lethality described above, the agents investigated manifest some striking qualitative differences in toxicity. Although the design of the test cages did not permit continuous observation, it was possible to make intermittent examinations of general behavior. On the basis of these observations it was apparent that the toxicity of racemic amphetamine and D(−) ephedrine could be qualitatively differentiated from that of the other ephedrine isomers employed. For example, pilo erection was commonly observed in mice which had been treated with either racemic amphetamine or D(−) ephedrine and then placed in either isolation or aggregation. In contrast, little pilo erection was observed in animals subjected to similar environmental conditions after having been treated with L(+) ephedrine, D(−) pseudoephedrine, or L(+) pseudoephedrine.

Animals injected with lethal doses of either racemic amphetamine or D(−) ephedrine and then placed in an isolated environment demonstrated tremors, loss of righting reflex, clonic and tonic convulsions, and acute respiratory failure leading to death. These animals invariably died within 1 hour after drug administration, usually in the throes of a convulsion. On the other hand, there appeared to be two distinct causes of death in mice treated with lethal doses of either of the above agents and then placed in a crowded environment. Those animals that died within the first hour succumbed to convulsions (as described above), whereas exhaustion due to prolonged hyperactivity or repeated episodes of clonic convulsions was the apparent cause of death in animals that expired 1 or more hours after drug administration. These mice lost their righting reflex, appeared comatose, and experienced progressive respiratory difficulty until apnea occurred and death ensued.

In contrast to the above, the pattern of toxicity observed in mice treated with L(+) ephedrine, D(−) pseudoephedrine, or L(+) pseudoephedrine was essentially the same for both environmental situations. Death was caused by acute respiratory failure preceded by clonic or tonic convulsions, and always occurred within 1 hour after drug administration.

An explanation for the failure of L(+) pseudoephedrine to demonstrate an "aggregation" phenomenon is not readily available. However, a number of preliminary experiments conducted in our laboratory, *e.g.*, determination of the behavioral changes accompanying drug administration, low frequency electroshock seizure threshold studies, and examination of drug-induced alterations in levels of central nervous system activity measured by chemoshock techniques indicate that several major quantitative and qualitative differences exist among these four ephedrine isomers. It is anticipated that further investigation into the exact nature of central stimulation induced by these agents will help elucidate the findings reported herein.

REFERENCES

(1) LaPidus, J. B., Tye, A., Patil, P., and Modi, B. A., *J. Med. Chem.*, **6**, 76(1963).
(2) Gunn, J. A., and Gurd, M. R., *J. Physiol. London*, **97**, 453(1940).
(3) Chance, M. R. A., *J. Pharmacol. Exptl. Therap.*, **87**, 214 (1946).
(4) *Ibid.*, **89**, 289(1947).
(5) Lasagna, L., and McCann, W. P., *Science*, **125**, 1241 (1957).
(6) Burn, J. H., and Hobbs, R., *Arch. Intern. Pharmacodyn.*, **113**, 290(1958).
(7) Swinyard, E. A., Clark, L. D., Miyahara, J. T., and Wolf, H. H., *J. Pharmacol. Exptl. Therap.*, **132**, 97(1961).
(8) Litchfield, J. T., and Wilcoxon, F., *ibid.*, **96**, 99(1949).

Colorimetric Microdetermination of Plasma Free Fatty Acids

By DONALD C. KVAM, J. G. SCHMIDT, D. A. RIGGILO, and D. G. GALLO

The application of a colorimetric method to the determination of free fatty acids in plasma is described. The method is simple, sensitive, and yields results comparable to those obtained with the titrimetric procedure.

THE MEASUREMENT of unesterified fatty acid levels in biological samples is usually accomplished by microtitration of the acidity present in suitably prepared extracts (1). Such microtitration techniques require special equipment and even with care and experience may give variable results. Ayers reported a colorimetric method for the estimation of fatty acids based upon the finding that copper or cobalt salts of monocarboxylic acids dissolve in chloroform to give colored solutions (2). Iwayama subsequently developed an essentially new procedure, also based upon the solubility of the copper salts of C_{10}–C_{22} monocarboxylic acids in chloroform, but its sensitivity was not great enough to be useful for the determination of plasma free fatty acids (3). The sensitivity of the latter procedure was greatly increased by Duncombe, who measured the concentration of copper in the chloroform solution with diethyldithiocarbamate (4). We have adapted this colorimetric method to the determination of plasma free fatty acids following their extraction by the technique of Dole and Meinertz (1). The resulting procedure is simple, very sensitive and reproducible, and uses commonly available laboratory equipment.

Received November 18, 1963, from Mead Johnson Research Center, Evansville, Ind.
Accepted for publication January 10, 1964.

EXPERIMENTAL

Reagents.—The following reagents were used.

Extraction Solution.—Mix 1 *N* H_2SO_4, hexane, and isopropyl alcohol in the volume ratio 20:200:780.

Cupric Nitrate Reagent.—Mix 450 ml. of 1 *M* triethanolamine, 50 ml. 1 *N* acetic acid, and 500 ml. 5% cupric nitrate solution.

Sodium Diethyldithiocarbamate, 0.1%.—Dissolve 100 mg. $(C_2H_5)_2NCS_2Na \cdot 3H_2O$ in 100 ml. normal butanol (prepare fresh daily).

Stock Standard Fatty Acid Solution.—Dissolve 56.8 mg. stearic acid in 100 ml. of hexane.

Working Standard Fatty Acid Solution.—Dilute 10 ml. of stock standard to 100 ml. with hexane. This solution contains 0.2 μmoles/ml.

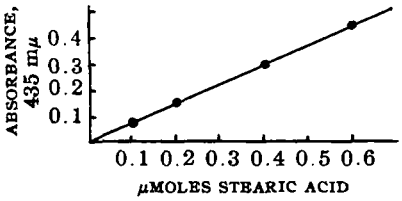

Fig. 1.—Typical standard curve.

Procedure.—The extraction of the plasma free fatty acids is accomplished as described by Dole and Meinertz (1), except that hexane rather than heptane is used as the hydrocarbon solvent due to its lower boiling point.[1] Transfer 0.25 ml. plasma to a 20 × 150 mm. screw-cap culture tube. Screw caps having Teflon liners should be used throughout. Add 0.75 ml. of distilled water and 5.0 ml. of the extraction solution. Shake the mixture well and allow to stand 5 minutes. Add 2.0 ml. of distilled water and 3.0 ml. of hexane, shake well, and allow the two phases to separate.[2] Transfer 4.0 ml. of the upper layer to a clean 20 × 150 mm. screw-cap culture tube and evaporate the solvent using gentle heat and a stream of nitrogen. Dissolve the residue in 5.0 ml. of chloroform and add 2.5 ml. of the cupric nitrate reagent. Shake well and centrifuge for 5 minutes at 2000 r.p.m. Carefully remove the upper aqueous layer by suction and transfer 2.0 ml. of the chloroform phase to a clean 10 × 75 mm. cell. Care must be taken at this point to avoid carrying over any of the aqueous phase, which contains a large amount of copper. Add 0.1 ml. of 0.1% sodium diethyldithiocarbamate, mix well, and determine the absorption of the solution at 435 mμ using a reagent blank which has been carried through the entire procedure. Maximum color develops immediately and is stable for over 2 hours. Deviations in the plasma and reagent volumes described are permissible, providing the ratios between reagents are not altered.

Standard Curve.—Transfer 0.5, 1.0, 2.0, and 3.0 ml. aliquots (equivalent to 0.4, 0.8, 1.6, and 2.4 μmoles free fatty acid per ml. plasma) of the working standard fatty acid solution to clean 20 × 150 mm. screw-cap culture tubes. Add hexane to a total volume of 3.0 ml., 3.0 ml. of distilled water, and 5.0 ml. of extraction solution. Shake well and allow to stand 5 minutes. Transfer 4.0 ml. of the upper layer to clean 20 × 150 mm. screw-cap culture tubes and carry through the remaining procedures as described above.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical standard curve obtained by absorbance *versus* μmoles stearic acid. Although we have observed only small variations in extinction, the standard curve should be checked daily for best results.

As reported by Ayers (2), the copper salts of monocarboxylic acids shorter than C-8 and of di- or tricarboxylic acids are not soluble in chloroform and do not interfere with the procedure. The copper salts of phospholipids are somewhat soluble and will interfere when present in large amounts. Synthetic *l*-dipalmitoyl lecithin and cephalin, obtained from California Corporation for Biochemical Research, were found to have, respectively, approximately 25 and 50% of the molar absorption of stearic acid. For this reason an extraction technique which discriminates against the phospholipids is required. When excessive amounts of phospholipids are present in the sample, the double extraction procedure of Dole and Meinertz (1) may be necessary.

TABLE I.—THE EFFECT OF EPINEPHRINE INJECTION ON PLASMA FREE FATTY ACIDS IN RATS

Epinephrine Dose,[a] mg./Kg./i.p.	No. Rats	Plasma Free Fatty Acids, μmoles/ml.	% Increase
Control	25	0.43 ± 0.03[b]	
0.02	10	0.57 ± 0.05	33
0.10	15	0.74 ± 0.05	72
0.50	10	0.92 ± 0.10	114

[a] Expressed as the base. [b] Standard error of the mean.

The results obtained with the colorimetric procedure are comparable to those obtained by the titrimetric method. In one study the free fatty acid concentration of five different samples of rat plasma was determined by both methods. The group mean was 0.61 μmoles/ml., with a standard deviation of 0.11 as determined colorimetrically, while the results of the titrimetric analysis gave values of 0.63 ± 0.06 μmoles.

Due to the extreme insolubility of fatty acids in aqueous media, it has been difficult to carry out recovery studies. However, the addition of stearic acid dissolved in a small amount of isopropanol to plasma has yielded recovery values averaging 95%.

The colorimetric method as described has been applied to the determination of plasma free fatty acids in both dogs and rats. It reflects accurately the changes produced by manipulations known to decrease or increase the level of plasma free fatty acids. For example, it is well accepted that epinephrine has the ability to cause large increases in these levels (5). Table I presents the results obtained 15 minutes following the intraperitoneal injection of various quantities of *l*-epinephrine bitartrate in saline into rats of the Sprague-Dawley strain. The control free fatty acid level as well as the increases in plasma free fatty acids in response to graded doses of *l*-epinephrine are similar to the results obtained by Maickel *et al.* in the same strain using the microtitration procedure (6). Similar results have been obtained with dogs and cats. The convenience and high sensitivity of this colorimetric procedure make it an extremely useful technique for the study of changes in plasma free fatty acids under a variety of experimental conditions.

[1] When five samples of a pooled rat plasma were extracted with heptane as the hydrocarbon solvent and the fatty acids determined by the above procedure, a value of 0.31 ± 0.016 μmoles/ml. was obtained. This compares favorably with the value of 0.32 ± 0.007 μmoles/ml. obtained with the same plasma when hexane was utilized in the extraction mixture.

[2] To aid in transferring the hexane phase, an additional 0.5 to 1.0 ml. of hexane may be added carefully after the phases have separated following shaking. This has no effect on the final result, provided the same volume is added to all tubes.

REFERENCES

(1) Dole, V. P., and Meinertz, H., *J. Biol. Chem.*, 235, 2595(1960).
(2) Ayers, C. W., *Anal. Chim. Acta*, 15, 77(1956).
(3) Iwayama, Y., *J. Pharm. Soc. Japan*, 79, 552(1959).
(4) Duncombe, W. G., *Biochem. J.*, 88, 7(1963).
(5) Havel, R. J., and Goldfien, A., *J. Lipid Res.*, 1, 102 (1959).
(6) Maickel, R., Sussman, H., Uamada, K., and Brodie, B., *Life Sci.*, 2, 210(1963).