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Recent Advances in the Application of Micromethods to the Identification of Organic Compounds

By

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With 7 Figures

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Part I. — The Microdetection of Fatty Acids

The present discussion will be restricted to a summary of the work that has been done during the past few years in the author's laboratory in the application of micromethods to the identification of organic compounds. Some of this work has been published[1, 2] but the main parts of this presentation on the characterization of aliphatic carboxylic acids and the use of tetrazolium salts as microchemical reagents have only recently been completed.

Proof of Identity

The determination of the identity of an unknown organic compound involves proof that the unknown bears complete similarity to one of the large number of organic compounds described in the literature. Thus the proof that *A* is identical to *B* from an operational point of view means that when *A* and *B* have been purified by several fractionations they exhibit substantially identical physical and chemical properties.

Some proofs of identity are more rigorous than others, depending upon the number and kind of properties that have been determined and compared. For example, in routine work the proof that *A* and *B* are identical is made frequently after one or two tests that are based on the formation of a colored complex or a precipitate, or generally on the similarity of behavior of *A* and *B* towards a reagent *C*. Such practice is admissible in routine work with certain reservations. For example, if an acidic solid is isolated from a natural product and is identified on the basis of color tests as *dl*-tartaric acid and it melts at 204° to 206° with some decomposition, such identification is tentative. For a definite proof according to the author's point of view it would be necessary to show that the melting point does not change on further fractionation or when admixed with known pure *dl*-tartaric acid. The same proof may be effected by preparing a derivative such as the *p*-nitrobenzyl ester or the amide of the compound and determining their melting points. In general, proof of identity on the basis of one physical constant and one color reaction (particularly of liquid compounds), though admissible in a number of instances, is not regarded as conclusive. The reason for this is that few color reactions are specific for a particular organic compound.

For absolute proof of the identity of any organic compound the author believes that besides the usual color classification reactions, data on at least two constants should be available, one of which preferably should be the melting point or some other constant of a derivative of the compound under investigation. The reasons for this thesis will be clarified by the discussion in the following sections.

Systematic Determination of Identity

The systematic determination of identity involves the following three steps:

1. Is the unknown a pure compound or a mixture? It is seldom that one meets with an unknown which is a relatively pure compound, unless it is handed in a test tube, vial or bottle as an "unknown" to the student. The first step, therefore, involves the determination of the number of components in the unknown and the isolation and purification of the various components before any of the other steps are taken.

2. Once one or more relatively pure compounds have been isolated, it is possible by following one of the standard schemes of qualitative analysis to assign the unknown first to a large group of compounds on the basis of solubility, elements present, and reaction to indicators. Then stepwise tests restrict the classification to fewer and fewer possibilities, until by correlation of all the data available a provisional assumption can be made that the compound on hand bears similarity to a compound listed in the literature.

3. Finally the provisional assumption is confirmed or discarded by reactions of the unknown with definite reagents whereby so-called derivatives are obtained, which are then compared with the melting points of the same derivatives described in the literature for the compound provisionally assumed to be identical with the unknown.

Modification of the Systematic Approach

The systematic approach is usually modified in the hands of the experienced worker, particularly when the quantities of the isolated unknown are in the milligram range, or when it is extremely difficult to isolate a pure substance. The experienced worker can often identify a substance without going through the systematic steps outlined above. For example the melting phenomena observed through the microscope on a hot stage[3] frequently give hints regarding the probable nature of the material, particularly when its origin and use are known. In such cases it is possible to modify the systematic procedure by elimination of some of the steps such as the analysis for elements and the solubility tests. Often the practice in such cases is to perform one or two color tests and eliminate the derivatization step. In the author's opinion this is admissible in routine work when the origin and use of the substance sought is known. However, since most of the color or precipitation tests are not specific it appears that for a rigorous proof at least one or two constants are required.

The use of micromethods in the identification of organic compounds is indicated not only when the quantity of material under investigation is limited to a few milligrams, but also when the amount of material is large, for obvious reasons. When the quantity is large it is relatively simple to separate a mixture into pure components and then determine the constants and identity of each. However, with microquantities there is a natural tendency to avoid isolating pure substances. In such cases the melting or boiling points are wide ranges of temperature which can hardly be called physical constants. This is particularly true when an attempt is made to identify organic substances in microgram quantities.

The Identification of Microquantities of Organic Substances

When the amount of material under investigation is a few milligrams it is possible to adapt the classical steps of the systematic determination of identity to small quantities. The work of *Schneider*[4], *Cheronis* and *Entrikin*[5] and *Cheronis*[2], outlines procedures by which all the identification steps can be carried out with milligram quantities. Furthermore, the work of *Feigl* and his collaborators[6] on "spot tests" constitutes a worthy contribution which may be employed to great advantage as an integral part of a scheme of identification, particularly when dealing with microquantities.

The advance of chromatographic procedures within the past years has provided an excellent tool for the detection and separation in a mixture of microgram quantities of substances that are extremely difficult or impossible to identify in such concentrations by other simple procedures.

The advances in the microidentification of organic compounds described in the present paper deal with the microderivatization of fatty acids and the detection of a number of reducing functions of organic compounds in microgram quantities. However, it should be pointed out that the determination of R_f values may serve as constants in micro identification work. For example, let it be assumed that one deals with 5 ml of an aqueous solution containing a mixture of 100 mg of two alcohols and a carboxylic abid. The separation of the mixture into pure components and the determination of their physical constants would be extremely difficult even for the most experienced microchemist. However, it is possible to make a tentative identification of the three components by functional group tests and then to convent a part of the mixture into the 3.5-dinitrobenzoates of the alcohols and another into the 2.4-dinitrophenylhydrazides as described in this paper. Small droplets of the solutions containing 2 to 5 micrograms of the derivatives are chromatographed alongside the same derivatives from the pure compounds tentatively assumed to be identical with the unknowns. If the spots of the developed chromatograms have travelled the same distance and are similar in other respects, the identification may be considered as rigorous as if the components of the mixture had been fractionated and the boiling or melting point of each had been determined.

Microderivatization of Fatty Acids

The primary objective of the investigation on fatty acids[7] was the study of their known derivatives and the development of new ones that could be prepared with microquantities and produced and identified on a paper chromatogram.

Table 1 represents the total number of derivatives described in the literature for the derivatization of carboxylic acids:

Table 1. Derivatives Described in the Literature for the Derivatization of Carboxylic Acids

	General Nature of Derivatives	Number of different derivatives within each group
I.	Amides and Substituted Amides (Amides, Anilides, Toluidides, Xenylamides etc.)	36
II.	Hydrazides and Ureides (Hydrazides, Phenylhydrazides, Monothioureides, etc.)	13
III.	Esters (p-Nitrobenzyl, p-Bromophenacyl, etc.) ...	9
IV.	Salts and Miscellaneous Derivatives (Benzylammonium, S-Benzylthiuronium, Hydroxamates, etc.)	18
	Total number of derivatives	76

The principal requirements for suitable derivatives were first that they could be prepared in quantities of one milligram or less, and second that their presence on a chromatogram could be readily detected. The reason for the second requirement is shown in Table 2. Inspection of the table will disclose that the indicators which are employed to locate the spots are pH indicators, ammoniacal silver nitrate, and ferric chloride, all of which have definite defects.

Table 2. Characterization and Separation of Fatty Acids by Paper Chromatography

Type of Fatty Acids	Derivative Chromatographed	Solvent System	Indicator for Location of Spots	Literature Reference
Lower	Free acids or Na, NH_4 etc., salt	1-BuOH-NH_3-H_2O	Bromocresol Green	[8, 9, 10]
Lower	Free acids or Na, NH_4 etc., salt	1-BuOH-NH_3-H_2O	Bromophenol Blue	[11, 12]
Lower	Free acids or Na, NH_4 etc., salt	1-BuOH-$EtNH_2$-H_2O	$Ag(NH_3)_2^+$ $(H_2O)_n$	[13, 14]
Lower and Higher	Free acids or Na, NH_4 etc., salt	1-BuOH-$(Et)_2$-NH-H_2O EtOH-NH_3-H_2O	Bromophenol Blue Chlorophenol Red Bromocresol Purple and CH_2O	[15, 16, 17]
Lower	Hydroxamates	1-BuOH-AcOH-H_2O C_6H_6-AcOH-H_2O	$FeCl_3$	[18, 19, 20] [21]
Lower and Higher	Hydroxamates	1-BuOH-H_2O Et-Acetate-H_2O	$FeCl_3$	[22]
Lower and Mixtures ...	Hydrazides	i-AmOH-Collidine-H_2O	$Ag(NH_3)_2^+$ BuOH	[23]

Generally polynitrophenyl derivatives of amino acids[24], alcohols[25] and carbonyl compounds[26] are detectable in microgram quantities by paper chromatographic procedures. Therefore the *p*-nitrobenzyl and the 2.4-dinitrobenzyl esters and the 2.4-dinitrophenylhydrazides were selected for investigation.

The *p*-nitrobenzyl esters of the fatty acids C_1 to C_4 and C_6 melt at 31°, 78°, 31°, 35° and 43° respectively, while the derivatives of C_8 to C_{10} are oils. Therefore they were rejected as unsuitable. The 2.4-dinitrobenzyl esters were investigated. Only the acetate was reported in the literature[27];

attempts to prepare the derivatives of the acids C_3 to C_7 gave a solid melting at 66° to 67° for the propionic and oils for the others. Therefore the 2.4-dinitrobenzyl esters were abandoned.

Table 3. Dinitrophenylhydrazides of Fatty Acids Reported in the Literature

Fatty Acid	Melting Point C°	Literature Reference
C_1	183–184	28
C_2	197–198	28
	197–198	29
C_{12}	110–111	30
C_{14}	118	30
C_{16}	120–121	30
C_{18}	123	30

The 2.4-dinitrophenylhydrazides of the fatty acids reported in the literature are shown in Table 3.

The methods for the preparations of these derivatives were investigated, a satisfactory method was developed and the pure derivative of the acids C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{12}, C_{14}, C_{16} and C_{18} were prepared, analyzed, and their melting points determined. These are tabulated in Table 4. Of these, eight (asterisked) were not previously reported in the literature and for those reported no analytical data were given.

Table 4. 2.4-Dinitrophenylhydrazides of Fatty Acids

Acid	Carbon Atoms	2.4-Dinitrophenylhydrazides			
		Melting Point	Difference °C**	Calculated N %	Found N %
Acetic	C_2	198–199 (197–198)		23.3	23.3
*Propionic	C_3	189–190	9	22.0	22.1
*Butyric	C_4	165	24	20.9	21.1
*Valeric	C_5	131–132	34	19.9	20.1
*Caproic	C_6	117–118	14	18.9	18.3
*Heptanoic .... (Oenanthic)	C_7	108–109	10	18.1	17.8
*Caprylic	C_8	107–108	1	17.3	16.7
*Nonanoic .... (Pelargonic)	C_9	108–109	1	16.6	16.3
*Capric	C_{10}	115–116	8	15.9	16.2
Lauric	C_{12}	117–118 (110–111)	2	14.7	14.9
Myristic	C_{14}	119–120 (118)	2	13.7	13.3
Palmitic	C_{16}	118–119 (120–121)	1	12.8	12.5
Stearic	C_{18}	120–121 (123)	2	12.1	12.1

* Derivatives not previously reported.

** Difference in melting temperatures between adjacent members of the series.

Inspection of the column which shows the differences in the melting temperatures of adjacent members of the series (Table 4) shows that there is sufficient spread for the first seven members, but negligible between C_8 and C_9, and small differences between C_{12} and C_{18}.

Chromatographic Characterization

Holman[31] has reviewed the chromatographic analysis of fatty acids and related substances by elution analysis, frontal analysis, displacement analysis, partition chromatography and paper chromatography. *Asselineau*[32], *Govindarajan*[33], *Jacobsberg*[34], and *Kaufmann*[35] have reviewed the application of paper chromatographic methods to the analysis of carboxylic acids.

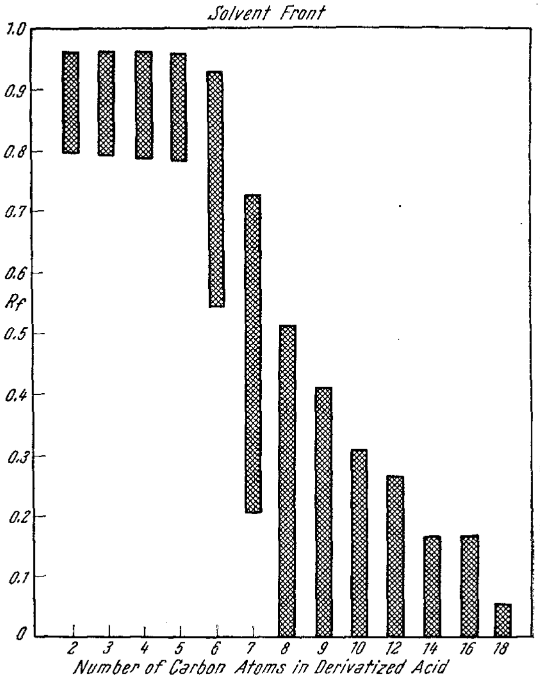

Fig. 1. Composite of several paper strip chromatograms of the 2.4-dinitrophenylhydrazides of saturated normal aliphatic carboxylic acids with 0.05 M potassium benzoate saturated with methyl ethyl ketone as solvent, 100 minutes at 25° (test tube method)

In order to determine the best solvent system a variety were investigated as shown in Table 5. The pure 2.4-dinitrophenylhydrazides were used on separate strips and then the strips used to prepare a composite

Table 5. Solvent Systems Investigated for Development of Paper Chromatograms of 2.4-Dinitrophenylhydrazides of Fatty Acids

1. Acetone-HCl-H_2O
2. Acetone-HCl-NH_3
3. 1-Butanol-H_2O-NH_3
4. 1-Butanol-acetic-H_2O
5. Benzene-acetic-H_2O
6. CCl_4-acetic-H_2O
7. $CHCl_3$-isopropyl alcohol-potassium benzoate-water
8. Collidine-water
9. Collidine-t-Amyl alcohol-H_2O
10. Methanol-water
11. Phenol-isoamyl alcohol-H_2O
12. Pyridine-isoamyl alcohol-H_2O
13. t-Butyl alcohol-buffer-p_H 11.0
14. Methyl ethyl ketone-H_2O-NH_3
15. Methyl ethyl ketone-H_2O-HCl
16. Buffered solutions at various p_H

chromatogram. For the characterization of microgram quantities of a relatively pure fatty acid or a fatty acid admixed with a small amount of impurity the solvent system is not too critical. The acid is converted to the 2.4-dinitrophenylhydrazide, then 1 to 3 μg can be placed on a strip and developed *alongside* of the same derivative from a pure fatty acid which is provisionally assumed to be identical with the unknown. Comparison of the R_f values of the two spots affords the same comparison as that of the values of their melting points. Similarly a composite spot made with 1 μg of each derivative when properly developed serves as a mixed melting point.

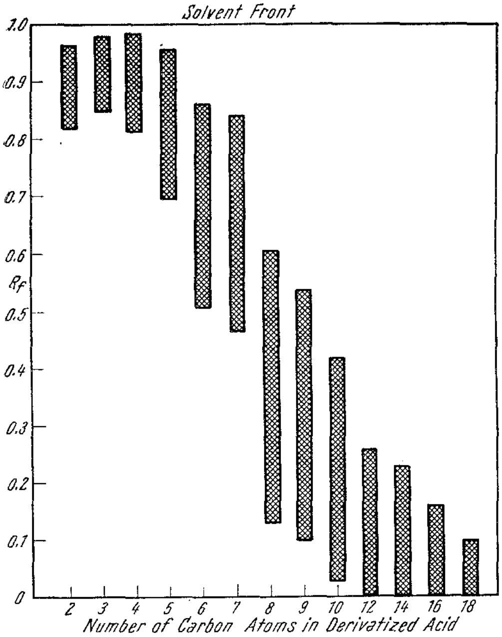

Fig. 2. Composite of several paper strip chromatograms of the 2.4-dinitrophenylhydrazides of saturated normal aliphatic carboxylic acids with pH 8.6 sodium hydroxyde-boric acid buffer saturated with methyl ethyl ketone as solvent, 100 minutes at 25° C (test tube method)

However the separation of the 2.4-dinitrophenylhydrazides of all the fatty acids by chromatographic procedures is not an easy matter. Inspection of the fatty acids listed in Table 4 indicates that adjacent members of the homologous series differ only by a CH_2 group and that many have relatively high molecular weights; hence difficulty in their separation should be anticipated.

It was generally found that solvent systems containing a high proportion of organic solvent, whether acid, neutral or basic (which are commonly used in chromatographic procedures) gave little or no separation. As the aqueous component of the solvent system was increased the derivative of the lower molecular weight acids moved more rapidly than those of the higher acids, and "tailing" increased. Fig. 1 shows a composite of paper strip chromatograms of the 2.4-dinitrophenylhydrazides of the fatty acids using 0.05 M potassium benzoate saturated with methyl ethyl ketone. By using various buffers from pH 8.6 to pH 12.0 saturated with methyl ethyl ketone it was found possible to study the effect of pH on the various R_f values of the 2.4-dinitrophenylhydrazides. Fig. 2 shows a chromatogram of the derivatives at pH 8.6, Fig. 3 at pH 11.0, Fig. 4 at pH 11.6, and Fig. 5 at pH 12.0*.

It was found that the optimum amount of the sample to be placed on the strip is about 1 to 3 μg and the optimum pH between 11.2 and 11.6. Fig. 6 illustrates a sharp separation of the 2.4-dinitrophenylhydrazides of the C_2 to C_9 fatty acids from those of C_{12} to C_{18} homologs, using a buffer of pH 11.6. Fig. 7 represents the identification of a three-component mixture.

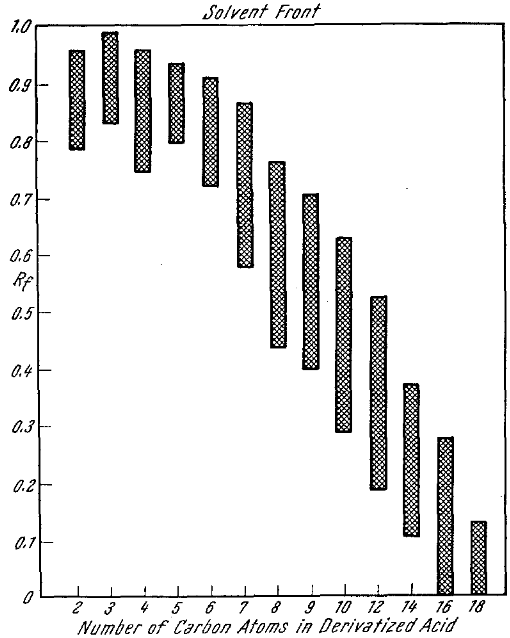

Fig. 3. Composite of several paper strip chromatograms of the 2.4-dinitrophenylhydrazides of saturated normal aliphatic carboxylic acids with pH 11.0 sodium hydroxide-dibasic sodium phosphate buffer saturated with methyl ethyl ketone as solvent, 100 minutes at 25° C (test tube method)

Operational Procedures for the Preparation and Chromatography of the 2.4-Dinitrophenylhydrazides of Fatty Acids

A mixture of 0.30 mg of capric acid and 0.30 mg of palmitic acid is neutralized with 0.16 mg of sodium carbonate in 0.5 ml water in a 6-inch test tube. The mixture is evaporated on a steam bath and dried in an oven at 130° for 30 minutes. The tube is cooled, charged with 0.5 ml thionyl chloride, provided with a microcondenser and heated for 15 minutes at 55°. The condenser is removed and suction is applied to the contents to remove the thionyl chloride. A solution of 1.21 mg 2.4-dinitrophenylhydrazine in 3.5 ml of benzene is added into the tube, the microcondenser replaced and the mixture refluxed for 20 minutes, then cooled and extracted twice with 0.5 ml portions of concentrated hydrochloric acid, 10 per cent hydrochloric acid and water. The final volume of benzene is about 2.5 to 3.0 ml.

The test tube ascending technique[36], [37] is employed using strips 0.5 inches wide and 18 cm length of Whatman filter paper No. 1 and pencil marks are made at points 1 cm and 14 cm from one end of the strip. A sample of the solution 5 to 10 μl (or sufficient volume containing 3 to 4 μg of the derivative) is applied at the 1-cm mark in the usual manner and the strip

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* Figures 1 to 6 inclusive do not represent optimum separations which can be achieved since the objective was to study the effect of pH with larger quantities of material (50 micrograms) than are employed in separations (Figure 7, 1—3 μg).

is placed in a tube (22 × 175 mm) containing 0.7 ml of buffer p_H 11.2 saturated with methyl ethyl ketone. The tube is stoppered and allowed to stand in a vertical position until the solvent reaches the 14-cm pencil mark, when the strip is removed and dried in an oven at 100°. The spots of 2.4-dinitrophenylhydrazides are detected with the unaided eye, but more readily with reflected or transmitted ultraviolet light. When the paper is sprayed with 1 N sodium hydroxide the spots become brown. The spots of capric and palmitic acid 2.4-dinitrophenylhydrazides are completely separated and correspond to control spots.

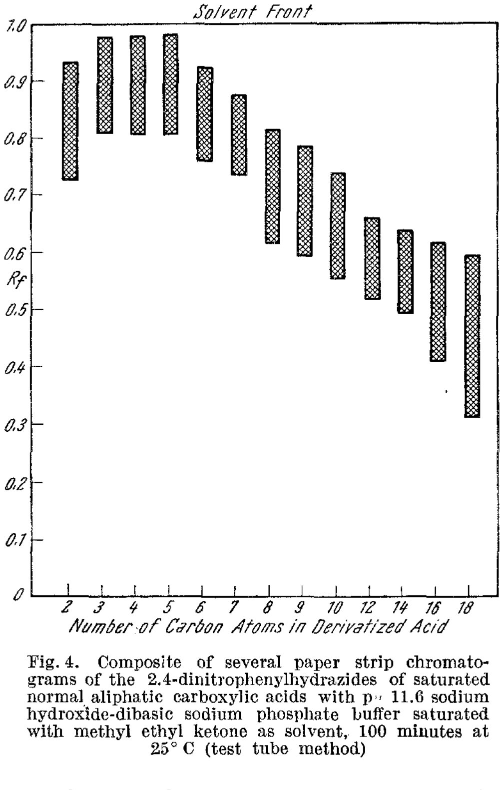
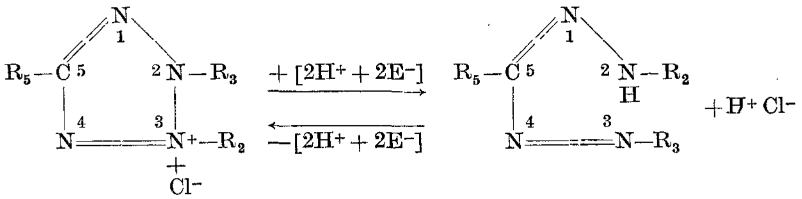


Fig. 4. Composite of several paper strip chromatograms of the 2.4-dinitrophenylhydrazides of saturated normal aliphatic carboxylic acids with p_H 11.6 sodium hydroxide-dibasic sodium phosphate buffer saturated with methyl ethyl ketone as solvent, 100 minutes at 25° C (test tube method)

Part II. —The Use of Tetrazolium Salts for Microdetection of Reducing Functions in Organic Compounds*

Tetrazolium salts first prepared by *Pechman* and *Runge*[38] and extensively investigated by *Kuhn* and *Jerchel*[39] were proposed by *Lakon*[40] for testing the viability of seeds. The usefulness of these reagents was pointed out by *Dutcher*[41] who had interrogated *Lakon* in 1945 as a member of the American Intelligence Branch in Germany. Subsequently, *Porter*, *Durrell* and *Romm*[42], *Mattson*, *Jensen* and *Dutcher*[43], *Waugh*[44] and *Cottrell*[45, 46] confirmed the observations of *Lakon* and indicated that tetrazolium salts might be useful as



(1)

Tetrazolium Salt.
Soluble in Water
and Colorless.

Formazan.
Insoluble in Water
and Colored.

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* This part of the work is by *N. D. Cheronis* and *H. Stein.*

reagents to detect differences in the viability of seeds and other tissues. This outstanding property is due to the fact that tetrazolium salt solutions on reduction by hydrogenases or generally by reductants yield colored formazan as shown in equation (1).

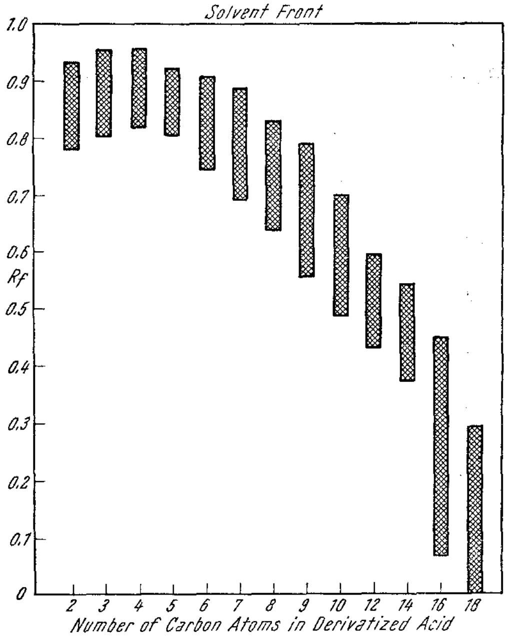


Fig. 5. Composite of several paper strip chromatograms of the 2.4-dinitrophenylhydrazides of saturated normal aliphatic carboxylic acids with pH 12.0 sodium hydroxide-di-basic sodium phosphate buffer saturated with methyl ethyl ketone as solvent, 100 minutes at 25° C (test tube method)

The application of tetrazolium salts to the study of carcinomatous tissues was made by *Straus*, *Cheronis* and *Straus*[46], who showed that neoplastic tissue reduces these salts in vivo or vitro at a greater rate. As a result of this a prodigious amount of work on the properties of these reagents has been accumulating in the literature.

Use of Tetrazolium Salts as Redox Indicators

The work of this laboratory has been directed first towards preparing a series of tetrazolium salts which in redox reactions yield colors in the green, blue and violet regions of the spectrum. Secondly, the study of their properties has been undertaken, and, thirdly, through such studies the most sensitive of these compounds have been selected to be used as microchemical redox indicators. It should be pointed out that in equation (1) the arrows do not indicate a reversible reaction since in aqueous systems the reaction is irreversible.

The utilization of tetrazolium salts to detect sugars was pointed out by *Mattson* et al.[43] who stated that sugars reduced solutions of tetrazolium salts above pH 11.0. Later *Mattson* and *Jensen*[47] published a quantitative colorimetric estimation of reducing sugars. The calculated pH of the solutions employed is about pH 13.4. However, the author states that alkalinity is one of the less critical factors.

Other investigators such as *Weiner*[48], *Wallenfels*[49] and *Trevelyan*[50] have published tests for detection of reducing sugars both in solution and by paper chromatography by means of triphenyl tetrazolium chloride but no information is given as to the optimum pH or the limits of identification.

Stability of Tetrazolium Salt Solution at High pH

Whether tetrazolium salts can be used as reagents at alkaline pH's depends on their stability. It was shown in this laboratory[51] that, at

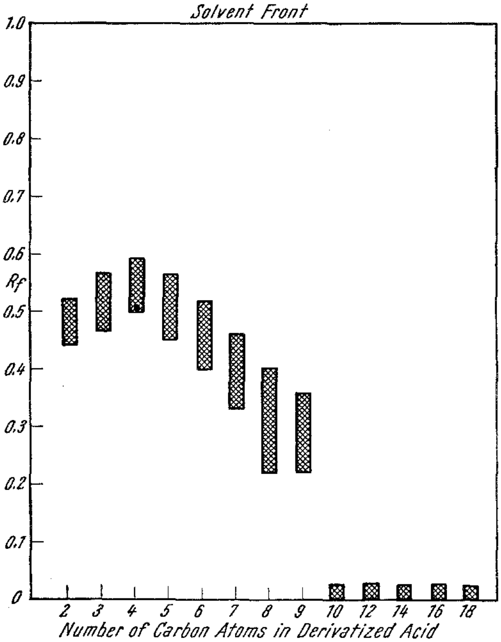


Fig. 6. Paper chromatogram of the 2.4-dinitrophenylhydrazides of saturated normal aliphatic carboxylic acids with p 11.6 sodium hydroxide-dibasic sodium phosphate buffer as solvent, 5 hours at 25° C (chamber method; solvent front traveled 29 cm)

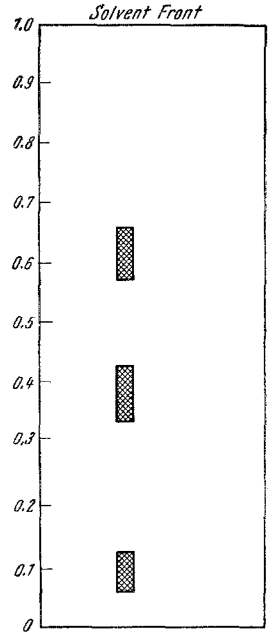


Fig. 7. Paper Chromatogram of the 2.4-dinitrophenyl hydrazides of a mixture of capric, lauric and palmitic acids. Development with buffer pH 11.6 saturated with methylethyl ketone for 10 hrs. 25° C. Solvent front 25 cm

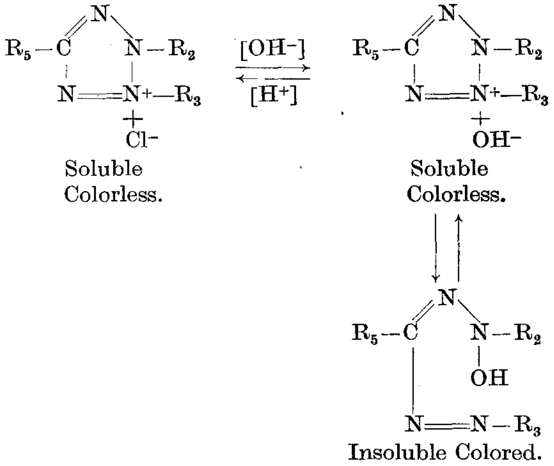
Table 1. Color Production by Basic Solutions of Triphenyl Tetrazolium Chloride

Conc. M TTC*	pH	Color Formation on Boiling
0.15	10.2	Color
0.06	11.85	Color
0.03	12.20	Color
0.003	12.40	None
0.03	NaOH 6 N	cold (red precipitate)

* Molar concentration of triphenyl tetrazolium chloride.

alkaline pH tetrazolium salts without addition of a reductant develop colors similar to those which appear when they are acted upon by the reductant. This is shown in Table 1.

The results indicate that the stability of the salts is a function not only of the pH but also of the concentration of the quaternary base of the salt. It can be shown that as the concentration of the hydroxyl ion increases, the following reaction takes place until finally the quaternary tetrazolium hydroxide at high concentration rearranges to an insoluble colored isomer:



On the basis of this work it was possible to determine the threshold of stability of alkaline triphenyl tetrazolium chloride solutions on boiling. The results are summarized in Table 2.

Table 2. Determination of the Threshold Stability of Aqueous Solutions of Triphenyl Tetrazolium Chloride at Alkaline p_H's

Tetrazolium Salt Solution		Volume of Reagents				
			Total			
ml	M 10^{-3}	0·3N NaOH ml	H_2O ml	Volume ml	pH	Color Formation on Boiling
0.1	7.5	0.3	0	0.4	12.6	Color
0.1	5.0	0.3	0.2	0.6	12.53	Faint
0.1	3.75	0.3	0.4	0.8	12.50	None
0.1	3.33	0.3	0.5	0.9	12.48	None
0.1	3.0	0.3	0.6	1.0	12.40	None
0.15	4.5	0.3	0.55	1.0	12.30	None
0.20	6.0	0.3	0.50	1.0	12.28	Faint
0.1	3.75	0.3	0.4	0.8	12.50	Color
0.1	3.0	0.3	0.5	0.9	12.45	None

The data indicate that a solution of triphenyl tetrazolium chloride containing 1 mg/ml is stable on boiling at pH 12.45. This is the threshold of its stability and thus can serve as a sensitive reagent.

The test as it has been developed[51, 52, 53] for the detection of reducing functions of organic compounds is as follows:

To 0.1 ml of 1% solution of the tetrazolium salt in a small tube, 0.3 ml of 0.3 N sodium hydroxide solution and 0.5 ml of water are added. The mixture is just boiled and examined for the development of color. Only a very light yellow colored solution is permissible. About 0.1 ml of the unknown solution containing 1 to 20 μg of the reductant is added and the solution boiled for 30 seconds. A colored solution depending on the type of tetrazolium salt employed results within 30 seconds. The color should be permanent and not fade. If a color develops and then fades it indicates that the redox color formation is below the limits of practical experimental conditions. In such cases the concentration of the reductant is increased. For example glucose with triphenyl tetrazolium chloride gives a color that fades at 5 μg/ml, but a permanent color at 10 μg/ml. When the concentration of the reducing function is 2 to 3 times the identification limit, the color formation is large and insoluble formazan separates out and the solution becomes cloudy. In such cases dropwise addition of acetone gives an intense colored solution.

Table 3. Microdetection of Sugars with Triphenyl Tetrazolium Chloride and Comparison with Methods Based on Reduction of Cupric Ions

Sugar	Limits of Detection in μg/ml		
	Triphenyl Tetrazolium Reagent	Cupric Ion* (*Fehling's*)	Cupric Ion* (*Benedict's*)
Rhamnose	8—10	30	15—20
Arabinose	8—10	30	15—20
Xylose	7—10	30	15—20
Lyxose	7—10	30	15—20
Fucose	8—10	30	20—25
Glucose	7—10	30	15—20
Mannose	10—12	30	20—25
Galactose	7—10	30	15—20
Fructose	7—10	30	15—20
Maltose	10	40	15—20
Lactose	8—10	40	15—20
Cellobiose	10—12	30	15—20
Melibiose	10—12	30—40	15—20
Amygdalin	10	100	None 100
Trehalose	None	None	None 100
Sucrose	50—100 on hydrolysis	Slightly with 100	Slight 100
Raffinose	25—30	40	100

* Solution heated to boiling for 1 minute, allowed to stand for 5 minutes then observed.

Micro Identification of Sugars with Triphenyl Tetrazolium Chloride

The test as described was applied to a large number of sugars. Triphenyl tetrazolium chloride was employed and for comparison both *Fehling*'s and *Benedict*'s solutions were used. The results are tabulated in Table 3. The data indicate that the tetrazolium reagent gives more sensitive results than those based upon the reduction of cupric ions. However, from the point of view of ease of observing color changes in the tetrazolium reagents at the lower limits there is much to be desired. Many people are not able to detect the slight color change from yellow (negative) to pink (positive). For this reason and also for other applications a systematic search was made for tetrazolium salts which were not only more sensitive but also gave better visible colors on reduction, such as blue, green and violet.

New Tetrazolium Salts

Tables 4, 5 and 6 give a summary of the tetrazolium salts synthesized in this laboratory for the past four years. A detailed discussion is being published elsewhere. For the present discussion the data as given in these tables will suffice. Of the 32 compounds listed, 27, to the best of our knowledge, have not been previously reported or studied. Table 4 lists 15 monotetrazolium salts beginning with the triphenyl and increasing in complexity of the radicals attached in the *3*- and *5*-positions.

Table 4. Nature of Radicals in Monotetrazolium Salts. R_2 = phenyl in all [compare equation (1), p. 934]

	R_3	R_5	Color on Reduction	Limits of Detection in Micrograms of Glucose
1.	Phenyl	Phenyl	Red	7—10
2.	*o*-Tolyl	Phenyl	Red	2—3
3.	*m*-Tolyl	Phenyl	Red	10
4.	Phenyl	*p*-Anisyl	Violet—Red	5
5.	*o*-Tolyl	*p*-Anisyl	Violet—Blue	4
6.	*m*-Tolyl	*p*-Anisyl	Violet—Red	5
7.	*p*-Tolyl	*p*-Anisyl	Red	5
8.	Phenyl	Piperonyl	Red	200
9.	*o*-Tolyl	Piperonyl	Red	4
10.	*m*-Tolyl	Piperonyl	Red	25
11.	*p*-Tolyl	Piperonyl	Violet—Red	4
12.	*o*-Anisyl	Phenyl	Red	5
13.	*o*-Anisyl	Piperonyl	Violet—Red	5
14.	*p*-Anisyl	Piperonyl	Violet—Blue	5
15.	2.5-Dimethoxyphenyl	Phenyl	Violet	5

It should be noted that the compounds containing ortho substituents in the radicals attached in the *3*-position exhibit greater sensitivity. Also these compounds exhibited greater solubility (not shown in the table) when compared with the para- or meta-isomer.

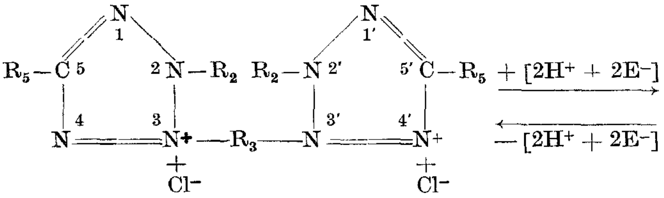
In the compounds listed in Table 5 the substituents of positions *3*- and *5*- are more complex. The *o*-biphenyl radical in the *3*-position provides a greater sensitivity to redox reactions as compared to the *p*-isomer and also to related compounds. Introduction of a nitro group

Table 5. Monotetrazolium Chlorides. Nature of Radicals in Monotetrazolium Salts. R_2 = phenyl in all

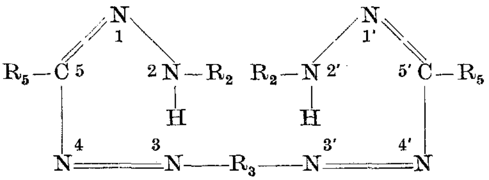
R_3	R_5	Color on Reduction	Limits of Detection in Micrograms of Glucose
16. α-Naphthyl	Phenyl	Violet	3
17. β-Naphthyl	Phenyl	Red	Unstable above p_H 10
18. *o*-Biphenyl	Phenyl	Violet—Red	2
19. *o*-Biphenyl	*m*-Nitrophenyl	Orange—Red	Unstable above p_H 10
20. *p*-Biphenyl	Phenyl	Violet—Red	5
21. *p*-Biphenyl	*m*-Nitrophenyl	Orange—Red	Unstable above p_H 10
22. Phenyl*	Furfuryl	Red	200
23. *o*-Tolyl	Thienyl	Red	20

* Previously reported.

Table 6. Ditetrazolium Chlorides



Ditetrazolium Salt.
Colorless



Diformazan.
Colored

Nature of Radicals in Ditetrazolium Salts. R_2 = phenyl in all

	R_3	R_5	Color on Reduction	Limits of Detection in Micrograms of Glucose
24.	4.4′-Diphenylene . .	Phenyl	Violet—Red	Unstable at p_H 12
25.	4.4′-Diphenylene . .	*m*-Nitrophenyl	Violet—Red	Unstable at p_H 12
26.	*m*-Phenylene	Phenyl	Violet	Unstable at p_H 12
27.	3.3′-Dimethoxy-4.4′-Diphenylene* .	Phenyl (BT)	Blue—Purple	4—5
28.	3.3′-Dimethoxy-4.4′-Diphenylene . .	*p*-Methoxyphenyl (*p*-Anisyl) (BTA)	Blue	1 or less
29.	3.3′-Dimethoxy-4.4′-Diphenylene . .	2.3-Dimethoxyphenyl	Orange—Red	5
30.	3.3′-Dimethoxy-4.4′-Diphenylene . .	3.4-Dimethoxyphenyl (Veratryl) (BTV)	Blue (Greenish)	1
31.	3.3′-Dimethoxy-4.4′-Diphenylene . .	Piperonyl (BTP)	Blue	2
32.	3.3′-Dimethoxy-4.4′-Diphenylene* .	Furfuryl (BTF)	Purple (Blue)	50
33.	3.3′-Dimethyl-4.4′-Diphenylene* .	Phenyl	Red—Violet	Unstable at p_H 12

* Previously reported.

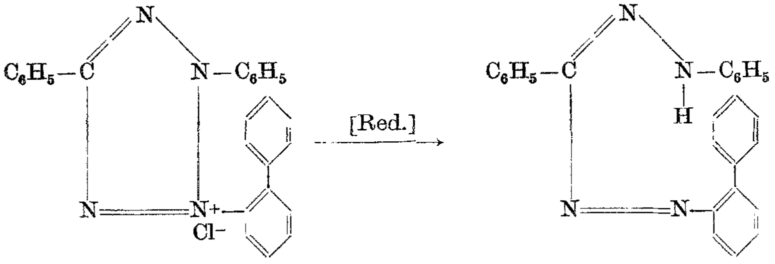
in the *o*-biphenyl and *p*-biphenyl radicals renders the tetrazolium salts unstable at the higher p_H's. Of all the monotetrazolium salts, the 2.5-diphenyl-3-*o*-biphenyl appears to be the best for the detection of reducing functions.

The ditetrazolium salts of the N—N type were known from the work of early investigators. Table 6 lists 10 ditetrazolium salts, of which 3 were previously reported. Of these three, No. 24, with a *p,p*-diphenylene radical in the *3*-position, is also called neotetrazolium, and No. 27, with a *p,p*-orthodianysidyl radical in the *3*-position, is also called Tetrazolium Blue. This name is unfortunate, because, as is shown in Table 6, a number of other ditetrazolium salts are blue. Therefore since these names most likely will become entrenched it is proposed to employ the name at the radical in the *5*-position with the new blue tetrazoliums. Thus No. 28 becomes *p*-anisyl tetrazolium blue or (BTA), No. 30: veratryl tetrazolium blue or (BTV).

Of all the ditetrazolium salts tested, the most useful for the microdetection of sugars is the *p*-anisyl tetrazolium blue. Table 7 summarizes data on the microdetection of various reducing sugars using the most sensitive monotetrazolium salt, biphenyl tetrazolium violet, and the most sensitive ditetrazolium salt, *p*-anisyl tetrazolium blue.

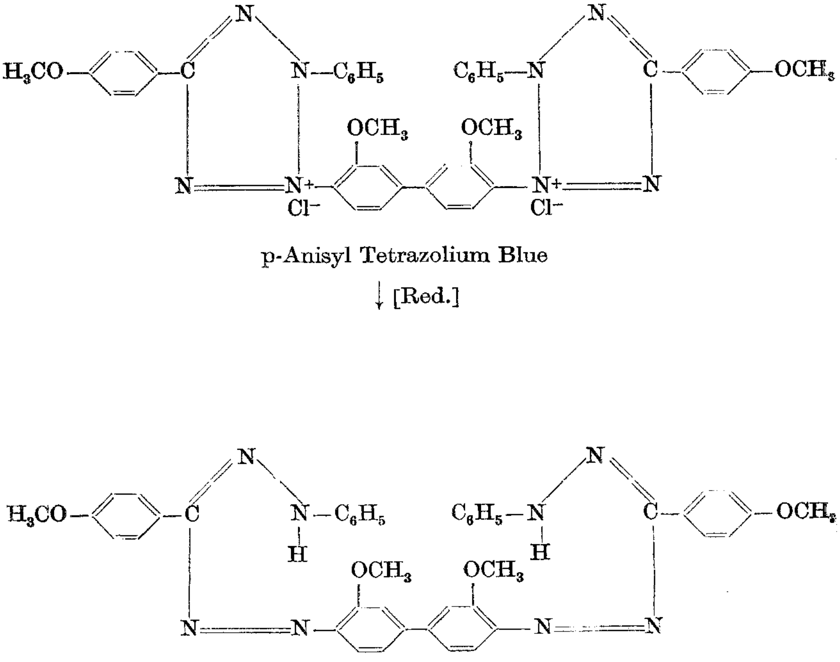
The work of determining the microdetection limits of other reducing functions of organic compounds with these selected reagents is in progress.

Table 7. Microdetection of Sugars with Biphenyl Violet and *p*-Anisyl Blue Tetrazolium Reagents



Biphenyl Tetrazolium Violet

Violet-Red Formazan



p-Anisyl Tetrazolium Blue

Deep Blue Diformazan

	Biphenyl Tetrazolium Violet (2.5-Diphenyl-3-*o*-biphenyl tetrazolium chloride)	*p*-Anisyl Tetrazolium Blue
	Limits of Detection in µg	
Rhamnose	2—3	1—2
Arabinose	2—3	1—2
Xylose	2—3	1—2
Lyxose	2—3	1—2
Fucose	2—3	1
Glucose	2	1
Mannose	3	1
Galactose	2—3	1
Fructose	2	1
Maltose	3	1
Lactose	2	1
Cellobiose	3—4	1—2
Melibiose	3—4	1—2
Amygdalin	3	1—2
Trehalose	None	None
Sucrose	None	None
Raffinose	7—8	3—4

Summary

The presentation is restricted to a summary of the work that has been done in the last few years in the application of micromethods to the identification of organic compounds. A critical discussion of the concept of the "proof of identity" is given in order to show that some "proofs" are more rigorous than others. For a rigorous proof of the identity of any organic compound the author believes that besides the usual color classification reactions, data on at least two constants should be available, one of which, if possible, should be the melting point (or some other constant) of a derivative of the compound under investigation. The steps of the systematic determination of identity are reviewed and their modifications by the experienced worker are discussed. In the proof of identity of microquantities there is a natural tendency to avoid isolating pure substances for determination of constants. However, the procedures developed for the derivatization of milligram quantities together with color group reactions permit rigorous proof. With microgram quantities it is often possible to prepare a derivative and use the R_f value as a constant; this is illustrated by the microderivatization of fatty acids.

The work on the derivatization of fatty acids and development of a method for their characterization using a few micrograms was done in collaboration with *Sol Cohen*. Of the 76 different kinds of compounds proposed in the literature for the derivatization of fatty acids the *p*-nitro-

benzyl and 2.4-dinitrobenzyl esters and the 2.4-dinitrophenylhydrazides were investigated. The latter were selected, and suitable procedures were developed for their preparation using microquantities of fatty acids. Pure derivatives of the fatty acids C_2 to C_{10} and C_{12}, C_{14}, C_{16} and C_{18} were prepared, analyzed, and their melting points determined. Of the 13 derivatives 8 are reported for the first time. The chromatographic properties of the 2.4-dinitrophenylhydrazides of the fatty acids were studied and a satisfactory method of resolution developed.

The development of new reagents for the microdetection of reducing functions in organic compounds was done in collaboration with *H. Stein*. A brief review of the development of tetrazolium salts as redox reagents is given. A brief survey of the properties of 33 monotetrazolium and ditetrazolium salts, 28 of which were synthesized in this laboratory during the past 4 years, is added. Two of these are 7 to 10 times more sensitive than the well known triphenyltetrazolium chloride, when compared on the basis of their limits of detection of microgram quantities of reducing sugars.

Zusammenfassung

Die Ausführungen beschränken sich auf eine Zusammenfassung der Untersuchungen, die von uns in den letzten zwei Jahren über die Anwendung von Mikromethoden zur Erkennung organischer Verbindungen ausgeführt wurden. Das Konzept der Identifizierung einer Substanz wird kritisch besprochen, um zu zeigen, daß die Sicherheit der Identifizierung von der Auswahl der Verfahren abhängt. Der Verfasser ist der Meinung, daß der strenge Beweis der Identität irgend einer organischen Verbindung außer der üblichen Klassifizierung durch Farbreaktionen die Angabe von wenigstens zwei physikalischen Konstanten enthalten sollte. Von diesen Konstanten sollte, wenn möglich, eine den Schmelzpunkt (oder irgend eine andere Konstante) eines Derivates der untersuchten Substanz darstellen. Die einzelnen Schritte der systematischen Bestimmung der Identität werden kurz besprochen und auf mögliche Abänderungen, die erfahrene Untersucher anwenden können, wird hingewiesen. Beim Arbeiten mit Mikromengen zeigt sich eine Neigung, die Isolierung von reinen Substanzen zwecks Bestimmung von Konstanten zu vermeiden. Dabei ist zu bedenken, daß ein strenger Beweis durch Verwendung der Mikroverfahren zur Herstellung von Derivaten möglich ist, wenn die dadurch gewonnenen Konstanten die mittels der Farbreaktionen erhaltenen Befunde ergänzen. Mit Mikrogramm-Mengen ist es häufig möglich, ein Derivat zur Bestimmung des chromatographischen R_f-Wertes herzustellen, wie dies im Falle der Fettsäuren gezeigt wird.

Die Darstellung von Fettsäurederivaten und die Entwicklung eines Verfahrens für die Kennzeichnung der letzteren unter Benutzung von wenigen Mikrogramm wurde in Zusammenarbeit mit *Sol Cohen* durchgeführt. Von den 76 verschiedenen, in der Literatur zur Herstellung von Fettsäurederivaten vorgeschlagenen Verbindungstypen wurden die *p*-Nitrobenzyl- und 2,4-Dinitrobenzylester und die 2,4-Dinitrophenylhydrazide untersucht. Der letztgenannte Verbindungstypus wurde ausgewählt und geeignete Verfahren zur Herstellung von Derivaten mit Mikromengen von Fettsäuren wurden ausge-

arbeitet. Reine Derivate der Fettsäuren mit 2 bis 10, 12, 14, 16 und 18 Kohlenstoffatomen wurden hergestellt, analysiert und ihre Schmelzpunkte bestimmt. Acht von den 13 Derivaten wurden zum erstenmal beschrieben. Die chromatographischen Eigenschaften der 2,4-Dinitrophenylhydrazide wurden studiert und eine zufriedenstellende Methode für ihre Trennung ausgearbeitet.

In Zusammenarbeit mit *H. Stein* wurden neue Reagenzien für den Nachweis reduzierender Funktionen in organischen Verbindungen gefunden. Die Entwicklung von Tetrazoliumsalzen als Redoxreagenzien wird kurz besprochen und durch eine Übersicht über die Eigenschaften von 33 Mono- und Ditetrazoliumsalzen, von denen 28 in den letzten vier Jahren im Laboratorium des Verfassers dargestellt wurden, ergänzt. Zwei dieser Salze sind 7- bis 10mal empfindlicher als das bekannte Triphenyltetrazoliumchlorid hinsichtlich der Nachweisgrenzen (Mikrogramm-Mengen) von reduzierenden Zuckern.

Résumé

L'exposé se limite à un résumé des travaux effectués par l'auteur pendant ces dernières années sur l'application des microméthodes à l'identification des composés organiques. Le concept de la «preuve de l'identité» est soumis à une discussion critique qui montre que certaines «preuves» sont plus rigoureuses que d'autres. Pour prouver rigoureusement l'identité d'un composé organique quelconque, l'auteur pense que la classification habituelle par réactions colorées doit être complétée par les valeurs numériques d'au moins deux constantes physiques. L'une de ces constantes devrait être, si possible, le point de fusion d'un dérivé du composé soumis à l'examen (ou une autre de ses constantes). L'auteur examine les phases de la détermination systématique de l'identité ainsi que leurs éventuelles modifications par un travailleur expérimenté. Lorsqu'il s'agit de prouver l'identité de microquantités on a généralement tendance à éviter l'isolement de substances pures pour la détermination des constantes.

Il est cependant possible d'établir une preuve rigoureuse d'identité en faisant simultanément appel aux techniques qui ont été mises au point pour la préparation de dérivés à l'échelle du milligramme et aux réactions colorées de groupes. A l'échelle du microgramme, il est souvent possible de préparer un dérivé et de considérer la valeur de R_f comme une constante, ce cas est illustré par la formation à l'échelle microchimique de dérivés des acides gras.

Ce travail sur les acides gras a été effectué en collaboration avec *Sol Cohen* ainsi que la mise au point d'une méthode permettant de les caractériser sur quelques microgrammes. Parmi les 76 différentes espèces de composés qui ont été proposés dans la littérature pour la formation de dérivés des acides gras, les recherches ont porté sur les esters *p*-nitrobenzylique et 2,4-dinitrobenzylique ainsi que sur les 2,4-dinitrophénylhydrazides. Ce sont ces derniers qui ont été choisis et leur préparation a été mise au point à partir de microquantités d'acides gras. On a ainsi préparé des dérivés purs des acides gras de C_2 à C_{10} ainsi qu'en C_{12}, C_{14}, C_{16}, et C_{18}; ils ont été soumis à l'analyse et leurs points de fusion ont été déterminés. 8 d'entre ces 13 dérivés sont signalés pour la première fois. On a étudié les propriétés chromatographiques des 2,4-dinitrophénylhydrazides et on a pu mettre au point une méthode permettant de les séparer de façon satisfaisante.

L'étude de réactifs nouveaux pour la détection des fonctions réductrices des composés organiques a été effectuée en collaboration avec *H. Stein*.

On donne un aperçu sur la mise au point des sels de tétrazolium comme réactifs oxydoréducteurs; il est complété par la description rapide de 33 sels de monotétrazolium et de 22 sels de ditétrazolium; la synthèse de 28 d'entre eux a été réalisée pendant les 4 dernières années dans le laboratoire de l'auteur. Deux de ces sels ont une sensibilité 7 à 10 fois plus élevée que celle du chlorure de triphényltétrazolium bien connu (identification des sucres réducteurs à l'échelle du microgramme).

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