two exponential terms whose decay constants are given by

$$\frac{-\{1/\bar{K} + E_0\} \pm \{(1/\bar{K} + E_0)^2 - 4p'_m/k_1\}^{1/2}}{2/k_1} \quad (12)$$

In certain favorable cases these transients could also be employed in deducing velocity constants. For example, if k_{-1} were very large compared to $(k_2 + k_1E_0)$, one decay constant would tend to zero, and the other to $-k_{-1}$, while if k_2 were very large compared to $(k_{-1} + k_1E_0)$, one decay constant would tend to $-k_1E_0$, and the other to $-k_2$.

With modern, high speed computing devices it is quite feasible to find by trial the unique set of values for the parameters needed to reproduce a given experimental concentration time curve for any component of the reaction system. This is easily done with an analog computer where the programming is direct and simple. However, the usefulness of the procedure may be limited in situations where there are wide differences in order of magnitude between the various parameters, *e.g.*, E_0 and S_0.

U. S. NAVAL MEDICAL RESEARCH INSTITUTE
BETHESDA, MARYLAND

Indicators for the Paper Chromatography of Lipids[1]

BY HELMUT K. MANGOLD, BEVERLY G. LAMP AND HERMANN SCHLENK

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The inclusion reaction has received considerable attention in recent years. One of the basic principles resulting from research in this field is that shape rather than chemical character of a molecule determines whether or not it can be included by a certain host. The complexing reaction can be carried out in minute amounts such as are used in practical paper chromatography. This is easily demonstrated by spraying a filter paper bearing a spot of octadecane with α-cyclodextrin and subsequently exposing it to iodine vapors. The area covered with hydrocarbon remains white, while the remainder of the paper turns bluish purple. This occurs because of different reactivities of the free and of the complexed α-dextrin. Free α-dextrin reacts with iodine to form a deeply colored inclusion complex very similar to that resulting from starch with iodine. In fact, the two reactions are closely related[2] and the same phenomenon can be observed with starch on a spotted paper. Comparative examination proved α-dextrin to be the preferable indicator in actual chromatograms. When the α-dextrin is already occupied by a guest molecule, in the above case the octadecane, it is no longer available for the iodine reaction which produces color. Accordingly, the spot of hydrocarbon stays white. This holds for other compounds having low chemical reactivity besides hydrocarbons; for example, esters, ethers or other types of molecules that react with α-dextrin. Long chain fatty acids, alcohols and esters fall into this category. Their chemical characteristics are not pronounced so that it is difficult to find indicators of a sensitivity adequate for use in paper chromatograms. Although various suggestions have been made to solve these difficulties,[3] paper chromatography does not hold its due place among analytical tools in the lipid field.

α-Dextrin can be used successfully on chromatograms of fatty alcohols, acids, methyl or ethyl esters and monoglycerides. Diglycerides and triglycerides do not respond to this indicator method, since they do not react readily with α-dextrin. However, they can be detected by splitting them into their components after chromatographic separation. The necessary hydrolysis is carried out *in situ* enzymatically by means of commercial pancreatin. In this way it is possible to make visible the separated di- and triglycerides on the paper.

Two other indicators were found to be applicable to certain types of lipids. Iodine vapors have been used for detecting a variety of substances[4] but not for higher fatty acids. In the lipid series we found it specific for spotting unsaturated components. For instance, oleic acid and its derivatives appear as brown spots on chromatograms when exposed to iodine vapors. The limitation of the method is given by the fact that symmetrical oleodipalmitin cannot be located unambiguously in actual chromatograms. A more specific reagent is lead tetraacetate which is applicable to the chromatography of monoglycerides. The reagent hydrolyzes in air to form brown lead dioxide; with α-monoglycerides it undergoes the normal glycol splitting reaction and yields colorless lead compounds.[5] Consequently, monoglycerides appear as white spots on a brown background in chromatograms sprayed with lead tetraacetate solution. For the detection of di- and triglycerides it can be used after splitting them by the above-mentioned enzymatic hydrolysis.

Complexing is the most universal method and is certainly applicable to many other compounds besides the lipids.

Table I shows R_f values obtained by chromatographing model mixtures and individual lipids for identification. Different solvent systems were used in ascending technique with silicone impregnated paper, *i.e.*, a reversed phase chromatography.

Whatman No. I paper was modified according to procedures reported in the literature. We found impregnation with silicone[6] to be the most versatile preparation for our purpose. Since silicone is not washed out by the developing solvents as hydrocarbons are from impregnated papers, the same paper can be used for reversed phase chromatography in the second dimension. Uniform coating is easily

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TABLE I

R_f VALUES[e] OF LIPIDS IN DIFFERENT SOLVENT SYSTEMS[f]

	R_f Acetic acid–water	R_f Tetrahydrofuran–water 3:2	R_f Chloroform–methanol 1:3
Satd. alcohols[a]	75% HAc		
Dodecyl	0.63		
Tetradecyl	.53		
Hexadecyl	.40		
Octadecyl	.28	<0.2	0.8–0.9
Unsatd. alcohols[a,c]			
Oleyl	0.40		
Satd. fatty acids[a]	85% HAc		
Lauric	0.74		
Myristic	.65		
Palmitic	.50		
Stearic	.36	<0.2	0.9–1
Unsatd. fatty acids[a,c]			
Oleic	0.52		
Linoleic	.64		
Linolenic	.73		
Satd. methyl esters[a]	85% HAc		
Laurate	0.52		
Myristate	.38		
Palmitate	.29		
Stearate	.16	<0.2	~0.5–0.7
Unsatd. methyl esters[a,c]			not suitable for sepn.
Oleate	0.32		
Linoleate	.41		
Linolenate	.51		
Satd. α-monoglycerides[a,d]			
Monolaurin		0.85	
Monomyristin		.70	
Monopalmitin		.49	
Monostearin	>0.9	.32	0.9–1
Unsatd. α-monoglycerides[a,c,d]			
Mono-olein		.45	
Monolinolein		.65	
Monolinolenin		.72	
Satd. 1,3-diglycerides[b]			
Dipalmitin			0.71
Unsatd. 1,3-diglycerides[c]			
Diolein			0.74
Satd. triglycerides[b]			
Trilaurin			0.45
Trimyristin			.28
Tripalmitin			.20
Tristearin	<0.1	<0.1	< .1
Unsatd. triglycerides[c]			
Triolein			0.24
Trilinolein			.36
Trilinolenin			.51
sym.-Diacid triglycerides[b]			
Oleodipalmitin			0.30
Oleodistearin			.22
Myristodistearin			< .1
Glycerol[d]	0.66	<1.0	0.60

Indicator method: [a] α-dextrin–iodine; [b] pancreatin, α-dextrin–iodine; [c] iodine; [d] lead tetraacetate. [e] Calculated by dividing the distance from starting line to center of spot by the distance from starting line to solvent front. [f] Ratios are vol./vol.

achieved and the quality of the paper is not changed during periods of six weeks storage. Still the R_f values are not reproducible enough to be used for characterizing an individual component. It was regularly found that they increased slightly with higher ascending fronts of the solvent. Also, it is advisable to use freshly prepared solvent mixtures to have a well controlled composition of the system.

From Table I it is seen that the R_f values increase with shorter chain length and higher unsaturation. Related to a saturated compound, the increment for one double bond is approximately equal to that found for shortening the chain length by one ethylene group. Therefore, it is impossible to separate oleic and palmitic acids, or diolein and dipalmitin. This reflects similar experiences in column chromatography,[7] displacement chromatography,[8] and also in countercurrent distribution.[9] By combining indicator method *c* with method *a*, oleic acid can be identified. Palmitic acid migrates at the same rate and its presence, superimposed on oleic acid, remains undetected. The separation and distinction of the pair can be carried out by exposing the chromatogram to bromine vapors. Subsequently the chromatogram is run in the second dimension in the same solvent which separates brominated and saturated acids. This has been carried out with oleic and palmitic acids and will be reported in detail for a variety of unsaturated acids in a forthcoming paper.

Besides chromatographing lipids groupwise, as compiled in Table I, several other combinations have been separated. Mono-, di- and triglycerides, having identical acid components, were separated in chloroform–methanol. Unsaturated monoglycerides mixed with the corresponding free acids were chromatographed, for the monoglycerides in tetrahydrofuran–water (the acids stay behind), and in acetic acid–water for the acids (the monoglycerides migrate with the solvent front). Cetyl alcohol, palmitic acid and its methyl ester were separated in 85% acetic acid. These examples illustrate the use of the table for choosing a working system for such mixtures.

It is impossible at the present time to chromatograph on paper a multicomponent mixture of closely related glycerides as they occur in natural mixtures, and it is still necessary to reduce the number of components. For example, coconut fatty acids could be chromatographed successfully only after they had been hydrogenated.

The procedures described already have proved their value where separation has been achieved without being intended. A commercial sample of margarinic acid was separated into two spots, representing palmitic and stearic acids. The R_f value of authentic margarinic acid[10] is between the values

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of these two acids. Commercial ethyl stearate contained free stearic acid. The presence of methyl esters could be shown in supposedly pure acids. Unsaturated triglycerides prepared according to accepted procedures[11] and having correct iodine values contained triglyceride, diglyceride, monoglyceride and methyl ester. Monoglycerides distilled in small lots in high vacuum contained free acid. These examples establish the method as useful for proving the identity and purity of lipids without appreciable loss of material.

Experimental

Paper.—Whatman No. 1 was cut in strips 12.5 cm. wide and 44 cm. long across the fiber and dried for 1 hour at about 200°. They were drawn through a solution of 5% silicone in ether (Dow Corning 200 fluid, viscosity 10 cs.), air-dried, and stored in a desiccator over calcium chloride. After such treatment, the paper is only slightly water repellent.

Lipids.—The amounts of lipid applied to the paper should be adjusted to the particular problem. Calibrated capillaries were used for measuring the desired amount of lipid solution in ether. It is advisable to start with small amounts (20 γ) which reveal the major components. In subsequent runs, higher amounts (up to 500 γ) should be used. Spots of major components with similar R_f values will then overlap but minor components having sufficiently different R_f values are easily detected.

Experimental Conditions.—The chromatograms were developed in ascending technique at approximately 20° within 16 hours to a height of about 20 cm. The paper strips were suspended by threads inside Pyrex glass cylinders (15.5 × 46 cm.). Modeling clay served for sealing glass plates to the tops of the chambers.

Indicator Methods

(a) **α-Dextrin-Iodine.**—α-Dextrin was prepared in this Laboratory.[12] The chromatograms were sprayed with a solution of 1% α-dextrin in 30% ethanol and air-dried. They were then placed into a humidifying chamber at room temperature for 1 hour. Humidity facilitates the subsequent iodine reaction which was carried out with iodine vapors in a dry container. Spots of saturated alcohols, fatty acids, esters and monoglycerides remain white, whereas the corresponding unsaturated compounds, which were white in the beginning, turn yellow or even brown after prolonged treatment. The background is violet due to the α-dextrin–iodine complex. The color fades within a few days but can be brightened up again by repeating the iodine treatment. Amounts of about 20 γ of acids or monoglycerides can be demonstrated on actual chromatograms by this technique.

(b) **Pancreatin.**—Di- and triglycerides are accessible to procedure a when split enzymatically into free acids and monoglycerides. The chromatograms were sprayed with a solution of 1% pancreatin (Takamine Labs., Clifton, N. J.) in water and incubated for about 24 hours at 37° in a moisture chamber. After air-drying they were subjected to the above α-dextrin–iodine technique. About 200 γ of a saturated triglyceride can be detected.

(c) **Iodine.**—Unsaturated alcohols, acids, esters and glycerides can be detected by exposure to iodine vapors. The spots are deep yellow or brown on a white background. Less than 20 γ of unsaturated material can easily be detected in the chromatogram.

(d) **Lead Tetraacetate.**—The air-dried paper strips were sprayed with a solution of 1% lead tetraacetate in absolute benzene. White spots on a brown background of lead dioxide are characteristic for monoglycerides or free glycerol. The sensitivity is 50 to 100 γ of monoglyceride in actual chromatograms.

UNIVERSITY OF MINNESOTA
THE HORMEL INSTITUTE
AUSTIN, MINNESOTA

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Preparation of β-Methylglutamic Acid[1]

BY D. C. MORRISON

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β-Methylglutamic acid was desired as a metabolite antagonist, and its synthesis was, therefore, undertaken. This compound has been prepared by Smrt and Sorm,[2] by a Schmidt reaction, and the α- and γ-methyl isomers were also obtained. The latter two amino acids had previously been synthesized by Gal, Avakian and Martin, and by Fillman and Albertson, respectively.[3,4]

It seemed logical to suppose that the β-methyl isomer might be available by an extension of the glutamic acid synthesis of Snyder, Shekelton and Lewis,[5] involving the condensation of ethyl acetamidomalonate with methyl acrylate. The reaction between ethyl acetamidomalonate and ethyl crotonate was studied, and the intermediate addition product was isolated as a solid of m.p. 73.5–74.5°. Contrary to the results obtained in the case of the γ-methyl isomer, loss of a carbethoxy group was not observed.

The intermediate was hydrolyzed and decarboxylated by heating with hydrochloric acid, furnishing a solution from which *dl*-β-methylglutamic acid could be obtained A second *dl*-form of the amino acid may be present in the mother liquors but it was not observed. The β-methylglutamic acid gave an N-benzoyl derivative which crystallized from water as a monohydrate.

In the corresponding synthesis of glutamic acid from methyl acrylate, previous workers did not isolate the addition product, but hydrolyzed the reaction mixture directly. This preparation was repeated, using ethyl acrylate, and the intermediate isolated and found to be N-acetyl-α-carbethoxyglutamic acid diethyl ester, a solid of m.p. 81–82.5°.

Experimental

N-Acetyl-α-carbethoxy-β-methylglutamic Acid Diethyl Ester.—A solution of 21.7 g. (0.1 mole) of ethyl acetamidomalonate in 150 ml. of absolute ethanol was treated with a solution of 400 mg. of sodium in 10 ml. of absolute ethanol. This mixture was treated slowly with 18.7 ml. (0.15 mole) of ethyl crotonate, and then refluxed 9 hours and left overnight. To the solution was added 5 ml. of acetic acid and 20 ml. of water, and most of the solvent removed by vacuum distillation.

The residue was steam distilled under water-pump vacuum for one hour, leaving a light brown oil in a colorless solution. After ice-cooling for one hour, crystals formed, and soon all of the oil had solidified. The crystals were filtered, washed with water, and dried and weighed 17.72 g. The filtrates, on concentrating and seeding, gave a second crop of 2.29 g. The total yield was 20.01 g. (60.5%) though more was still present in the filtrates. This compound was recrystallized several times from water, and then had m.p. 73.5–74.5°.

Anal. Calcd. for $C_{15}H_{25}NO_7$: C, 54.38; H, 7.55. Found: (I): C, 54.03; H, 7.02. Found (II): C, 54.25; H, 7.03.

β-Methylglutamic Acid.—For hydrolysis, 5 g. of the ester was refluxed overnight with 50 ml. of concentrated hydro-

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