Effect of Phospholipases and Lipase on Submitochondrial Particles*

(Received for publication, December 18, 1970)

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SUMMARY

1. Exposure of submitochondrial particles to phospholipases impaired the rate of oxidation as well as phosphorylation. Phospholipase A and phospholipase C were more inhibitory to phosphorylation and to NADH oxidation than to succinate oxidation.

2. At low concentrations of phospholipase A the inhibition of phosphorylation was prevented to a considerable extent by the presence of large amounts of bovine serum albumin preferably during both digestion and assay. At larger concentrations of phospholipase A only slight protection was observed by addition of serum albumin.

3. Exposure of submitochondrial particles to phospholipase C from Clostridium welchii in the presence of Mg++ or Mn++ inhibited the 32Pi-ATP exchange and the P:O ratio by about 30 to 60%. Addition of coupling factors restored phosphorylation associated with oxidation in these particles. Additional exposure of the digested particles to phospholipase C from Bacillus cereus in the presence of Zn++ further depressed the 32Pi-ATP exchange which was no longer restored by coupling factors. Addition of phospholipids partially restored the 32Pi-ATP exchange in these extensively digested particles. Similar effects were noted after prolonged exposure to phospholipase C from B. cereus alone, provided both Mn++ and Zn++ were present.

4. Oxidative phosphorylation was also severely impaired after exposure of submitochondrial particles to phospholipase C from B. cereus in the presence of Mn++ and Zn++. A partial restoration of the P:O ratio was achieved by reconstitution with phospholipids.

It was shown in the preceding paper (1) that phosphorylation and particularly ion transport properties of mitochondria are damaged by exposure to phospholipase A or phospholipase C. In contrast, respiration remained remarkably unimpaired. It has been known for some time (c.f. References 2, 3) that respiration in submitochondrial particles is quite sensitive to a variety of P-lipases, while relatively little information is available on the effect of different lipases on phosphorylation processes. There appears to be no documentation that impaired phosphorylation has been restored by addition of P-lipids.

Since several coupling factors as well as dehydrogenases appear to be present on the matrix side of the inner mitochondrial membrane and therefore available at the surface of submitochondrial particles (4) it was of particular interest to study the effect of a variety of lipases and to explore the possibility as to whether some of their effects are reversible by addition of P-lipids.

It is the purpose of this paper to report on the effect of various P-lipases on submitochondrial particles and to show that, as in the case of the resolution of coupling factors, gentle treatment of particles appears to be a feasible approach to the problem of P-lipid topography and reconstitution.

EXPERIMENTAL PROCEDURE

Preparations—Preparation of mitochondria, particles, P-lipase C from Clostridium welchii, and P-lipid suspensions were as described in the preceding paper (1). F1 (5), F4 (6), OSCP from F4 (7) and OSCP from particles (8), and F2 (9) were prepared as described. A slant of Bacillus cereus (NCTC $945) was provided by Dr. L. Hager. The bacteria were grown in 2 liters at 30° for 20 hours in a medium containing per liter 5 g of yeast extract (Difco), 10 g of NaCl, and 10 g of Bactotryptone. P-lipase C was purified from the supernatant by a modification of described methods (10, 11). To each 100 ml of supernatant 51.5 g of ammonium sulfate were added. After 1 hour of stirring at 25° the mixture was centrifuged for 1 hour at 15,000 × g at 4°, the precipitate was taken up in 100 ml of water, dialyzed against distilled water (until free of NH4), lyophilized (160 mg), and stored at −20°. A solution of 32 mg in 20 mm Pi was adjusted to pH 7.5 and passed through a Sephadex G-50 column (1.5 × 20 cm) which was equilibrated with 20 mm Pi, pH 7.5. The most active fractions were combined and stored at −20° in 50% glycerol. Lipase from Candida cylindracea was a commercial preparation (MV) from the Meito Sangyo Company, Japan, which was kindly donated to us by Dr. F. Mattson.

* This work was supported by Public Health Service Research Grant CA-08864 from the National Institutes of Health.
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1 The abbreviations used are: F1, F2, F3, F4, coupling factors 1 (ATPase), 2, 3, 4, 5, respectively; OSCP, oligomycin sensitivity-conferring protein; SMP, submitochondrial particles prepared by sonication of bovine heart mitochondria in the presence of pyrophosphate; Gw, Clostridium welchii; Bc, Bacillus cereus; CCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
Effect of Lipases on Submitochondrial Particles

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Effect of Lipase A on Submitochondrial Particles—In contrast to its effect on mitochondria, lipase A inhibited markedly the oxidation of succinate and NADH in submitochondrial particles. Since phosphorylation was even more sensitive to lipase digestion than oxidation, the P:O ratio was severely decreased (Fig. 1). As in the case of mitochondria, addition of bovine serum albumin protected considerably. To avoid the complicating feature of the inhibition of respiration, a more detailed study was carried out on the effect of lipase A on the 32P-ATP exchange. It can be seen from Fig. 2 that at low lipase A concentrations the inhibition of phosphorylation was overcome to a considerable extent by serum albumin, particularly if it was present during digestion as well as during assay. Even in the presence of relatively large amounts of lipase A (1 μg per mg of SMP) the recovery of activity was about 25%. Noteworthy is the failure of egg lecithin to activate phosphorylation under these conditions, a finding significant in relation to subsequent studies with lipase C which revealed an effect of egg lecithin.

Also of interest is the observation that SMP are less sensitive to lipase A than mitochondria tested under similar conditions (1).

Effect of Lipase C (C.W. and B.C.) on Respiration of SMP—in contrast to the lack of an inhibitory effect of lipase C from C. welchi on the respiration of mitochondria, the oxidation of NADH in SMP was markedly inhibited by lipase C treatment. As shown in Table I, oxidation of NADH was more severely impaired than oxidation of succinate, while oxidation of ascorbate remained unchanged under the same conditions of digestion. Similar effects were noted with lipase C (B.C.). For the purpose of comparison both enzymes were tested in the presence of low concentrations of Zn++. The greater sensitivity of NADH oxidation points to an essential role of lipids in close association with the surface of SMP.

RESULTS

Analytical Methods—Thin layer chromatography (12) of lipids was carried out on silica gel (F-256 from Merck) in chloroform-methanol-H2O (65:25:4). Lipids were detected by exposure to iodine vapors. Other procedures were as described in the accompanying paper (1). Reconstitution of particles with lipids and measurements of P:O ratio were performed as follows. The digested particles (300 μg of protein) were incubated with 750 μg of defatted, dialyzed bovine serum albumin and with lipids (as indicated in the tables) for 10 min at 30° in a final volume of 0.15 ml made up with 0.25 M sucrose. Then 40 μg of F1, 10 μg of OSCP, 230 μg of P1 containing 20 μmoles of potassium P1 were added and the mixture was sonicated in a Branson “sonic 5 x 5” for 20 sec. After an incubation for 10 min at 30°, the reconstituted particles (0.3 ml) were transferred to a microcell of a Gilson oxigraph, Gilson Medical Electronics, Middleton, Wisconsin (at 26°) and respiration was initiated by addition of 0.4 ml of a mixture (pH 7.4) containing 7 units of hexokinase, 6 μmoles each of ATP and MgSO4, 20 μmoles of glucose, 3.5 μmoles of Tris-SO4, 20 μmoles of succinate, 700 μg of defatted, dialyzed bovine serum albumin, and 10⁶ cpm of 32P. The reaction was terminated by withdrawing the sample and adding it to a centrifuge tube which contains 0.1 ml of 50% trichloroacetic acid.
Effect of P-lipase C on respiration in submitochondrial particles

SMP (2.5 mg of protein) were treated with 22 μg of P-lipase C (C.W.) or with 2.5 μg of P-lipase C (B.C.) for 60 min at 25° in the presence of 2.5 μmoles of Tris-Cl, pH 7.4, 0.05 μmole of ZnSO4, and 0.165 mM sucrose in a final volume of 0.25 ml. The reaction was stopped by addition of 0.5 μmole of EDTA, and samples corresponding to 250 μg of particles were assayed in a Gilson oxygraph in the presence of 20 mM KPi, pH 7.4.

The presence of 2.5 pmoles of Tris-Cl, pH 7.4, 0.05 pmole of ZnSO4, and the presence of 20 mM KPi, pH 7.4.

Results showing that the presence of 250 ng of particles were assayed in a Gilson oxygraph in stopped by addition of 0.5 μmole of EDTA, and samples corresponding to 250 μg of particles were assayed in a Gilson oxygraph in the presence of 20 mM KPi, pH 7.4.

The presence of 20 mM KPi, pH 7.4.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

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Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.

Effect of P-lipase C (C.W.) on SMP and on SMP Obtained from particles treated with P-lipase C + lipase + coupling factors + OSCP.
P-lipid content (Table II). These doubly exposed particles, which had only about 30 to 40% of the P-lipid content of SMP, were capable of catalyzing a rate of phosphorylation after addition of coupling factors which was only slightly lower than that of untreated SMP. The lack of a more extensive depletion of P-lipids after the second exposure to P-lipase C and the dependency on coupling factors following P-lipase treatment will be discussed later.

Effect of Lipase on P-lipase C-treated SMP—P-lipase C is known to cleave the linkage between the diglyceride and phosphocholine of lecithin (14). Organically bound phosphorus was released into the medium, whereas little diglyceride could be detected. Since the diglycerides, as will be shown later, remained in the membrane, the possibility was considered that they could substitute for P-lipids in whatever function the latter serve in oxidative phosphorylation. To test this possibility a search was made for a lipase which would cleave the remaining diglyceride in the membrane. Pancreatic lipase proved unsuitable because of side reactions presumably due to contamination with proteolytic enzymes. However, purified lipase from C. cylindracea was found to have no effect on control SMP but markedly inhibited phosphorylation (either P:O or 32P-ATP exchange) of the particles that had been exposed to P-lipase C (Table III). The rate of oxidation of succinate was not significantly altered by the treatment with lipase. Attempts to restore phosphorylation by addition of P-lipids were partially successful and can be seen from Table IV.

Effect of P-lipase C (B.c.)—P-lipase C (B.c.) has been reported to have a broader substrate specificity than P-lipase C (C.w.) and is capable of cleaving cardiolipin in the presence of low concentrations of Zn++. In later experiments it was found that prolonged exposure to P-lipase C from B. cereus alone was quite effective provided both Mn++ and Zn++ were present. In general it appears from thin layer chromatography that the degree of inhibition was related to the extent to which phosphatidylycholine and phosphatidyl-ethanolamine were degraded in the membrane.

Specificity of P-lipid Effect on 32P-ATP Exchange—Various

**Table V**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Egg lecithin</th>
<th>Pi plus OSCP plus bovine serum albumin</th>
<th>Pi plus OSCP plus bovine serum albumin plus lecithin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control particles</td>
<td>81</td>
<td>68</td>
<td>71</td>
</tr>
<tr>
<td>Particles treated with P-lipase C (C.w.)</td>
<td>37</td>
<td>34</td>
<td>45</td>
</tr>
<tr>
<td>Particles treated with P-lipase C (C.w.) (Zn++ added at 30 min)</td>
<td>26</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>Particles treated with P-lipase C (D.o.) (Zn++ added at 30 min)</td>
<td>25</td>
<td>36</td>
<td>31</td>
</tr>
<tr>
<td>Particles treated with P-lipase C (C.w.), plus Zn++ added at 30 min</td>
<td>6</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>Particles treated with P-lipase C (O.w.) and P-lipase C (D.o.) in presence of 12 mM EDTA</td>
<td>80</td>
<td>72</td>
<td>75</td>
</tr>
</tbody>
</table>

It can be seen from Fig. 3 that addition of small amounts of P-lipase C from B. cereus greatly accelerated the loss of ability of SMP to catalyze 32P-ATP exchange. Addition of Zn++ alone increased somewhat the action of P-lipase C (C.w.), but P-lipase C (B.c.) was much more effective. Addition of EDTA during digestion eliminated the inhibitory effect due to the combined action of both P-lipases.

Partial Restoration of 32P-ATP Exchange Following Sequential Treatment with Both P-lipase C Preparations by Addition of P-lipases—It can be seen from Table V that either P-lipase C from C. welchii or from B. cereus partially inactivated the 32P-ATP exchange while sequential addition of both resulted in over 90% loss of exchange activity. After digestion with both P-lipase C preparations, addition of coupling factors had little effect, while there was 3- to 4-fold stimulation of the 32P-ATP exchange on addition of egg lecithin. Neither bovine serum albumin nor coupling factors had much effect either in the presence or absence of egg lecithin. It can be seen that if the digestion with both P-lipases was carried out in the presence of EDTA the exchange rate remained unimpaired.

In later experiments it was found that prolonged exposure to P-lipase C from B. cereus alone was quite effective provided both Mn++ and Zn++ were present. In general it appears from thin layer chromatography that the degree of inhibition was related to the extent to which phosphatidylycholine and phosphatidyl-ethanolamine were degraded in the membrane.

**Fig. 3.** Time course of the combined action of phospholipase from C. welchii and B. cereus. Experimental conditions were as described in the legend of Table V. Phospholipase C from C. welchii (C.w.) was present from the beginning of the experiment, the B. cereus enzyme (B.c.) was added after 30 min together with Zn++. In a control only Zn++ was added. One control without C. welchii was run with B. cereus being added after 30 min. Another control (see top line) was run with addition of both enzymes (C.w. + B.c. + Zn++) but in the presence of 12 mM EDTA.
TABLE VI

Restoration by phospholipids of 32P-ATP exchange in P-lipase-treated SMP

<table>
<thead>
<tr>
<th>Additions</th>
<th>32P-ATP exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMP</td>
</tr>
<tr>
<td>None</td>
<td>220a</td>
</tr>
<tr>
<td>Asolectin</td>
<td>150</td>
</tr>
<tr>
<td>Egg lecithin</td>
<td>210</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>200</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>0</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>225</td>
</tr>
<tr>
<td>F-CCP plus lecithin</td>
<td>5</td>
</tr>
<tr>
<td>Rutamycin plus lecithin</td>
<td>2</td>
</tr>
<tr>
<td>Cardiolipin plus lecithin</td>
<td>110</td>
</tr>
<tr>
<td>Cardiolipin plus phosphatidylethanolamine plus lecithin</td>
<td>220</td>
</tr>
</tbody>
</table>

*All results are averages of at least two experiments.

P-lipids were tested for the ability to stimulate the 32P-ATP exchange after P-lipase C digestion. In the experiment shown in Table VI sonicated egg lecithin was more effective than crude soybean P-lipids (asolectin) but usually asolectin was either equally or more effective. The results with different egg lecithin preparations were variable, the most reproducible results being observed with dialyzed asolectin suspensions (6). The 32P-ATP exchange restored by P lipids was sensitive to rutamycin and to F-CCP. Cardiolipin was not only ineffective but uncoupled the residual phosphorylation as well as phosphorylation in untreated particles. Of interest is the observation that mixtures of cardiolipin with other P-lipids were less inhibitory and in fact somewhat active in restoring phosphorylation.

Partial Restoration of Oxidative Phosphorylation after Digestion with P-lipase C (B.c.)—As mentioned above, P-lipase C from B. cereus effectively damaged the phosphorylation capacity of submitochondrial particles provided both Mn++ and Zn++ were present during digestion. Experiments documenting the restoration of oxidative phosphorylation to such digested particles by addition of P-lipids are shown in Table VII. In contrast to the 32P-ATP exchange, oxidative phosphorylation in the control particles was not inhibited by incubation with P-lipids but in fact slightly stimulated. This made it easier to evaluate the restorative capacity of P-lipids added to digested particles. With increasing amounts of P-lipase C, there was less residual phosphorylation after digestion, and the P:O ratio restored on addition of P-lipids was also somewhat lower. When the P:O ratio of the digested particles was above 0.1 the P:O ratio of the reconstituted particles was over 0.5. When the P:O ratio fell below 0.1, the P:O ratio after reconstitution was somewhat lower than 0.5 but the relative stimulation was much greater (8- to 10-fold) than in the less severely damaged particles.

The amount of P-lipids required for optimal stimulation (about 300 μg of soybean P-lipids) was much higher than expected from the extent of P-lipid digestion. This finding as well as the incomplete restoration of the P:O ratio may well be due to the fact that the composition of the soybean P-lipid mixture used for reconstitution was not optimal. This is furthermore borne out by the observation (Table VII, Experiment 2) that a preparation of lecithin isolated from the crude soybean P-lipids by thin layer chromatography was considerably less effective than the crude mixture. Isolated mixtures of P-lipids from bovine heart mitochondria were effective in restoring phosphorylation but thus far have not proved to be better than the mixture of soybean P-lipids.

Analysis of Products of P-lipase-digested SMP—Analysis of the P-lipids of SMP treated with P-lipases by thin layer chromatography revealed that after P-lipase A digestion, both phosphatidylcholine and phosphatidylethanolamine as well as cardiolipin were found in reduced amounts, while fatty acids and lysophosphatidylcholine and lysophosphatidylethanolamine appeared. Digestion with P-lipase C (C.w.r) resulted in a loss in phosphatidylcholine and in the appearance of diglycerides. There was also some decrease in phosphatidylethanolamine. If the action of P-lipase C was followed by treatment with lipase from C. cylindracea, the results were similar except that most of the diglycerides were converted to monoglycerides and fatty acids. No.
fatty acids or lyssolecithin were detectable when P-lipase C (C.w.) acted alone. After treatment with P-lipase C (B.c.), phosphatidylcholine and phosphatidylethanolamine disappeared almost completely and again diglycerides appeared. Of particular significance is the finding that there was no detectable disappearance of cardiolipin. It is therefore possible that the membrane-bound cardiolipin is either not susceptible or not accessible to the added lipolytic enzyme. These semiquantitative analyses are recorded only to emphasize that the specificity of P-lipase action should be monitored with each enzyme and substrate preparation since it appears likely that the specificity varies with different membrane preparations and with different physical states of the substrate P-lipid.

**DISCUSSION**

Effect of P-lipases on Respiration and Phosphorylation in SMP

---In view of the suggested importance of the asymmetric organization of the inner mitochondrial membrane (10), it is pertinent to emphasize the remarkable difference in the susceptibility of mitochondria (1) and submitochondrial particles to digestion with P-lipases. Whereas respiration was found to be relatively insensitive in mitochondria, it was highly sensitive in submitochondrial particles, NADH oxidation being considerably more sensitive than succinate oxidation. On exposure of SMP to P-lipase C, NADH oxidase activity was lost, while neither succinoxidase nor NADH dehydrogenase activity were markedly impaired. This indicates a high sensitivity of the NADH-Qo segment to lipolysis which has been previously observed with P-lipase A in studies where successful restoration of oxidative function by addition of P-lipids was reported (3). The advantage of using P-lipase C lies in the observation that the products of P-lipase A action on phospholipids (fatty acids and lyssolecithin) are particuarly toxic to phosphorylation while the products of P-lipase C (e.g., phosphatidylcholine and diglyceride) are not. Even large amounts of serum albumin had only a limited restorative effect against the combined effect of fatty acids or lysolecithin were not detectable when P-lipase C acted alone. After treatment with P-lipase C (B.c.), phosphatidylcholine and phosphatidylethanolamine, P-lipase C from B. cereus has been reported to cleave these P-lipids as well as cardiolipin provided Zn2+ was added (14). The action of these two enzymes on submitochondrial particles was therefore compared. It first appeared that the two preparations of P-lipase C were more effective together than either alone. Although this may still be true, conditions were found showing that exposure of SMP to P-lipase C (B.c.) alone could bring about a marked inhibition of the O2-Pi-ATP exchange which could be restored on addition of P-lipids. No claim is being made that the specificity of this enzyme, as recorded with pure P-lipids as substrates, is responsible for its greater effect on the membrane. This point is emphasized because of the observation that following digestion with P-lipase C (B.c.), cardiolipin was still present in the membrane. Thus the requirement for Zn2+ which was repeatedly demonstrated cannot be correlated to an enhanced range of action by P-lipase C, but appeared related to the extent of loss of phosphatidylcholine and phosphatidylethanolamine in the membrane as indicated by semiquantitative thin layer chromatography. It was therefore not too surprising that phosphatidylcholine as well as other P-lipids, rather than cardiolipin, had restorative activity.

Role of P-lipids in Phosphorylation—It seems remarkable that, as observed in some experiments, up to 70% of the P-lipids could be cleaved by exposure of both sides of the membrane to P-lipase C without impairing the capacity for oxidative phosphorylation. It can therefore be concluded that the hydrophobic portion of these P-lipids is not essential for phosphorylation. On the other hand the diglycerides, which remain in the membrane after digestion with P-lipase C, cannot be removed without causing complete loss of phosphorylating activity. Whether the diglycerides are involved directly in the coupling mechanism or whether their removal results in a disorganization of the protein components cannot be stated.

Phospholipids and Membrane Structure—It is of considerable interest for our understanding of membrane structure that digestion of P-lipids by both P-lipase A and C can take place in membranes that are functionally active. It appears, therefore, that both the hydrophobic and the hydrophilic components of P-lipids in native membranes are available to the added lipolytic enzymes. Since from studies reported elsewhere (19) we consider it unlikely that proteins penetrate deeply into the membrane, we conclude that those P-lipids that can be digested are present on the surface. This conclusion does not necessarily conflict with a P-lipid bilayer model of the membrane, since we found that there is an appreciable amount of P-lipids which appears inaccessible to P-lipases from either side of the membrane. This fraction may represent the true bilayer contribu-

*A. Loyter and C. Burstein, unpublished observations.*
tion of P-lipids to the membrane and may fulfill the function of the central bilayer proposed in the Danielli-Davson model or of the ion-impermeable layer postulated by Mitchell (cf. Reference 16).

Acknowledgments—We wish to thank Mr. Richard Morris for technical assistance during the early phases of this work. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone was a gift from Dr. P. G. Heytler of DuPont.

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