the four components of the equilibrium mixture was determined quantitatively. In both solvents it was found that equilibrium was established immediately and that within the accuracy of the analytical method (estimated at 5%) benzoyl trifluoroacetate was formed quantitatively. Furthermore, equimolar solutions of trifluoroacetic anhydride with lauric, furoic and phenylacetic acids had infrared spectra which indicated that only the acyl trifluoroacetate and trifluoroacetic acid were present, *i.e.*, the reaction was quantitative with these acids too. Therefore, the isolation of the acyl trifluoroacetates was undertaken and was easily effected by previously described techniques.[1]

Experimental

Benzoyl Trifluoroacetate.—To 525 g. (2.5 moles) of trifluoroacetic anhydride was added 244 g. (2.0 moles) of benzoic acid. This mixture was then stirred and heated under reflux for 30 minutes. The trifluoroacetic anhydride and acid were distilled *in vacuo* and the benzoyl trifluoroacetate flash distilled at 100° (0.5 mm.), yield 240 g. (55%). The infrared spectrum of this material was identical to that prepared from silver trifluoroacetate and benzoyl chloride.[1]

Lauroyl Trifluoroacetate.—To a solution of 20.0 g. (0.1 mole) of lauric acid in 100 ml. of dry methylene chloride was added 21.0 g. (0.1 mole) of trifluoroacetic anhydride. The methylene chloride was immediately distilled *in vacuo* and the lauroyl trifluoroacetate flash distilled at 160° (0.4 mm.), yield 17.1 g. (59%). The infrared spectrum of this preparation was identical with that of an authentic sample.

Reaction of Carboxylic Acids and Trifluoroacetic Anhydride.—Solutions of benzoic, furoic, lauric and phenylacetic acid were prepared in purified *n*-butyl ether.[3] Aliquots of each acid and trifluoroacetic anhydride were made up to volume to give equimolar concentrations of each at the 0.1 *M* level. Infrared spectra were obtained on a Perkin-Elmer Model 21 spectrophotometer. The strong trifluoroacetic anhydride band at 1873 cm.$^{-1}$ was absent from the spectrum of each mixture. In its place were the following acyl trifluoroacetate carbonyl bands: benzoyl trifluoroacetate, 1835 cm.$^{-1}$; furoyl trifluoroacetate, 1835 cm.$^{-1}$; phenylacetyl trifluoroacetate, 1850 cm.$^{-1}$; lauroyl trifluoroacetate, 1850 cm.$^{-1}$. These frequencies are in reasonable agreement with those previously reported for capillary layers of the mixed anhydrides.[1]

Reaction of Benzoic Acid and Trifluoroacetic Anhydride.—Mixtures were analyzed for each component in both acetonitrile and *n*-butyl ether for the system benzoic acid–trifluoroacetic anhydride. All measurements were made at 25–28°. Working curves were prepared from solutions of the pure components. Band intensities were measured for each solution at each analysis frequency to obtain corrections for mutual interference. Curvature of these optical density *vs.* concentration plots was too great to permit use of simultaneous equation solutions based on Beer's law. The method of successive graphical approximations was, therefore, used for determination of the concentrations of each of the components. The data obtained are summarized in Table I. The fact that an excess of trifluoroacetic acid over benzoyl trifluoroacetate was obtained is accounted for by some hydrolysis of trifluoroacetic anhydride by water introduced into the system while the solutions were processed; accordingly, one-half of the excess trifluoroacetic acid found over benzoyl trifluoroacetate (Table I) represents the molar quantity of water introduced into the system. If this correction is taken into account, 0.109 mole of product was accounted for in acetonitrile out of 0.107 mole originally introduced. Similarly in *n*-butyl ether the analytical procedure accounted for 0.086 mole out of 0.084 mole introduced. It should also be mentioned that there was no evidence of disproportionation of benzoyl trifluoroacetate during any of these experiments.

TABLE I

REACTION OF BENZOIC ACID AND TRIFLUOROACETIC ANHYDRIDE

Compound	Analysis bands, cm.$^{-1}$ Aceto-nitrile	Analysis bands, cm.$^{-1}$ *n*-Butyl ether	Moles added Aceto-nitrile	Moles added *n*-Butyl ether	Moles found Aceto-nitrile	Moles found *n*-Butyl ether
$CF_3COOCOCF_3$	1872	1873	0.035	0.057	None	0.028
C_6H_5COOH	714	708	0.066	0.025	0.036	None
$C_6H_5COOCOCF_3$	1832	1835	None	None	.030	.027
CF_3COOH	688	695	None	None	.043	.031
H_2O			0.006	0.002		
Total moles			0.107	0.084	.109	.086

(3) A. T. Blomquist and A. F. Ferris, THIS JOURNAL, 73, 7412 (1951).

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Further Studies on the Enzymatic Phosphorylation of Riboflavin[1]

BY SASHA ENGLARD[2]

RECEIVED JULY 13, 1953

In a previous communication,[3] ADP[4] was found to participate in the flavokinase reaction which catalyzes the phosphorylation of riboflavin to yield FMN. The inability to detect myokinase which catalyzes the reaction 2 ADP $\rightleftarrows$ ATP + AMP, in the enzyme preparations, plus the fact that the relative efficiencies of ATP and ADP in FMN synthesis are the same throughout purification, led to the conclusion that ADP participated directly in the synthesis of FMN. In view of the widespread acceptance that in most transphosphorylation reactions involving the adenylic acid system only the terminal phosphate of ATP can be directly transferred,[5] the problem was reinvestigated, and myokinase activity was detected even in the purest flavokinase preparations. The finding of such a contaminant in the flavokinase preparations sug-

TABLE I

Each tube contained approximately 7.5 μM. of a preparation derived from Sigma Ba-ADP which consisted of 1.11 μM. AMP, 5.51 μM. ADP, and 0.66 μM. ATP; 1 mg. of flavokinase at an activity of 213,375 μM. of tris-(hydroxymethyl)-aminomethane buffer at *p*H 7.41 and 1×10^{-3} *M* $MgSO_4$ or 6×10^{-4} *M* $ZnSO_4$. The final volume of the reaction mixture was 5.0 ml. The tubes were incubated for 2 hr. at 33.5°, after which time they were immersed in a boiling water-bath for 5 min. prior to filtration through Whatman No. 2. A 3-ml. aliquot of each filtrate was diluted to 25 ml. with water and these solutions passed through Dowex-1 anion exchange columns according to the method of Cohn and Carter [THIS JOURNAL, 72, 4273 (1950)]. Results are recalculated on the basis of the 5.0-ml. final volume of the original reaction mixture.

	Mg^{++} and boiled enzyme, μM.	Mg^{++} and enzyme, μM.	Zn^{++} and enzyme, μM.
ΔAMP	+1.44	+1.65	+0.03
ΔADP	−2.96	−3.11	+ .14
ΔATP	+1.53	+1.58	+ .03

(1) Supported in part by a grant from the Prentiss Fund of Western Reserve University.

(2) U. S. Public Health Pre-doctoral Fellow of the National Institute of Arthritis and Metabolic Diseases. Now at McCollum-Pratt Institute, Baltimore 18, Md.

(3) E. B. Kearney and S. Englard, *J. Biol. Chem.*, 193, 821 (1951).

(4) The following abbreviations are used: AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; IMP = inosine monophosphate; FMN = flavin mononucleotide.

(5) S. P. Colowick, in Summer and Myrback, "The Enzymes," Vol. II, Part I, Academic Press, Inc., New York, N. Y., 1951, p. 114.

TABLE II

Expt. no.	Conditions	mμ M. FMN[a] synthesized in 2 hr.
1	1×10^{-3} *M* $MgSO_4$, 1.8×10^{-3} *M* ATP	230.2
	1×10^{-3} *M* $MgSO_4$, 1.6×10^{-4} *M* ADP	104.8
	6×10^{-4} *M* $ZnSO_4$, 1.8×10^{-3} *M* ATP	328.3
	6×10^{-4} *M* $ZnSO_4$, 1.6×10^{-4} *M* ADP	0.0
2	1×10^{-3} *M* $MgSO_4$, 2.8×10^{-4} *M* ATP	153.3
	1×10^{-3} *M* $MgSO_4$, 2.8×10^{-4} *M* ATP, 4.5×10^{-4} *M* AMP	82.9
	1×10^{-3} *M* $MgSO_4$, 2.8×10^{-4} *M* ATP, 4.8×10^{-4} *M* IMP	142.3
	6×10^{-4} *M* $ZnSO_4$, 2.8×10^{-4} *M* ATP	57.7[b]
	6×10^{-4} *M* $ZnSO_4$, 2.8×10^{-4} *M* ATP, 4.5×10^{-4} *M* AMP	51.9
	6×10^{-4} *M* $ZnSO_4$, 2.8×10^{-4} *M* ATP, 4.8×10^{-4} *M* IMP	63.5
3	1×10^{-3} *M* $MgSO_4$, 2.0×10^{-4} *M* ATP	146.9
	1×10^{-3} *M* $MgSO_4$, 2.0×10^{-4} *M* ATP, 4.5×10^{-4} *M* AMP	68.5
	1×10^{-3} *M* $MgSO_4$, 2.0×10^{-4} *M* ATP, 6.0×10^{-4} *M* adenosine	144.0
4	6×10^{-4} *M* $ZnSO_4$, 3.0×10^{-4} *M* ATP	38.7
	6×10^{-4} *M* $ZnSO_4$, 3.0×10^{-4} *M* ATP, 4.5×10^{-4} *M* AMP	41.6
	6×10^{-4} *M* $ZnSO_4$, 3.0×10^{-4} *M* ATP, 6.0×10^{-4} *M* adenosine	39.0

[a] Standard assay conditions and analytical determination of FMN as previously described,[1] except that the reaction was run at *p*H 7.41 and at 33.5°. [b] The lower synthesis in the presence of Zn^{++} is due to the fact that in the presence of Zn^{++} the saturation level for ATP is increased. At higher concentrations of ATP, Zn^{++} is more efficient than Mg^{++} (expt. 1 and unpublished data).

gested that ADP probably participated in the reaction by prior conversion to ATP. Moreover, the previously observed inhibitory effect of AMP[1] which was interpreted as suggesting a competition with ATP for the active site on the enzyme surface by virtue of its NH_2 group on the 6-position of the purine ring, could be re-evaluated as a competition for ATP to form inactive ADP and thus lowering the effective concentration of the phosphate donor for the synthesis of FMN. To determine whether or not ADP participated directly in the reaction and also in order to elucidate the mechanism of the AMP inhibition it was necessary to obtain a system devoid of myokinase activity. In the presence of Zn^{++} which can replace Mg^{++} as an activator of the flavokinase reaction,[1] the myokinase contaminant is virtually inactive as indicated in Table I.

The effect of Zn^{++} on the myokinase activity is probably inhibitory in nature, since in other experiments it has been shown that the myokinase is still active in the absence of added metal, although this activity can be increased by the addition of Mg^{++}. Table I also shows that the boiled control in the presence of Mg^{++} reached the same equilibrium over the 2-hr. incubation period, as the unheated sample. Similar reports on the resistance of myokinase to boiling have appeared previously.[6,7] The inability to detect myokinase with the method previously used can be attributed to the unusual resistance of myokinase to treatments which generally denature most proteins. The method previously used was based on the quantitative determination of AMP with Schmidt deaminase[8] which catalyzes the specific deamination of AMP to IMP. It was ascertained by measuring the AMP content, of what was thought to be a protein-free filtrate, that preincubation of AMP and ATP with a flavokinase preparation did not result in a loss of AMP which would have occurred had myokinase been present. It is now probable in view of the unusual high resistance of myokinase to denaturating treatments, that the so-called protein-free filtrate was contaminated with myokinase. Hence, the previously established equilibrium, which resulted from incubatiug ATP and AMP with a flavokinase preparation contaminated with myokinase, probably reverted back to the initially added concentration of components by virtue of the irreversibility of Schmidt deaminase

$$\text{IMP} \xleftarrow{\text{Schmidt deaminase}} \text{AMP} + \text{ATP} \underset{\text{Myokinase}}{\rightleftharpoons} 2\text{ADP}$$

Using a highly active preparation of flavokinase, the ability of ADP to act as a phosphate donor was re-examined both in the presence of Mg^{++} and Zn^{++}. The results obtained are outlined in Table II, exp. 1.

It is clear from this experiment that in the presence of Mg^{++}, ADP was about 45% as effective as ATP, in the presence of Zn^{++} ADP was inactive as a phosphate donor in the synthesis of FMN. Moreover, whereas in the presence of Mg^{++} AMP as previously reported exerted a 46% inhibition, no such inhibitory effect was detected in the presence of Zn^{++} (Table II, expt. 2). IMP, for which no enzymatic transphosphorylation from ATP has as yet been reported, does not inhibit the synthesis of FMN even in the presence of Mg^{++} (Table II, expt. 2). It could be argued, however, that Mg^{++} may be required for the binding of AMP to the ATP site on the flavokinase surface and actually as previously suggested this binding through the 6-NH_2 group of the purine ring is an essential part of the mechanism of its inhibition. A similar mechanism of inhibition has been proposed to explain the inhibitory effects of adenine and its derivatives in contrast to compounds of the inosine series, on the phosphorylation of pyridoxal.[9]

If such a mechanism were involved, adenosine

(6) S. P. Colowick and H. M. Kalckar, *J. Biol. Chem.*, **148**, 117 (1943).

(7) A. Kornberg and W. E. Pricer, Jr., *ibid.*, **193**, 481 (1951).

(8) H. M. Kalckar, *J. Biol. Chem.*, **167**, 461 (1947).

(9) J. Hurwitz, *Biochim. Biophys. Acta*, **9**, 496 (1952).

which does not participate in the myokinase reaction would be expected nonetheless to inhibit the phosphorylation of riboflavin in the presence of Mg^{++}. However, as can be seen from Table II, expt. 3, whereas AMP inhibited the synthesis of FMN by 53.5%, no inhibition by adenosine was observed. Thus, on the basis of this analogy, it appears less likely that the absence of Mg^{++} is responsible for the loss of AMP inhibition in the presence of Zn^{++}. The higher AMP inhibition noted here as compared to expt. 2 is due to the slightly lower relative ATP concentration and to the use of unpurified ATP solutions which probably already contain some AMP. As expected neither AMP nor adenosine inhibited in the presence of Zn^{++} (Table II, expt. 4).

The experimental results outlined here are highly suggestive that the observed activity of ADP as a phosphate donor in the presence of Mg^{++} is due to contaminating myokinase activity. The remote possibility that ADP acts as a direct phosphorylating agent only in the presence of Mg^{++} has not been excluded. The competitive inhibition of AMP observed in the presence of Mg^{++} can be attributed to a competition with riboflavin for ATP to form ADP which in itself cannot donate phosphate, thus lowering the effective ATP concentration of the reaction mixture.

Although the maximal velocity with ADP in the presence of Mg^{++} is about 50% of that with ATP, this does not constitute an unequivocal argument to conclude that the activity of ADP is direct and not due to its prior conversion to ATP by myokinase. A possible inhibitory effect of ADP on the flavokinase reaction may very well lead to such fortuitous results. Neither does the maximal velocity with ADP equal that with AMP in the activation of crude muscle phosphorylase (Dr. S. P. Colowick, personal communication). Yet with purified muscle phosphorylase ADP does not retain its activating effect, suggesting that its stimulatory effect in the crude system was due to its prior conversion to AMP by myokinase.

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Cyclopentadienylsilane Derivatives

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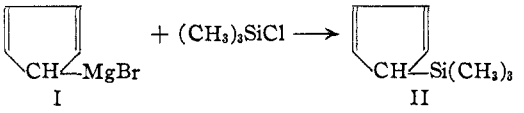
RECEIVED JUNE 19, 1953

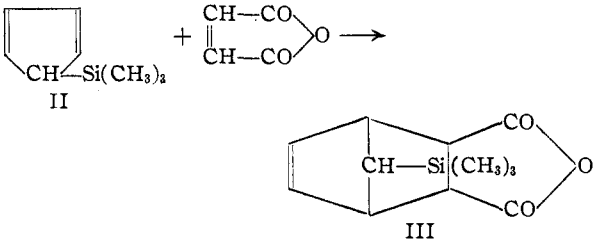
Cyclopentadienylsilane derivatives have hitherto not been reported in the literature. Several routes are possible for the preparation of these derivatives, among them the use of alkali metals to form the corresponding salts which then could react further with chlorosilanes. However, some of these salts are spontaneously inflammable on exposure to air.[1]

The Grignard method offered a convenient and safe way to arrive at these compounds. Cyclopentadienylmagnesium bromide (I) was prepared according to the method of Grignard and Courtot[2] using an exchange reaction between cyclopentadiene and ethylmagnesium bromide. This Grignard compound I then was treated with various chlorosilanes. The reaction of I with trimethylchlorosilane resulted in the formation of cyclopentadienyltrimethylsilane (II).



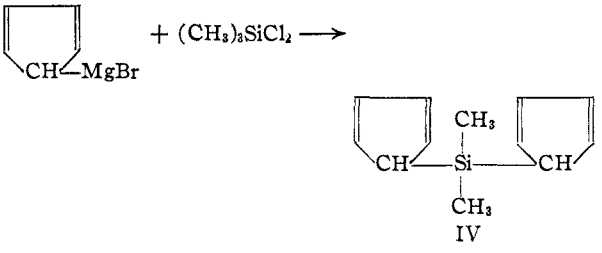
I II

Since a silicon analysis alone was not sufficient to decide between a monomeric or dimeric structure, a molecular weight determination in dioxane indicated the existence of the monomeric form. In addition, a crystalline Diels–Alder adduct III with maleic anhydride gave further proof that the conjugated double bond system in II was still intact



III

The reaction of cyclopentadienylmagnesium bromide with dimethyldichlorosilane led to the isolation of two products from which bis-(cyclopentadienyl)-dimethylsilane (IV) was identified.



IV

Another product, distilling at 80–83° at 0.7 mm., was obtained in a yield of 11%. It had a silicon content of 16.6% and gave a positive chlorine test. However, no definite structure was assigned as yet to this product.

Experimental

Cyclopentadienylmagnesium Bromide (I).—To an ethylmagnesium bromide solution, prepared from 200 g. of ethyl bromide was added 500 cc. of benzene and the ether removed by distillation.

One hundred twenty-one grams of cyclopentadiene, obtained by slow distillation from dicyclopentadiene, was added slowly to the ethylmagnesium bromide solution. It was then added for 1.5 hours at 60°. Ethane evolution occurred during the heating period. Cyclopentadienylmagnesium bromide formed a dark colored, clear solution.

Cyclopentadienyltrimethylsilane (II).—Half of the above Grignard solution was added gradually to a solution of 99.5 g. of trimethylchlorosilane in 150 cc. of benzene. The reaction mixture was then refluxed for 15.5 hours. The inorganic precipitate was filtered off and washed with benzene. The solvent was removed from the filtrate and the residual liquid vacuum distilled. The product distilled at 43–44° at 19 mm. as a colorless liquid which darkened on prolonged exposure to air. The yield was about 45%.

Anal. Calcd. for $C_8H_{14}Si$: Si, 20.3; mol. wt., 138. Found: Si, 19.7; mol. wt. 135.

The 3,6-*endo*-Trimethylsilylmethylene-1,2,3,6-tetrahydrophthalic Anhydride (III).—Two and two-tenths grams of maleic anhydride was added to a solution of 3.1 g. of cyclopentadienyltrimethylsilane in 10 cc. of benzene at room temperature. An instantaneous exothermic reaction set in. The reaction mixture was allowed to stand at room tempera-

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(1) J. Thiele, *Ber.*, **34**, 68 (1901).

(2) V. Grignard and C. Courtot, *Compt. rend.*, **158**, 1763 (1914).