Gas Chromatographic Determination of Pyruvic and Lactic Acids and Krebs Cycle Components

Esterification and Recovery

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▶ In addition to the components of the tricarboxylic acid (TCA) cycle, including the α- keto-, α-hydroxy-, and α,β-unsaturated acids, lactic, and pyruvic acids were esterified in good yields with diazomethane. Gas chromatographic separation on diethyleneglycol succinate gave single peaks for the esters of pyruvic, lactic, malonic, fumaric, succinic, malic, α-ketoglutaric, *cis*-aconitic and citric acids, in order of retention times. Oxalacetate gave a broad flat peak beyond citrate. The only peak observed for the methylation product of glutamic acid was identified as that of methanol. At column temperatures above 115° C. for pyruvate and above 90° C. for fumarate thermal decomposition of the esters occurred and a methanol peak appeared. Recoveries of pyruvate, lactate, malonate, fumarate, succinate, and malate were determined. The methylation of mixtures of lactic and pyruvic acids in varying proportions led to quantitative recoveries of each of the methyl esters.

THE METHYL ESTERS of the stable (non-keto) acids of the TCA cycle, as well as the unsaturated acids, fumaric and aconitic have been identified by gas chromatography (*10, 11*). Similarly, the methyl esters of lactic and pyruvic acids have been separated from other acids (*2, 6*), and from acids of the TCA cycle including α-ketoglutarate (*2, 13*). These reports differ in the order of elution of the esters, the number of peaks given by pyruvate and α-ketoglutarate, and in the apparent isomerization of dimethyl fumarate. Rumsey *et al.* (*13*) used a column containing 15% diethyleneglycol succinate on Chromosorb W at 135° C. and observed two peaks for methyl pyruvate. Other than the emergence of methyl lactate before methyl pyruvate, the observations of Alcock (*2*) with a diethyleneglycol adipate column appeared similar to those of Gee (*6*) using a neopentylglycol succinate column. The two peaks reported by Alcock (*2*) for α-ketoglutarate were not observed by Rumsey *et al.* (*13*).

For these investigations, the methyl esters were prepared from the acids by a variety of methods including: methanol–HCl (*6, 10*), thionylchloride (*6, 10*) methanol–H_2SO_4 (*11, 13*), methanol–boron trifluoride (*2, 10*) and diazomethane (*10, 11*). The method used appeared to be dependent on the particular esters of interest and the reported applicability of the methylating agent to the acid in question.

For the long chain acids, Stoffel *et al.* (*16*) found methanol–HCl to give quantitative yields of the methyl esters, and Schlenk (*14*) observed that the quantitative esterification of long chain fatty acids by diazomethane required the addition of methanol. These observations did not appear applicable to the short chain acids. For the esterification of butyric, valeric, and caproic acids, Vorbeck (*17*) found diazomethane to give better yields than methanol–HCl, or methanol–BF_3. Luke *et al.* (*10*) also used the method of Roper and Ma (*12*) for methylation of the di- and tricarboxylic acids. McKeown and Read (*11*), however, reported that low yields were obtained when the methylation was carried out in ether solution and that the addition of methanol enhanced the yield.

Eistert (*5*) had suggested that diazomethane could add to esters of unsaturated, conjugated, dicarboxylic acids—e.g., fumaric, to form pyrazoline dicarboxylic esters, and that on heating, the pyrazoline could decompose to form cyclopropane derivatives. McKeown and Read (*11*) regarded the formation of 4,5-dicarbomethoxyl–pyrazoline as the probable explanation for the isomerization of dimethyl fumarate as reported by Luke *et al.* (*10*).

Eistert (*5*) had also indicated that with diazomethane, the α-hydroxyl group could be methylated and that methyl pyruvate could further react to give ethylenic acid. Burchfield's (*3*) comment that "the use of diazomethane should be avoided unless the mixture consists solely of saturated nonoxygenated acids such as succinic and glutaric" was consistent with these reports.

The diazomethane method of esterification, however, presented the advantage of producing nitrogen as the chief by-product which should eliminate problems of recovery of the ester that would be encountered in the other methods. In view of the previous observations, it was essential that the products formed from α-substituted, and unsaturated dicarboxylic acids on reaction with diazomethane be characterized.

Although some of the difference in the reports of the various workers may be related to column material, the work of Ackman *et al.* (*1*) suggests that thermal decomposition of the sample should be considered.

Other than the examination of the molar response of the β-ray argon detector toward straight chain α,ω-dicarboxylic fatty acids (*15*), there has been little indication of recovery or quantification of these materials.

Since our interest involved application of the method to the quantitative determination of these acids in biological systems, the chromatographic factors affecting the quantification of the esters were examined.

EXPERIMENTAL

In the initial preparation of larger quantities of the methyl ester, the method of Roper and Ma (*12*) was modified by direct addition of the free acid to an ethereal solution of diazomethane. The diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide ("Diazald," Aldrich Chemical Co.) by the procedure of De Boer (*4*).

In all cases 35 millimoles of free acid were added directly to the ethereal solution of diazomethane. An equivalent excess of diazomethane was allowed for each carboxyl group. On completion of the reaction, the ether was removed by placing the reaction vessel in a warm water bath.

The solid esters were recrystallized from ethanol. Where possible, the liquid esters were vacuum distilled. Since the products from the diazomethylation of glutamic and α-ketoglutaric acids decomposed on heating, they were not distilled, but the lower boiling components were removed under

Table I. Properties of Methyl Esters Prepared

Ester	B.P. °C.[b] This lab	B.P. °C.[b] Ref. (7)	M.P. °C. This lab	M.P. °C. Ref. (7)	Refractive index This lab	Refractive index Ref. (7)	Density This lab	Density Ref. (7)	Yield, % This lab	Yield, % Ref. (12)
Dimethylfumarate			101	102					89.6	...
Trimethyl citrate			78	79					89	54
Methyl pyruvate	135.35	137	[a]		$1.4108^{24°}$	$1.4046^{25°}$	$1.148^{20°}$	$1.154^{0°}$	71	...
Methyl lactate	144.2	144.8			$1.4124^{26°}$	$1.4156^{16°}$	$1.051^{25.5°}$	$1.119^{0°}$	69	...
Dimethylsuccinate	106^{1} mm.	192.8			$1.4120^{26°}$	$1.4197^{18°}$	$1.119^{26°}$	$1.1202^{18°}$	86.4	33
Dimethylmalate	164^{1} mm.	242			$1.4404^{26°}$	$1.4425^{20°}$	$1.222^{22°}$	$1.226^{20°}$	73.7	...

[a] 2,4-Dinitrophenylhydrazone deriv. m.p. 144° C. Reported 142–4° C. (9)
[b] Unless otherwise indicated, boiling points were determined at 760 mm. Hg.

Table II. Effect of Temperature on Retention Time and Peak Area of Methyl Esters

Ester	Temp., 65° C.	Recovery, %	Temp., 85° C.	Temp., 100° C.	Recovery, %	Temp., 125° C.	Recovery, %
Methyl pyruvate	26.6 (388)	97.4	17.6 (371)	10.7 (368)		5.3 (274)	
Methyl lactate	32.3 (522)	100.01	20.7 (469)	12.2 (464)		5.3 (399)	
Dimethylmalonate				26.6 (350)	98.8	14.0 (322)	
Dimethylfumarate			47.4 (565)	32.8 (453)	92.6	17.4 (370)	
Dimethylsuccinate				34.0 (318)		22.3 (322)	88.5
Dimethylmalate				60.0 (219)		46.3 (280)	92.0

Retention time in minutes. Values in parenthesis are peak areas in thousands of counts. Column: 8-ft. glass column packed with 15% DEGS on Gas-Chrom-P. Flash heater: 215° C., carrier gas, argon 8 p.s.i.g. (88 ml./min. at outlet). Detector: argon Sr^{90} at 215° C. Sample: 5 μl. (10^{-2} micromoles in acetonitrile). Recovery: calculated on the basis of the free acid used for the methylation and the assumption the commercial preparations were pure.

reduced pressures at temperatures below the decomposition points of the esters.

Melting points were determined on a Fisher-Jones apparatus and refractive indices on an Abbé 3L refractometer. Boiling points were determined by distillation at the indicated pressures and densities by the weight–volume method, corrected for temperature.

For esterification of individual acids in micromole quantities, 0.02 ml. or 25 mg. of the acid were dissolved in 10 ml. of diethyl ether and the ethereal solution of diazomethane was added with shaking until a permanent yellow color appeared. A 5-ml. excess of the diazomethane solution was then added. The ether and excess diazomethane were removed from the esters on a rotary evaporator at 20° C. under vacuum. The residue of methyl esters was dissolved in 2 ml. of acetonitrile (spectro grade, Eastman Organic Chemicals) for chromatographic analysis.

For the preparation of the esters in mixtures and for studies of gas chromatography separations, the liquid acids (0.02 ml.) and the solid acids (25 mg.) were mixed and dissolved in 100 ml. of diethyl ether for esterfication. The ethereal solution of the acids was contained in the first chilled (ice bath) receiving flask so that the diazomethane distilled directly into it. For convenience, the esterified mixture was stored overnight in dry ice. To ensure complete methylation of the poly-acids additional diazomethane was added if the solutions became decolorized. For chromatography, an 8-ft. "U"-shaped column (10-mm. I.D.) packed with 15% diethyleneglycol succinate (DEGS) on 80–100 mesh Gas-Chrom-P (Applied Science Laboratories) was used in a Barber-Colman Model 15 Gas Chromatography unit with an Argon Strontium-90 Detector. Peak areas were measured by the attachment of a Model CRS-1 Chromatography Readout System (Infotronics Co.).

For esterification, the acids used were: pyruvic acid (Eastman Organic Chemicals), lactic acid (91%), succinic acid (Baker analyzed), citric acid (California Biochemical, A grade), fumaric acid and malonic acid (California Foundation for Biochemical Research, purified grade), L-malic acid (Nutritional Biochemical), *cis*-aconitic acid and oxalacetic acid (K and K Laboratories) and L-α-ketoglutaric acid (Sigma).

Commercially available esters, methyl pyruvate, methyl lactate, dimethyl malate, dimethyl fumarate, dimethyl malonate, and dimethyl succinate (K and K Laboratories) were used for comparison of retention times and recovery.

RESULTS

In the initial studies, the methyl esters were prepared in sufficient quantities for characterization. The physical properties and yields of the esters examined have been shown in Table I. Although a mole excess of diazomethane was used, there was no indication of secondary reactions for methyl pyruvate. Likewise, even after recrystallization, the yield of dimethyl ester of fumaric acid was comparable with that of succinic acid and considerably better than the yield of dimethyl malate. Glutamic acid was included because of the possibility that the methyl ester would complicate the analysis of α-ketoglutarate. As no interference was observed, the material ($\alpha_D^{26°}$ 1.3919, D $1.059^{25°}$) was not further identified. Likewise, the product of methylation of α-ketoglutaric acid has not been further identified. The 2,4-dinitrophenylhydrazone derivative (m.p. 120° C.) was formed from the product recovered ($\alpha_D^{26°}$ 1.4003, D $1.109^{25°}$).

Since our interest was in the quantification of micromole quantities of the acids, the esters were prepared in micromole quantities for subsequent studies.

The effect of temperature on the retention time and relative recovery of the individual methyl esters has been shown in Table II. As the temperature increased, the apparent peak area for methyl pyruvate, lactate, malonate, and fumarate decreased. This was most evident for fumarate between 85 and 100° C. and for pyruvate between 100 and 125° C.

Not apparent from Table II, was the observation that at temperatures above 100° C. methyl pyruvate no longer appeared as a single peak but gave three peaks; one appearing before and one

after the main pyruvate peak. It was also observed, especially for methyl pyruvate and lactate, that if the diluted samples were permitted to be at room temperature for some period of time (48 hrs.), decomposition occurred and a chromatographic peak identified as methanol appeared. The other methyl esters also exhibited this apparent decomposition to give methanol. A similar decomposition of methyl pyruvate and of methyl lactate occurred if the flash heater temperature exceeded 225° C. In all of the trials using freshly prepared methyl pyruvate at column temperatures of less than 110° C., only one peak was observed. There was no evidence of the peak generally referred to as "pyruvate-2" (*2*).

For evaluation of the methylation and possible losses in transfer, individual samples of the prepared esters were compared with known esters. The per cent recovery shown in Table II was calculated on the basis of the free acid used for the methylation and the assumption that the commercial preparations were pure. At the temperatures used for this comparison, the individual commercial esters also exhibited only a single peak. Any loss on the column would be identical for the isolated esters.

Although good recoveries of both methyl pyruvate and methyl lactate could be obtained at column temperatures below 100° C., there was no indication that such recoveries could be achieved when either of the acids were in excess. Mixtures of pyruvic acid and lactic acid in reciprocal concentrations from 0 to 25 $\times$ 10^{-2} micromole, such that the total acid concentration was constant, were prepared for methylation. Each sample of the mixed esters was taken up in 5 ml. of acetonitrile, and 5 μl. of the sample applied to the column. The results from these observations have been shown in Figure 1. Except for the sample containing 30% pyruvate and 70% lactate, all counts were within 3% of the predicted values as calculated from various concentrations of lactate and pyruvate. These observations indicate the usefulness of the method for the analysis of these specific compounds.

The data in Table II were used as a guide in programming the column temperature (65–175° C.) for the resolution of mixtures of the prepared esters. In the temperature program shown in Figure 2, several compromises were involved. For vaporization of the higher boiling components, a flash heater temperature of 225° C. was required. Likewise, in order to decrease the retention time for the longer chain compounds, the initial temperature increase was so rapid as to be at ca. 112° C. by the time fumarate was eluted. Under these conditions the decomposition of some of the ester was apparent in the consistently appearing methanol peak. Since the appearance of methanol was always associated with the higher flash heater and column temperatures, it must not be assumed that recoveries from the temperature programmed mixture would be comparable to those reported for the individual components as shown in Figure 1 or Table II. These observations indicate that the programmed situation permits identification of the components in a mixture but for quantification, the conditions for the analysis, particularly temperatures, must be related to the individual component, or group of components.

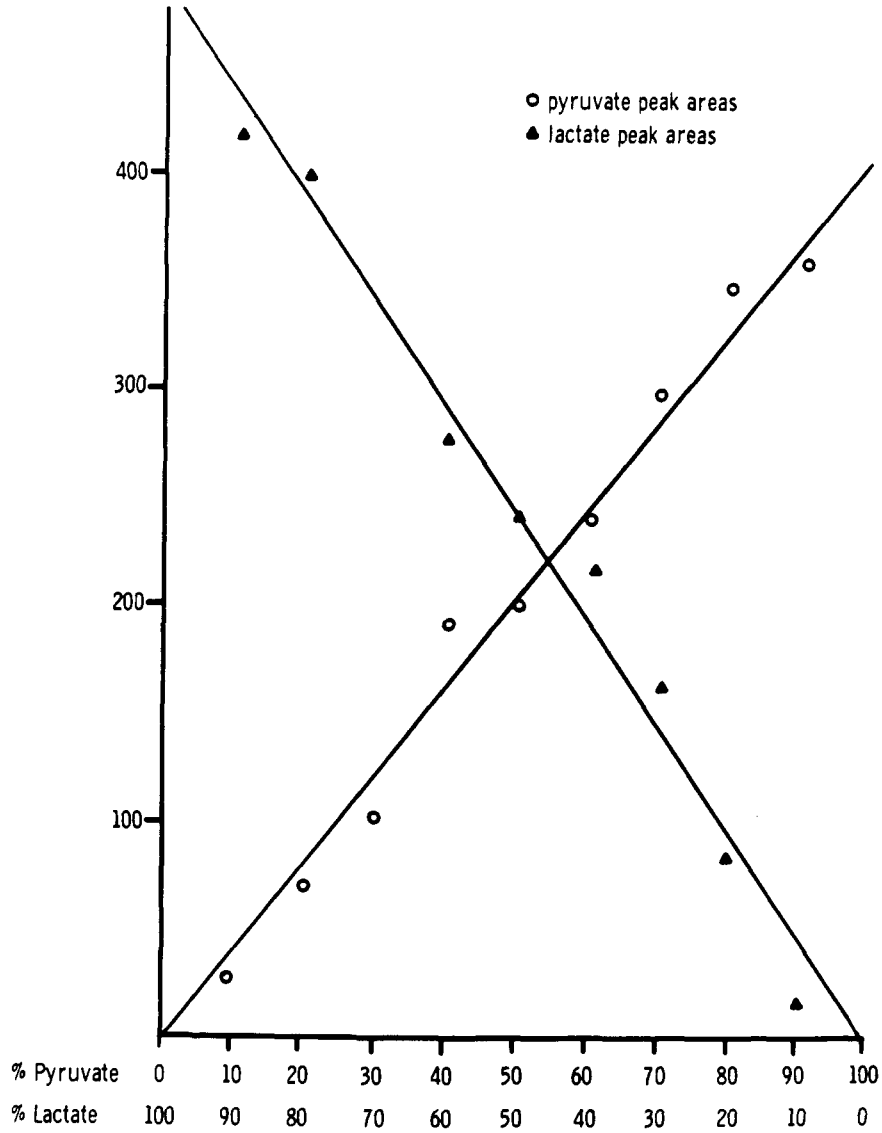

Figure 1. Recoveries of pyruvic and lactic acid as methyl esters

Relative peak areas in thousands of counts
Column temp. 85° C.
Injection port temp. 215° C.
Carrier gas (argon)
Flow rate 8 p.s.i.g. (88 ml./min.)
Sensitivity 1 $\times$ 10^{-7}
Attenuation 8

DISCUSSION

Of the esters prepared, dimethyl fumarate gave the lowest recovery. These observations are consistent with previous work (*11*) suggesting poor yields of dimethylfumarate. It is likewise in agreement with Vorbeck *et al.* (*17*) that the lower yields do not result from side reactions and there was no evidence of formation of addition products at the ethylene bond. Furthermore, even at column temperatures of 90° C., dimethyl fumarate (m. p. 102°) gave a methanol peak.

Except for Luke *et al.* (*10*), irrespective of the method of methylation, column packing material, or temperature programming, all the previously reported observations (*2, 6, 11, 13*) are in agreement with this report that dimethyl fumarate eluted as a single peak before methyl succinate. McKeown (*11*) has suggested that in the methylation Luke *et al.* (*10*) formed the 4,5-dicarbomethoxy-pyrazoline. If this ex-

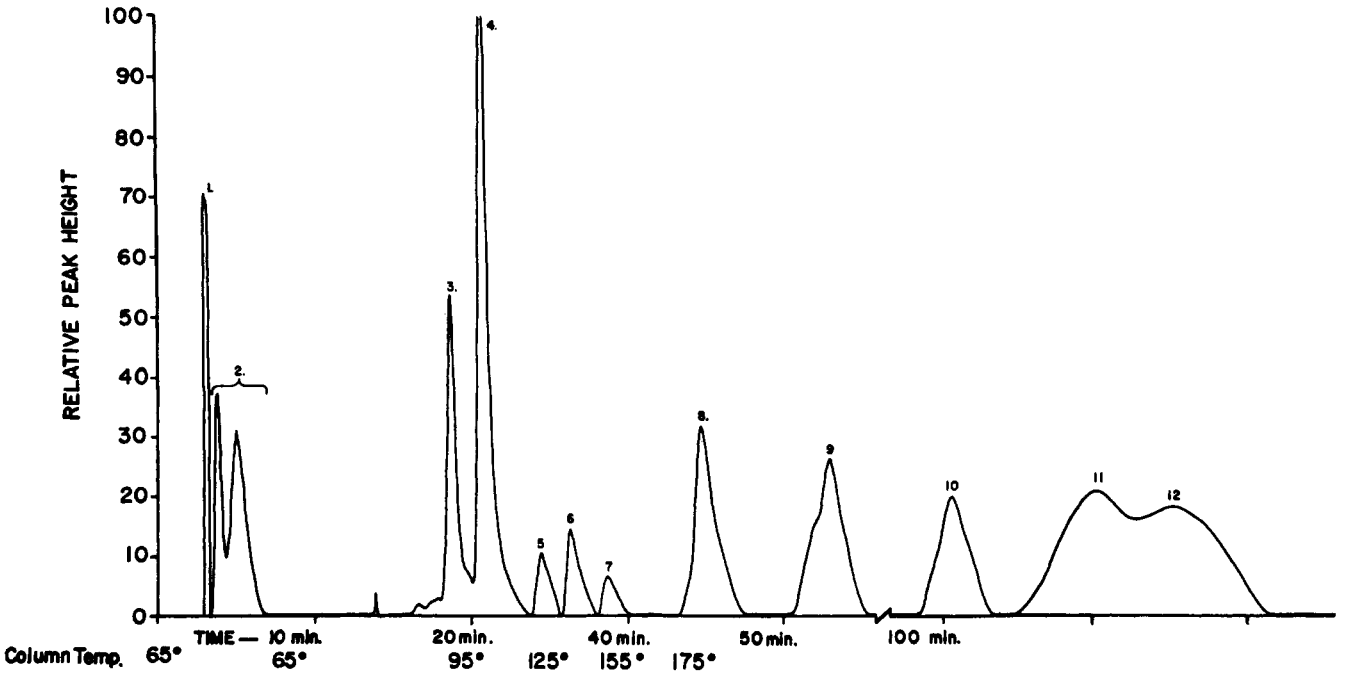

Figure 2. Programmed separation of methyl esters

Injection port 225° C., argon at 88 ml./min. Values in parenthesis are retention times relative to pyruvate, 20.7 min.

1. Methanol
2. Solvent (2 peaks)
3. Pyruvate (1.00)
4. Lactate (1.10)
5. Malonate (1.28)
6. Fumarate (1.38)
7. Succinate (1.78)
8. Malate (2.17)
9. α-Ketoglutarate (2.56)
10. *cis*-Aconitate (4.88)
11. Citrate (5.14)
12. Oxalacetate

planation is true, then it resulted from either reducing the volume in a stream of N_2, or from drying with Na_2SO_4. The results herein are not in agreement with McKeown (*11*) that reduced temperatures (−70° C.) are required to prepare dimethyl fumarate. Likewise, we were unable to confirm their (*11*) observation that better yields were obtained with methanol as a solvent for the methylation. Their use of 10% acetic acid in methanol to destroy the excess diazomethane may have contributed to the differences observed. No difficulties in methylation were encountered when the ether solution containing the diazomethane was cool, excess diazomethane was used, and the ether and unreacted diazomethane were removed in a rotary evaporator at 20° C. It was also observed that even in dry solvents, when the esters are in low concentration, prolonged standing at room temperature should be avoided.

Since both Gee (*6*) and Rumsey (*13*) used methanol to dilute their sample, any methanol resulting from the high temperature of the injection port would not have been observed. The second pyruvate peak reported by Rumsey (*13*) quite probably resulted from the column temperature used.

Although Rumsey *et al.* (*13*) found some overlapping with dimethylmalate peaks, they report the dimethyl ester of α-ketoglutarate as a single peak which is in agreement with our observations. Alcock (*2*), however, found 2 peaks for α-ketoglutarate differing in proportion from one preparation to another. In addition, while this work is in accord with Gee (*6*) that the methyl pyruvate (b.p. 137° C.) elutes before methyl lactate (b.p. 144° C.), Alcock reports the reverse order. Although the column material was diethyleneglycol adipate as compared with neopentylglycol succinate and diethyleneglycol succinate the fact that the observations involved only the keto acids suggests that the differences do not result from the packing material but possibly from the use of chloroform as a solvent.

From the data, it is apparent that methyl esters of α-hydroxyl, α-keto, and conjugated unsaturated acids can be prepared by diazomethane. The characterization and recovery of methyl lactate as determined by peak area would indicate that the α-hydroxyl group was not esterified. Likewise, the characterization and recovery of methyl pyruvate is not consistent with the formation of secondary reaction products (*3*, *5*). Quite apart from the method of preparation, it is apparent that the equilibrium for methyl pyruvate is on the side of hydrolysis products, as evidenced by the appearance of the methanol peak at column temperatures above 100° C. Since heat decomposition of methyl esters of α-ketoglutaric and glutamic acid precluded distillation, it is not surprising that the methanol peak appeared with α-ketoglutarate and that methanol has been the only peak observed on gas chromatography of the dimethyl ester of glutamic acid. The latter observation indicated that application of this method to biological preparations would not be complicated by the α-aminodicarboxylic acids.

Although the observations on the resolution of the methyl ester of oxalacetic acid were not in complete agreement with those of Alcock (*2*), they are in accord with the conclusion that the resolution was not satisfactory. The problem may be one of esterification as Alcock has suggested, however, it may be inherent in the character of oxalacetic acid which is also an α-keto acid.

These observations suggest that for quantification of α-substituted dicarboxylic acids, the problem is not so much one of method of methylation as of selection of the derivative to be used. The recent report of Horii *et al.* (*8*) suggests that the trimethylsilyl derivatives might be useful for these acids.

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An Improved Method for the Determination of Orthophosphate Suitable for Assay of Adenosine Triphosphatase Activity

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▶ **In view of poor recovery and lack of reproducibility and stability frequently experienced in the assay of orthophosphate (Pi) by the method of Martin and Doty, the method has been modified in three respects: protein is precipitated with ClO_4^- at pH 1.5–1.8; the precipitate is removed prior to formation of phosphomolybdic acid; phosphomolybdic acid is measured, after extraction into isobutanol–benzene (1:1), in the unreduced (yellow) form. Optimal concentrations of H_2SO_4 and molybdate have also been determined. The method developed is superior in precision and accuracy, as well as sensitivity, to those presently available, and the product measured is stable for at least 48 hours. The procedure was applied, with satisfactory results, to the determination of the adenosine triphosphatase activity of extracts of muscle tissue and proteins isolated from such tissue.**

ORTHOPHOSPHATE (Pi) is almost always determined as phosphomolybdic acid, usually after reduction (*5*, *18*, *24*). Duval (*4*) aptly remarks on the "very numerous modifications" of the method, and of "profound contradictions on the subject of the optimal concentration of molybdic acid, of acidity, and of the temperature of reduction." He further states that if reduction is carried out at room temperature, as is commonly done, the blue color is unstable. Kondrashova *et al.* (*14*) likewise comment on the "existence of many modifications [and] the absence of a single one that is sufficiently satisfactory." Our experience fully confirms these statements.

The object of the investigation presently described was to determine conditions which are optimal for the determination of Pi and compatible with the presence of adenosine-5′-triphosphate (ATP), proteins and various salts. It was found necessary to consider the following factors: the agent used for precipitation of the protein, the necessity of removing the (precipitated) protein, the form of the phosphomolybdate during extraction and measurement, the $[H^+]$, and the concentration (aqueous) of molybdate.

EXPERIMENTAL

All spectra were obtained with a Bausch and Lomb Spectronic 505 recording spectrophotometer. Other absorbance measurements were made with a Beckman DU spectrophotometer which was calibrated for wavelength and absorbance (*12*). The level of phosphorus is expressed as final concentration in mg. of P/liter. Except where otherwise indicated, the unit of absorptivity a is $(cm)^{-1}$ (mg. P/liter)$^{-1}$. When phosphorus was extracted from an aqueous into an organic phase, the absorptivity figures refers to the concentration of phosphorus in the latter phase. In such cases the absorptivity is "apparent" in the sense that the extraction was assumed to be quantitative and the volume of the organic phase was assumed to be unaffected by equilibration with the aqueous phase.

The reduced and unreduced forms of the colored phosphomolybdate product(s) are indicated by the superscripts B (blue) and Y (yellow), respectively. Additions of inorganic phosphate were in the form of KH_2PO_4, Fisher primary standard, dried at 110° C. for 24 hours. The levels of acids and salts are expressed as final concentrations in the aqueous phase.

Adenosine Triphosphatase Assay

To 1 ml. of enzyme solution containing from 10 to 400 μg. of myosin (*6*) in buffer A were added two ml. of substrate solution, containing ATP in buffer B (*24*). The samples were incubated in a water bath at 22° C. for five minutes. Some assays were carried out using the buffers suggested by Haga *et al.* (*9*).

RESULTS

I. Tests with Phosphomolybdate Blue. The procedure of Martin and Doty (*18*), as modified by Szent-Györgyi and Holtzer (*24*), was applied to a series of standard phosphate solutions. The absorbance spectrum of the blue product (Figure 1*A*) has a broad peak in the visible range, and peaks in the ultraviolet at 313 mμ and at 273 mμ. An absorptivity $a^B_{675} = 0.512$ was found, agreeing with Szent-Györgyi and Holtzer (*24*). However, fading of the extracts was observed. The fading rate increased with increasing concentration of Pi, being a first-order function of phosphate concentration up to 1.3 mg. P/liter, and a higher order function above this level. Furthermore, the rate of fading was not reproducible in different experiments. This could not be attributed to variability in reagents. Chilling the reduced extract in an ice bath improved color stability considerably, but the results were still too erratic to be acceptable. The substitution of *p*-semidine for stannous chloride, as recommended by Dryer *et al.* (*3*) was tried, but very little reduction to the blue product was obtained as measured by the color yield. The use of isoamyl acetate (iAA) as the extracting solvent, and addition of stannous chloride in the dry form, as reported by