dose was not excreted over 3 weeks. As in nonruminants, the major metabolite was produced by carboxyester hydrolysis; the principal fecal metabolite, however, was dimethyl phosphate. Milk contained no malathion or malaoxon, but had 0.11 p.p.m. of radioactive materials, most of which could not be identified. Blood metabolites were also examined.

WHEN malathion, *O,O*-dimethyl *S*-1,2-bis(carboethoxy)ethyl phosphorodithioate, is injected into mice, rats, or dogs, it is degraded rapidly, the principal reaction being hydrolysis of the ethyl ester bonds (*8, 9*). In insects the degradation is slower, and ethyl ester hydrolysis is somewhat less important, cleavage of a phosphate thioester bond being correspondingly more significant (*9*). Tissue residues following spray application of P^{32}-labeled malathion to calves were reported by March *et al.*, but no metabolites were identified (*10*). Milk and fat residues after spraying cows were reported by Claborn *et al.* (*6*); colorimetric assays on milk showed small residues which disappeared after 1 day.

Malathion is degraded to unknown products by cow rumen juice (*1*). Therefore, when malathion was fed to the cow, the metabolites produced might be unlike those found in nonruminant mammals. The extent of degradation might also be increased.

In the present study, malathion was fed at moderate levels to a lactating cow for 3 days, and the nature and extent of the metabolites produced were examined for 3 weeks.

[1] Present address, Pesticide Research Institute, London, Ontario, Canada.

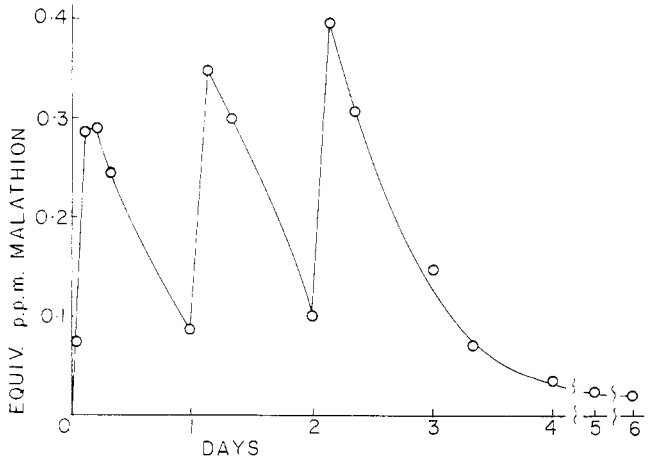


Figure 1. Total P^{32} in blood, expressed as p.p.m. of malathion

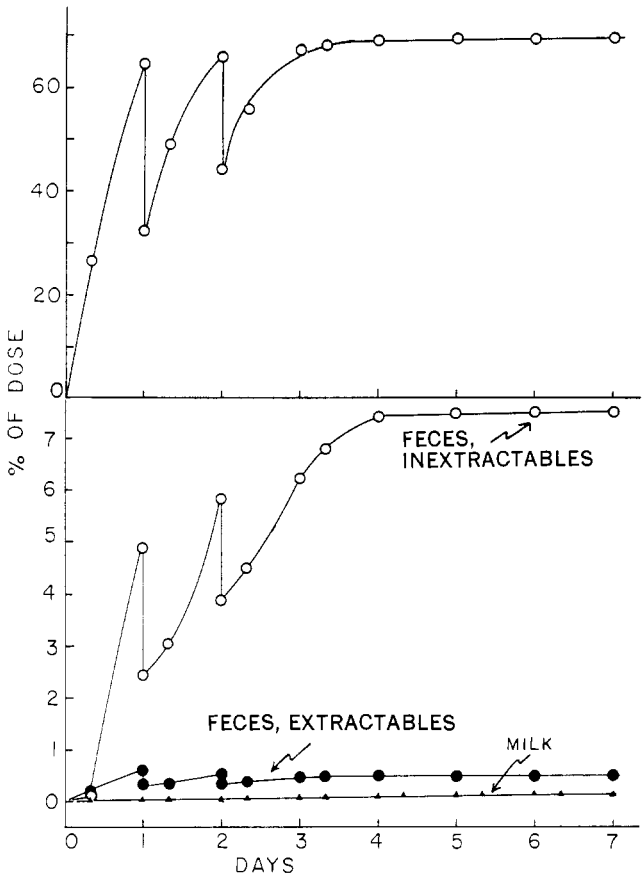


Figure 2. Cumulative per cent excretion of total P^{32} in urine

Top. In urine Bottom. In milk and feces ▶

Table I. Daily Output after 1, 2, and 3 Weeks

No. of Weeks	Malathion Equivalents, Mg.				
		Feces			
	Urine	Inextractables	Extractables	Milk	Total
1	0.944	0.353	0.013	0.037	1.347
2	0.052	0.468	0.012	0.021	0.553
3	0.051	0.197	0.023	0.011	0.282

1 mg. = 0.04% of dose; figures are for 7th, 14th, and 21st days.

Methods

Malathion labeled with phosphorus-32 was synthesized and purified by the method described previously (*9*), which utilizes an exchange reaction between labeled phosphoric acid, $H_3P^{32}O_4$, and phosphorus pentasulfide, P_2S_5 (*5*). The period of the final reflux was extended from 4 to 12 hours. The yield based on P_2S_5 was 87%. The product had an infrared spectrum identical with that of a sample provided by American Cyanamid Co. as 99+ % pure. The activity was 1.67 mc. per gram.

The malathion was administered to a 1252-pound lactating Guernsey cow for 3 days. Two gelatin capsules, each containing 374 mg. of compound in bran, were given daily, one immediately after the other. This represented 63 p.p.m. of the dry matter intake (26 pounds per day as concentrate, corn silage, and hay) or 1.3 mg. per kg. per day. The cow yielded about 20 pounds of milk per day. The cow was catheterized and held in a stall. The feces, urine, and milk were collected and measured at 8 A.M. and 4 P.M. daily, and aliquots taken for analysis. Blood samples were taken from the jugular vein into heparinized tubes at various intervals as indicated in Figure 1. Two further sets of samples were taken at 14 and 21 days.

Extraction Procedures. The extraction procedure was based upon that used by Anderson *et al.* (*3*) for Co-Ral. A 50-ml. sample of milk, or five-times diluted blood, or 50 grams of feces were mixed with 7 grams of Celite, and 200 ml. of acetone were added. The mixture was homogenized (Lourde's multimixer) in an iced 1-quart jar for 2 minutes, then filtered on a Büchner funnel through a No. 4 Whatman paper. The paper and precipitate were returned to the jar, 200 ml. of benzene were added, and the process was repeated. The jar was washed out with 50 ml. of benzene. The filtrates were combined and 200 ml. of water were added. The mixture was shaken in a separatory funnel and the phases were separated. The aqueous phase was filtered (Whatman 420), and the benzene phase was dried with sodium sulfate; in each case clear solutions resulted. This procedure gave recoveries of 85 to 95% of labeled malathion which had been added directly to milk, blood, and feces, the malathion being in the benzene phase.

To determine if the urine contained solvent-extractable materials, 100 ml. were extracted with 200 ml. of carbon tetrachloride, a portion of which was then counted.

Chromatographic Analysis. The water-extractable metabolites were separated by a modification of the Dowex 1 ion exchange resin technique of Plapp and Casida (*13*). The acetone of the acetone-water solution was substantially removed by vacuum before being added to the column. The successive pairs of solvents used in the gradient elution were:

1. 50 ml. of 0.01*N* HCl; 250 ml. of 0.1*N* HCl. 2. 50 ml. of 0.01*N* HCl plus 150 ml. of methanol; 50 ml. of 0.1*N* HCl plus 150 ml. of methanol. 3. 50 ml. of 0.1*N* HCl plus 150 ml. of methanol; 50 ml. of 0.1*N* HCl plus 150 ml. of methanol. 4. 50 ml. of 0.1*N* HCl plus 150 ml. of acetone; 50 ml. of conc. HCl plus 50 ml. of water plus 150 ml. of acetone.

Identification was achieved by cochromatography with known metabolites, which were detected by phosphorus assay (*2*). Malathion diacid: *O,O*-dimethyl *S*-(1,2-biscarboxy) ethyl phosphorodithioate; malathion monoacid: either *O,O*-dimethyl *S*-(1-carboethoxy 2-carboxy) ethyl phosphorodithioate, or *O,O*-dimethyl *S*-(1-carboxy 2-carboethoxy) ethyl phosphorodithioate; potassium dimethyl phosphate; potassium *O,O*-dimethyl phosphorothioate; and potassium *O,O*-dimethyl phosphorodithioate were kindly provided by American Cyanamid Co. Desmethyl malathion, *O*-methyl *O*-hydrogen *S*-(1,2-biscarboethoxy) ethyl phosphorodithioate, was prepared by refluxing potassium *O,O*-dimethyl phosphorodithioate with equimolar malathion in acetone overnight; the acetone was removed and water added. The material was extracted three times with hexane, and the aqueous preparation, which also contained residual potassium *O,O*-di-

methyl phosphorodithioate, was used for cochromatography.

The solvent-extractable materials in benzene-acetone were taken to dryness with a rotary evaporator, dissolved in hexane, and added to an alumina column (*9*). Lipides were eluted by hexane, malathion was eluted by benzene, and malaoxon by chloroform; methanol was used to elute any other products.

Samples were counted with a liquid counter which held 14 ml., except in the case of the ion exchange column, for which 1-ml. samples of the eluted fractions were placed in planchettes and counted with an end-window counter.

Cholinesterase determinations were made in triplicate. The whole blood, which has virtually no plasma cholinesterase (*4*), was diluted with an equal volume of water: then 1 ml. was added to a Warburg flask with 1.5 ml. of 0.4% sodium bicarbonate. The substrate in the side arm was 0.2 ml. of 2% acetyl choline bromide. Manometric assays were made at 38° C. under 5% carbon dioxide–95% nitrogen.

Results

Quantity of Metabolites. BLOOD. Figure 1 shows the total P^{32} in blood, expressed as parts per million of malathion. Zero time is the time of administration of the first dose. The surge in blood activity within the first hours after each dose is followed by an extremely rapid decline. If the blood volume is taken as 8% of the body weight (*7*), then the blood 1 hour after the first dose contains 18% of that dose. Of the total activity, the fraction extracted by solvents oscillated (not shown in figure); 3 hours after the first dose 50% was extractable, rising to 94% after 24 hours. Three hours after the second dose the fraction was 26%, rising to 60% after 24 hours from that dose; 3 hours after the third it was 8%, one day after that dose it was 46%, and by the next day (and thereafter) 100% was extractable.

Erythrocyte cholinesterase was studied for all samples up to 3 days, but no significant inhibition was observed.

EXCRETION RATES. Figure 2 shows that malathion metabolites were rapidly excreted in urine, 69% of the total being excreted 4 days after the first dose. After this the excretion rate dropped.

Figure 2 shows the excretion into milk and feces, which again was virtually complete at 4 days.

Table I shows the very small daily outputs after 1, 2, and 3 weeks; the feces by 2 weeks have become the principal excretion route.

The levels of activity in milk were relatively constant: They rose steadily to 0.11 p.p.m. (expressed as malathion) in 3 days, then dropped slowly to 0.07 p.p.m. at 1 week. At 2 weeks there were 0.03 p.p.m., at 3 weeks 0.01 p.p.m.

Table II. Water-Soluble Metabolities in Urine and Feces

	% of Total Water Solubles					
	Dimethyl phosphate	*Molothion monoacid*	*Malathion diacid*	*O,O-Dimethyl phosphoro-thioate*	*Desmethyl malathion*	*O,O-Dimethyl phosphoro-dithioate*
Urine						
8 hr.	2	66	12	9	9	2
1 day	2	55	26	7	8	2
3 days	2	56	30	7	5	0
4 days	3	15	54	15	9	4
Composite for week	2	63	17	11	7	0
Feces						
Composite for week	47	6	9	29	9	0

Zero time is time of administration of first dose. Each result is average of two determinations. Percentages based on total activity present in ion exchange column peaks, all of which were identified.

At 7 days, 77.2% of the dose was recovered, 69.0% of which was in urine, 8% in feces, and 0.2% in milk. In the next 2 weeks only another 0.02% was recovered from all sources. The remaining 22.8% must have become incorporated into body tissues. This figure would be a little reduced if the extraction techniques were poor, but because most of the activity was in urine, it was counted without extraction, and the amount remaining in the body must have been at least 20% of the dose. This represents incorporation of 42 mg. of phosphorus into the metabolic pool of a 568-kg. animal whose serum alone contained at least 1 gram of phosphorus (*7*), and whose phosphorus was undergoing rapid turnover in the secretion of milk, in which 7.6 grams of phosphorus per day were put out (*11*); the result is therefore reasonable.

Nature of Metabolites. URINARY. A negligible amount of urinary metabolites was extractable by carbon tetrachloride (as malathion or malaoxon would be)—from 0.00006 to 0.006% of the total. Table II shows that malathion monoacid was the principal urinary metabolite; malathion diacid became increasingly important with the later samples. Desmethyl malathion was a significant component, but dimethyl phosphate was of little importance (as contrasted with feces). The zero value recorded for *O,O*-dimethyl phosphorodithioate in the composite is of course anomalous; oxidation to the phosphorothioate may have occurred on standing.

FECAL. An average of 7% of the total fecal metabolites during the first week partitioned into benzene-acetone from water-acetone; clearly, degradation products were the main components. Table III shows that in the benzene-acetone fraction, 85% of the labeled material was malathion, but malaoxon was also present (12%). Table II shows that the principal degradation products were dimethyl phosphate and *O,O*-dimethyl phosphorothioate in strong contrast to the products in urine.

BLOOD. An average of 31% of the

Table III. Solvent-Extractable Materials in Blood and Feces

	% of Total	
Material	*Blood*	*Feces*
Soluble in hexane		
Hexane	0.3	2.0
Malathion	27.6	84.7
Malaoxon	15.3	11.7
Methanol eluate	0.4	1.6
Insoluble in hexane, soluble in ether		
Soluble in acetone-ether	55.8	...
Insoluble in acetone-ether (phospholipide)	0.6	...

Blood was extracted fresh each day. After first week, fractions partitioning into acetone-benzene from acetone-water were combined and analyzed further, with results shown.

total blood metabolites during the first week partitioned into benzene-acetone from water-acetone. Table III shows that of the benzene-acetone fraction, malathion (28%) and malaoxon (15%) constituted a minority of the total labeled material. Some labeled phospholipide was found (0.6%), but most activity (56%) was in an unidentified fraction, insoluble in hexane but soluble in ether and ether-acetone. This fraction probably represents a normal constituent of blood which has incorporated P^{32}. It may account for the 100% extractability observed on the 4th day, as it is unlikely that malathion or malaoxon would persist so long.

MILK. Only 29% of the radioactivity in milk was recoverable by the standard acetone-plus-benzene extraction; all of this partitioned into water-acetone from benzene-acetone, and therefore malathion and malaoxon probably were absent. All (102.0%) of the missing radioactivity was recovered from the residue from the extraction procedure by nitric acid digestion (concentrated nitric acid, 4 hours at 100° C.). Treatment with ether of the residue from extraction yielded only 1.3% of the missing radioactivity. It seemed probable

that the inextractable phosphorus was in casein; this protein is vigorously synthesized during lactation, and contains 0.8 to 0.9% of phosphorus (*11*, *12*). A sample of milk taken at 4 days was centrifuged to remove fat, and hydrochloric acid was added to pH 4.7, in order to precipitate the casein. The filtrate contained 76% of the radioactivity. The major fraction of the P^{32} in milk therefore is not casein; its identity is unknown.

Discussion

Because this study was carried out with one animal, the quantitative aspects of the results should only be tentatively accepted. However, it is clear that metabolism in the cow is substantially similar to that in the mouse, rat, and dog in that hydrolysis at the carboxyester bond accounts for most degradation. However, the fecal metabolites were mainly the product of phosphate ester hydrolysis, suggesting (but not proving) that rumen microorganisms degraded malathion by phosphatase action. If so, the reason that so little phosphatase products were found in the urine might be the fairly rapid uptake from the rumen that was observed, so that metabolism in the rumen was not very important.

Acknowledgment

The authors are grateful to Judy Engel and Lydia McBride for expert technical assistance. The malathion intermediates for cochromatography were kindly supplied by Shirley DuBreuil and R. W. Young of American Cyanamid Co.

Literature Cited

(1) Ahmed, K. A., Casida, J. E., Nichols, R. E., J. AGR. FOOD CHEM. **6,** 740 (1958).
(2) Allen, R. J. L., *Biochem. J.* **34,** 345 (1940).
(3) Anderson, C. A., Adams, J. M., MacDougall, D., J. AGR. FOOD CHEM. **7,** 256 (1959).
(4) Augustinsson, K. B., *Acta Physiol. Scand.* **15,** Suppl. 52 (1948).
(5) Casida, J. E., *Acta Chem. Scand.* **12,** 1691 (1958).
(6) Claborn, H. V., Radeleff, R. D., Beckman, H. F., Woodard, G. T., J. AGR. FOOD CHEM. **4,** 941 (1956).
(7) Dukes, H. H., "The Physiology of Domestic Animals," pp. 54, 62, Comstock Publishing Co., Ithaca, N. Y., 1947.
(8) Knaak, J. B., O'Brien, R. D., J. AGR. FOOD CHEM. **8,** 198 (1960).
(9) Krueger, H. R., O'Brien, R. D., *J. Econ. Entomol.* **52,** 1063 (1959).
(10) March, R. B., Metcalf, R. L., Fukuto, T. R., Gunther, F. A., *Ibid.*, **49,** 679 (1956).
(11) Maynard, L. A., "Animal Nutrition," p. 409, McGraw-Hill, New York, 1947.
(12) McMeekin, T. L., in "The Proteins," H. Neurath and K. Bailey, eds., Vol. **2A,** p. 389, Academic Press, New York, 1954.
(13) Plapp, F. W., Casida, J. E., *Anal. Chem.* **30,** 1622 (1958).

Received for review February 8, 1960. Accepted May 5, 1960. Approved for publication by the director of the Wisconsin Agricultural Experiment Station. Study supported in part by grants from American Cyanamid Co. and Regional Research Project NC-33.

INSECTICIDE RESIDUES

Fluorometric Method for Estimation of Residues of Bayer 22,408

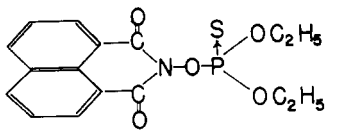
PAUL A. GIANG

Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

A rapid and sensitive fluorometric method for the estimation of microgram amounts of O,O-diethyl O-naphthalimido phosphorothioate (Bayer 22,408) is based on the principle that, when mixed with methanolic sodium hydroxide and dioxane in the presence of hydrogen peroxide, solutions of this compound fluoresce in proportion to concentration. The sensitivity of the method is 5 γ, and recoveries of the insecticide added to milk and to butterfat were over 90%. The method was also applied to the determination of Bayer 22,408 in plants.

ENTOMOLOGICAL interest in (*O,O*-diethyl *O*-naphthalimido phosphorothioate (Bayer 22,408) has created a need for a sensitive analytical method of detecting and estimating minute amounts of residues of this compound in animal tissues or on treated plants. Interest has centered especially on its toxic action on pests that are injurious and destructive to farm animals.

A technical grade sample of Bayer 22,408 was obtained as a brown powder from the Vero Beach Laboratories, Inc., Vero Beach, Fla. This was crystallized several times from *n*-hexane containing a little chloroform to give fine light yellow crystalline flakes melting at 160° to 161° C. The compound has the following structural formula:



It is not soluble in water but very soluble in most organic solvents, although only slightly soluble in petroleum ether.

Hornstein (*3*) reported that fluorometric methods may be worked out for a number of insecticides containing multiple-conjugated double bonds that possess a high degree of resonance stabilization. Anderson, Adams, and MacDougall (*1*) published a photofluorometric method for the determination of *O* - (3 - chloro - 4 - methylumbelliferone) *O,O* - diethyl phosphorothioate (Co-Ral, also known as Bayer 21/199), using 1*N* potassium hydroxide and heat.

In the development of a fluorometric method for the estimation of Bayer 22,-408 residues, it was found that this compound fluoresces weakly in methanolic sodium hydroxide solution. The fluorescence has a calamine blue color, and its intensity is greatly increased when dioxane containing a small amount of hydrogen peroxide is added to the reaction mixture. Work on the identification of the fluorescent compound is in progress.

Apparatus

Fisher Nefluoro-Photometer or equivalent.

Filter arrangement. Left sample filter, No. 440; light-source filter, No. 365; and right reference filter, No. 430.