J. Biochem., **69**, 155–167 (1971)

A Function of Cytochrome b_5 in Fatty Acid Desaturation by Rat Liver Microsomes

Nozomu OSHINO,* Yoshio IMAI and Ryo SATO
The Institute for Protein Research,
Osaka University, Osaka

Received for publication, June 8, 1970

On addition of NADPH to rat liver microsomes cytochrome b_5 assumes a steady-state reduction level, based on a balance between its reduction by NADPH and its autoxidation. Initiation of fatty acid desaturation by adding stearoyl-CoA (stearyl CoA) results in a rapid shift of the NADPH-supported steady state of the cytochrome in favor of more oxidation, and this shift is intensified by partially inhibiting microsomal NADPH-specific flavoprotein by $HgCl_2$. This suggests that increased utilization of reducing equivalents for desaturation is accompanied by a stimulation of reoxidation of cytochrome b_5. Actually, the magnitude of stearyl CoA-induced shift is roughly proportional to the fatty acid desaturation activity of the microsomes employed. Moreover, this shift is interfered with by cyanide which inhibits a terminal component ("cyanide-sensitive factor") of the desaturation system.

Since the reduction of microsomal bound cytochrome b_5 by NADH is much faster than the autoxidation of the cytochrome and the overall desaturation reaction, NADH causes complete reduction of cytochrome b_5 and no change in the reduction level is observed on addition of stearyl CoA. However, when NADH-cytochrome b_5 reductase is strongly inhibited by PCMS, stearyl CoA does lower the steady-state reduction level of cytochrome b_5 in NADH-treated microsomes. Stearyl CoA also stimulates the oxidation of cytochrome b_5 observable on exhaustion of the NADH added, and this stimulation is again prevented by cyanide.

With microsomes from which cytochrome b_5 has been removed to various extents by mild proteolytic digestion, the desaturation of stearyl CoA supported by ascorbate as electron donor is dependent on the content of remaining cytochrome b_5.

It is concluded that cytochrome b_5 in liver microsomes acts as an intermediary electron carrier which passes reducing equivalents from NADH, NADPH and ascorbate to the cyanide-sensitive factor where fatty acid desaturation *per se* probably takes place.

Microsomes prepared from rat epididymal adipose tissue contain a significant amount of cytochrome b_5, which plays a similar role in fatty acid desaturation.

* Present address: Johnson Research Foundation, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, U.S.A.

Liver microsomes catalyze the conversion of stearoyl CoA (stearyl CoA) and palmitoyl CoA (palmityl CoA) to corresponding Δ^9-monounsaturated fatty acids in a mono-oxygenase type reaction requiring both molecular oxygen and NADH or NADPH (*1–5*). Similar fatty acid desaturation also takes place in microsomes from adipose tissue (*3, 6*) and some other tissues (*7, 8*). The desaturation activity of liver microsomes has been studied extensively in relation to its responses to diabetes (*3, 9–11*), dietary conditions (*12–14*) and alterations in hormonal status (*9, 15–17*), but its enzymatic mechanism has not yet been fully understood.

In a previous paper (*4*), we reported several features of the enzymatic mechanism operating in the desaturation of stearyl CoA by rat liver microsomes. These include: *a*) In addition to NADH and NADPH, ascorbate can also serve as a weak electron donor. *b*) A cyanide-sensitive factor of unknown nature is involved in the system. *c*) The electrons from NADH, NADPH or ascorbate are utilized to reduce the cyanide-sensitive factor, which in the reduced form seems to act as the oxygen-activating site. *d*) Microsomal NADPH-specific flavoprotein, usually called NADPH-cytochrome *c* reductase (*18, 19*), is functional in the electron transfer from NADPH to the cyanide factor. *e*) Cytochrome P-450 (*20*), which is the key enzyme in may microsomal mono-oxygenase reactions (*21–23*), is not involved in the desaturation system. These findings have led us to suspect a possible role of cytochrome b_5 in the electron-transfer mechanism associated with fatty acid desaturation, because this cytochrome is the only microsomal component known to be reducible by all the three electron donors for desaturation.

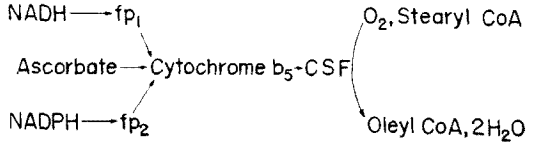

Fig. 1. Mechanism of stearyl CoA desaturation by rat liver microsomes. fp_1, NADH-cytochrome b_5 reductase; fp_2, NADPH-specific flavoprotein (NADPH-cytochrome *c* reductase); CSF, cyanide-sensitive factor.

This paper presents evidence that cytochrome b_5 in liver microsomes acts as an intermediary electron carrier which mediates the electron transport from NADP, NADPH and ascorbate to the cyanide-sensitive factor. This conclusion, coupled with current knowledge of the microsmal electron-transfer system, has permitted us to formulate a mechanism, shown in Fig. 1, for hepatic microsomal fatty acid desaturation. We also report that cytochrome b_5 is similarly involved in fatty acid desaturation by adipose tissue microsomes. A preliminary acount of this work has been presented (*24*).

EXPERIMENTAL PROCEDURE

Animals and Preparation of Microsomes– Male Sprague-Dawley rats, weighing 90 to 120 g, were fed a laboratory chow (Oriental Yeast Company, Osaka) for 7 to 10 days. Two days before sacrifice, the diet was removed at 4 pm and the animals were fasted until 4 pm the next day, when they were again allowed free access to the laboratory chow. The rats were killed at 10 am the next morning. These animals were referred to as "fed" rats. When microsomes of high desaturation activity were needed, the refeeding after 24 hr fasting was made on a high-carbohydrate diet, consisting of 62.9% dextrin, 15% sucrose, 15% casein, 4% salt mixture (Tanabe Amino Acid Research Foundation, Osaka), 2% cellulose powder, 1% vitamin mixture (Tanabe Amino Acid Research Foundation, Osaka), and 0.1% choline chloride. The animals thus treated were labeled "induced" rats and killed at 10 am as above. In some experiments, the refeeding was omitted and the animals were kept starved until sacrifice ("fasted" rats).

Liver microsomes were isolated from the above three types of rats as described previously (*4*) in 0.25 M sucrose containing 5 mM EDTA and 5 mM Tris-HCl buffer, pH 7.2. The activities of liver microsomes from "fed," "induced" and "fasted" rats to desaturate stearyl CoA were 0.4–3, 2–6 and 0.06–0.3 nmoles per min per mg of protein, respectively,

when NADPH was used as electron donor.

Epididymal adipose tissues were removed from "fed" and "induced" rats and homogenized with 9 volumes of 0.25 M sucrose. Microsomes were prepared from the homogenate under conventional centrifugal conditions.

Enzyme Assays—Desaturation of 1-^{14}C-stearyl CoA to oleate was assayed in 0.1 M Tris-HCl buffer, pH 7.2, as described previously (*4*), except that the reaction was started by adding 0.1 ml of a mixture containing buffer, ^{14}C-stearyl CoA and an electron donor (NADH, NADPH, NADPH-generating system, or ascorbate; final concentrations of pyridine nucleotide and ascorbate were 0.4 and 10 mM, respectively) to 0.4 ml of microsomal suspension in buffer, and incubation was carried at 30°C for 1.5 to 5 min. The concentration of microsomes and incubation time were selected so that the conversion of stearyl CoA to oleate would not exceed 40% to ensure the linearity of reaction. NADH- and NADPH-cytochrome *c* reductase activities were measured according to Dallner (*25*) and Phillips and Langdon (*19*), respectively, except that 0.1 M Tris-HCl buffer, pH 7.2, was used and the reaction was performed at 30°C. Cytochrome b_5 and P-450 were determined as described by Omura and Sato (*20*).

Measurements of Redox Behavior of Cytochrome b_5—The time course of oxidation-reduction reactions of cytochrome b_5 was followed mostly in a triple wavelength spectrophotometer constructed by Hagihara (*26*), though an Aminco-Chance dual wavelength spectrophotometer was also used in some experiments. In the Hagihara instrument, light beams of three different wavelengths are passed alternately through a sample cuvette (optical path, 1.0 cm), so that it is possible to record, as a function of time, the transmittance changes between two sets of wavelength pair in a turbid system. To follow the behavior of cytochrome b_5, the wavelengths were set at 555 nm (α-peak of reduced cytochrome b_5), 538 and 568 nm (isosbestic points ef the cytochrome). The transmittance change between 555 and 538 nm was recorded as a measure of the reduction level of cytochrome b_5, whereas that between 538 and 568 nm was simultaneously followed to check possible interference due to turbidity changes. However, no appreciable interference was detected in most experiments. When the Aminco-Chance spectrophotometer was nsed, the wavelengths were set at 424 nm (Soret peak of reduced cytochrome b_5) and 409 nm. The reaction mixture for these experiments contained a suitable amount of microsomes in 0.1 M Tris-HCl buffer, pH 7.2, and other necessary additions. The mixture was preincubated for 5 min to stabilize the turbidity fluctuation, and then recording was started at 30°C. Detailed experimental conditions are given in figure legends. Since the transmittance change recorded was usually less than 7%, it was regarded as proportional to the absorbance change.

Proteolytic Digestion of Microsomes—The digestion mixture contained liver microsomes from "fed" rats (25 mg of protein), 15 to 750 μg of crystalline *Bacillus subtilis* protease (Nagarse), 0.25 M sucrose, and 5 mM Tris-HCl buffer, pH 7.2, in a total volume of 3.0 ml. After incubation at 0°C for 15 hr under nitrogen, the mixture was transferred quantitatively to a centrifugal tube with the acid of 4 ml of 0.25 M sucrose-5 mM Tris-HCl buffer, pH 7.2, and centrifuged at 104,000 $\times g$ for 60 min. The pellet was resuspended in 0.25 M sucrose-5 mM Tris-HCl buffer, pH 7.2, and assayed for cytochrome b_5 and stearyl CoA desaturation.

Other Procedures—The other procedures used, including the method for protein determination, were conducted as described previously (*4*).

Biochemicals—Crystalline *B. subtilis* protease (Nagarse) was supplied from Nagase Company, Osaka. The γ-globulin fraction of rabbit antiserum against rat liver microsomal NADPH-spectific flavoprotein (NADPH-cytochrome *c* reductase) (*27*) and the γ-globulin fraction of rabbit antiserum against rat liver microsomal NADH-cytochrome b_5 reductase (*28*) were generous gifts from Dr. T. Omura. The sources of the other biochemicals were described in the previous paper (*4*).

RESULTS

Effect of Stearyl CoA on NADPH-reduced

Cytochrome b_5—When NADPH was added to an aerobic suspension of liver microsomes from "induced" rats, cytochrome b_5 was rapidly reduced as indicated by the upward deflection of the spectrophotometric trace in Fig. 2. The reduction did not, however, proceed to completion; instead, a steady state was established when about 80% of the total cytochrome was reduced. It was evident that this steady state was based on a balance between the rate of cytochrome b_5 reduction by NADPH and that of slow autoxidation of the cytochrome. Figure 2 also shows that the addition of a small amount of stearyl CoA at this stage of reaction caused an immediate decrease in the reduction level of cytochrome b_5 to a new steady state. The newly established steady state persisted only for a short time and gradually returned to the original 80% level, probably because of the consumption of the stearyl CoA in the desaturation reaction. A rough estimate based on the desaturation activity of the microsomes employed actually indicated that the stearyl CoA added could be desaturated within 1 min under these conditions. When all the NADPH added was used up, cytochrome b_5 was slowly brought back to the fully oxidized form owing to its autoxidizability. The observed shift of the steady state could be explained by assuming that the addition of stearyl CoA, *i.e.* the initiation of the desaturation reaction, stimulat-

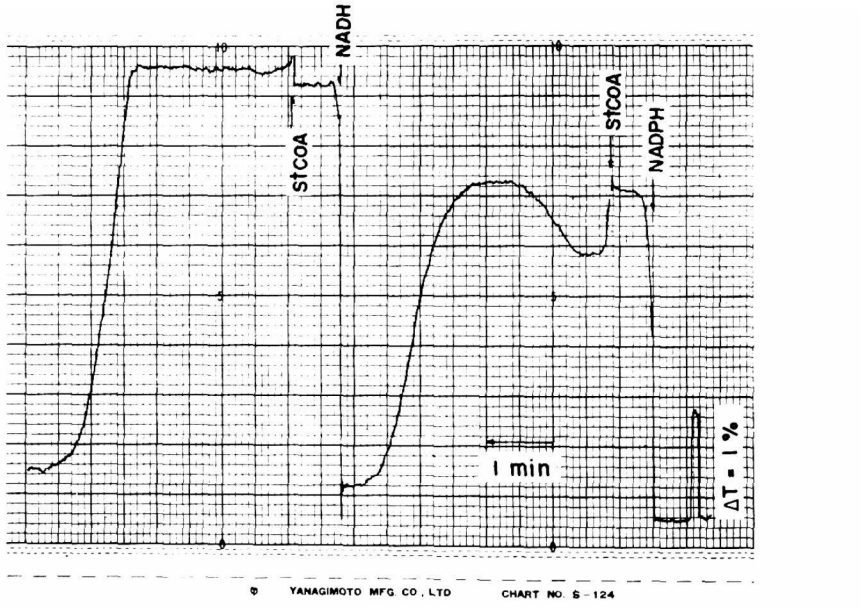

Fig. 2. Effect of stearyl CoA on steady-state reduction level of cytochrome b_5. The reaction mixture contained liver microsomes (2.4 mg of protein per ml) and 0.1 M Tris-HCl buffer (pH 7.3) in a volume of 2.5 ml. After preincubation at 30°C for 5 min, NADPH (79 nmoles), stearyl CoA (StCoA, 23.3 nmoles), NADH (91 nmoles) and again stearyl CoA (23.3 nmoles) were added in this order at the indicated time points. The redox behavior of cytochrome b_5 during the experiment was followed by recording the change in transmittance increment between 555 and 583 nm in the triple wavelength spectrophotometer as described in "EXPERIMENTAL PROCEDURE." The upward deflection of the trace represents the redcution of the cytochrome and time goes from right to left. The NADH- and NADPH-dependent desaturation activities of the microsomes used (from "induced" rats) were 7.1 and 6.2 nmoles of oleate formed per min per mg of protein, respectively, at 30°C.

ed the reoxidation of cytochrome b_5.

The ability to induce the decrease in the reduction level of cytochrome b_5 seemed to be specific for substrates of the desaturation system. Thus, the addition of stearate, oleate and oleyl CoA did not induce any change.

The left-hand side of Fig. 2 shows that NADH caused complete reduction of cytochrome b_5 and no effect was observable on addition of stearyl CoA. This finding will be discussed in a later section.

Effect of $HgCl_2$ on Stearyl CoA-induced Shift of Steady State—Since it has recently been shown that the reduction of microsomal bound cytochrome b_5 by NADPH is mediated by microsomal NADPH-specific flavoprotein (*27*), it was expected that partial inhibition of this flavoprotein would lead to a more intensive effect of stearyl CoA on the reduction level of cytochrome b_5. For this purpose, we used 0.032 mM $HgCl_2$ which caused partial inhibition of the NADPH-cytochrome *c* reductase activity of the flavoprotein as well as the NADPH-dependent desaturation of stearyl CoA, but did not affect the NADH- and ascorbate-dependent desaturation reactions.

It was thus found as expected that in the presence of 0.032 mM $HgCl_2$ the reduction of cytochrome b_5 by NADPH was slower and the steady-state reduction level was lower (about 60% reduction) than in the absence of $HgCl_2$ (Fig. 3). Furthermore, the shift induced by stearyl CoA was much more pronounced and the time required for consumption of the stearyl CoA was longer. It was also noticed that the onset of autoxidation of cytochrome b_5 was also delayed, because of slower utilization of NADPH under these conditions

The addition of anti-NADPH-specific flavoprotein antibody (the γ-globulin fraction of immunized rabbit serum) (*27*) to the system led to qualitatively similar events to the case of $HgCl_2$-treated microsomes. NADH reduced cytochrome b_5 completely even in the presence of $HgCl_2$ and stearyl CoA showed no effect.

Correlation between Stearyl CoA Effect and Desaturation Activity—It was found that there was a good correlation between the extent of stearyl CoA-induced shift of the steady state and the desaturation activity of the microsomal preparation employed. Thus, when liver microsomes from "fasted" rats, possessing a negligible desaturation activity, were used, stearyl CoA caused no change in the reduction level of cytochrome b_5 even in the presence of $HgCl_2$ (Fig. 4). With liver micro-

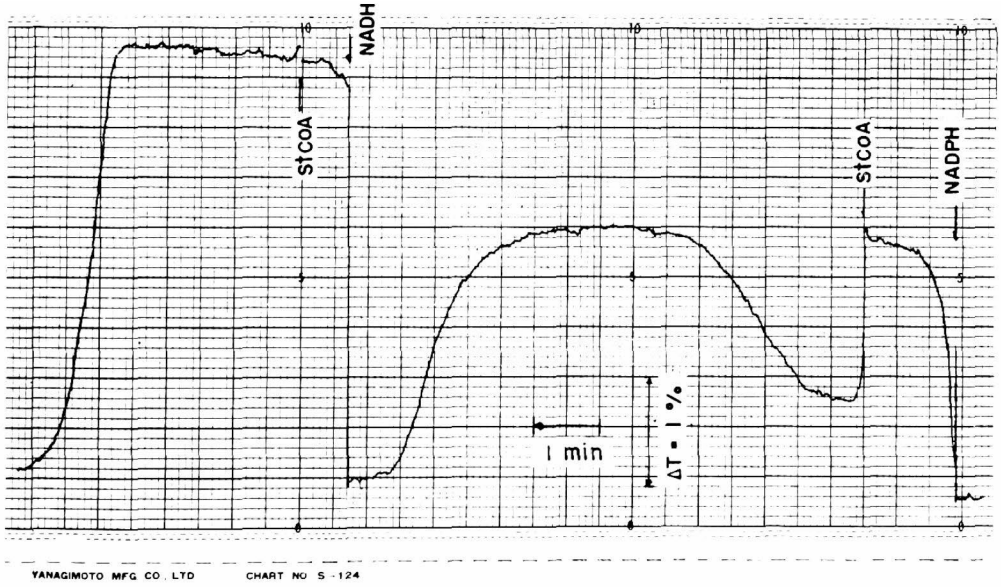

Fig. 3. Effect of stearyl CoA on steady-state reduction level of cytochrome b_5 in the presence of $HgCl_2$. The reaction conditions were exactly the same as in Fig. 2, except that 0.032 mM $HgCl_2$ was included in the reaction mixture.

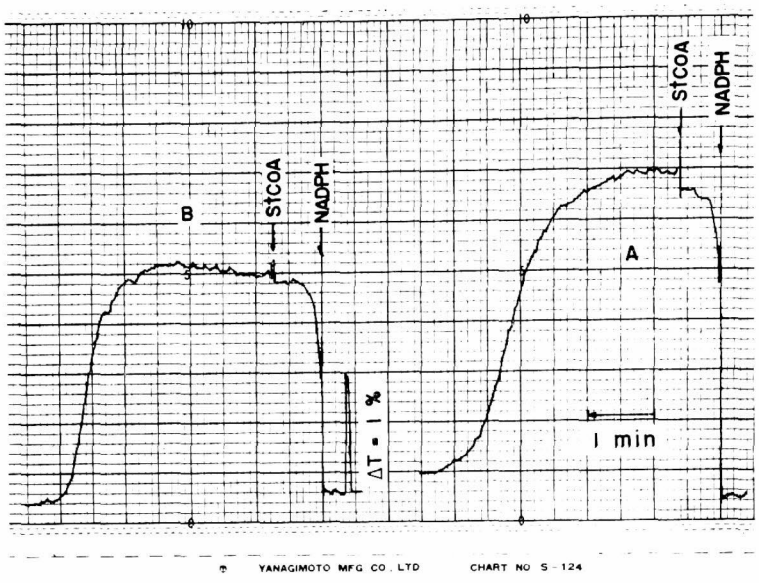

Fig. 4. Lack of effect of stearyl CoA on NADPH-supported reduction level of cytochrome b_5 in liver microsomes from "fasted" rats. The experiments were carried out essentially as described in Figs. 2 and 3, except that microsomes from "fasted" rats (2.2 mg of protein per ml) were used. The NADPH-dependent desaturation activity of the microsomes was 0.36 nmole of oleate formed per min per mg of protein at 30°C. *Experiment A*, in the absence of $HgCl_2$; *Experiment B*, in the presence of 0.032 mM $HgCl_2$.

somes prepared from "fed" rats, having a desaturation activity intermediary between "induced" and "fasted" microsomes, only a very slight decrease was observed and the effect was intensified by $HgCl_2$.

It seemed possible to explain all the results described so far by assuming that the addition of stearyl CoA to aerobic microsomes in the presence of NADPH to initiate the desaturation reaction stimulates the reoxidation of cytochrome b_5 and the stimulation is proportional to the desaturation activity. In other words, the reducing equivalents required for the desaturation site seems to be supplied from NADPH *via* cytochrome b_5.

Effect of Cyanide on Stearyl CoA Effect—We have previously reported that a cyanide-sensitive factor is involved, probably as the common oxygen-activating site, in all the NADH-, NADPH- and ascorbate-dependent desaturation reactions, and suggested that it is located in the terminal region of the desaturation system, probably after cytochrome b_5 which does not bind cyanide (*4*). This allowed the prediction that cyanide would inhibit the effect of stearyl CoA on cytochrome b_5. As shown in Fig. 5, the addition of NADPH to liver microsomes from "induced" rats in the presence of 0.02 to 4 mM KCN rapidly reduced cytochrome b_5 until the same steady-state level as in the absence of cyanide was reached. Contray to expectation, however, the addition of stearyl CoA at this stage of reaction induced as rapid decrease in the reduction level as in the absence ef cyanide, though sufficient concentrations of KCN were present to inhibit the desaturation activity almost completely. However, the steady state thus lowered returned to the original level within a much shorter period of time than expected from the amount of stearyl CoA added. Furthermore, the higher the cyanide concentration, the shorter was the time required for restoration of the original level. These results could be accounted for by assuming that the binding of cyanide to the

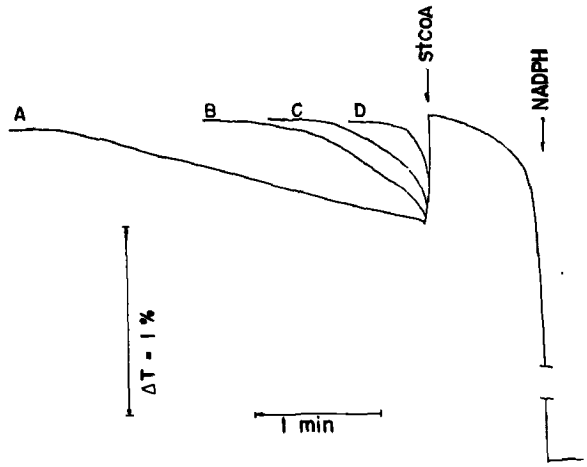

Fig. 5. Effect of KCN on stearyl CoA-induced shift of reduction level of cytochrome b_5. The reaction mixture contained liver microsomes (2.5 mg of protein per ml), 0.1 M Tris-HCl buffer (pH 7.2), and KCN (for concentration, see below) in a volume of 2.5 ml. After preincubation for 5 min at 30°C, NADPH (156 nmoles) and stearyl CoA (StCoA, 46.6 nmoles) were added in this order at the indicated time points. The redox behavior of cytochrome b_5 was followed in the triple wavelength spectrophotometer. The microsomes used (from "induced" rats) had a NADPH-dependent desaturation activity of 2.4 nmoles of oleate formed per min per mg of protein at 30°C. The KCN concentrations used were 0.02 mM (*Curve A*), 0.5 mM (*Curve B*), 1.0 mM (*Curve C*), and 4.0 mM (*Curve D*).

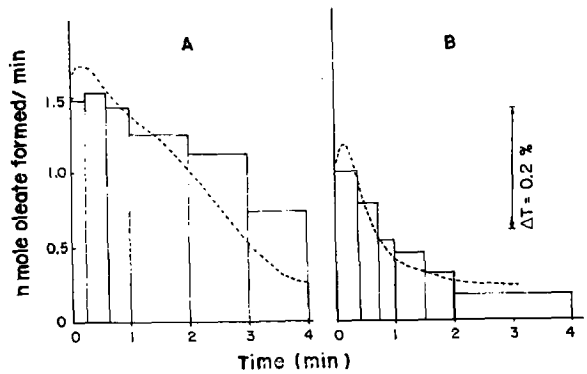

Fig. 6. Correlation between stearyl CoA-induced spectral change and rate of oleate formation. The spectrophotometric experiments were conducted as described in Fig. 5 both in the presence and absence of 0.5 mM KCN. Upward deflection of dotted line shows the *decrease* in the steady-state reduction level of cytochrome b_5. Stearyl CoA was added to the mixture at time zero. NADPH-dependent desaturation of stearyl CoA was measured under essentially the same conditions as for the spectrophotometric measurements, except that the total volume was scaled down to 0.5 ml and microsomal concentration was 1.2 mg of protein per ml. *Experiment A*, without KCN; *Experiment B*, with KCN.

cyanide-sensitive factor is a relatively slow reaction which takes place only when the substrate is supplied. At any rate, it could be concluded that the cyanide-sensitive factor was actually located after cytochrome b_5. Azide showed an effect similar to cyanide, though less effective than the latter.

Correlation between Shift of Reduction Level and Oleate Formation—To confirm that the decrease in the reduction level of cytochrome b_5 is actually due to an increased utilization for the desaturation reaction of reducing equivalents pooled in the cytochrome, an experiment was designed to correlate the reduction level and the formation of oleate from stearyl CoA. The results are shown in Fig. 6. In this figure, the upward deflection of the dotted line represents the decrease in the steady-state reduction level of cytochrome b_5 recorded in the spectrophotometer, and the vertical bars show the amounts of oleate (nmoles per min) formed during the indicated time interval. Time zero is the time when stearyl CoA was added to NADPH-treated microsomes under aerobic conditions. For technical reasons, the concentration of microsomes used for the desaturation assay was reduced to half that used for the spectrophotometric measurement. Therefore, the restoration of the original level was attained before oleate formation ceased. Nevertheless, it could be seen that there was a beautiful parallelism between the rate of oleate formation and the change in the reduction level of cytochrome b_5 during the process of characteristic inhibition by cyanide. In the presence of cyanide the parallelism was more clear. It could be concluded that the change in the reduction level of cytochrome b_5 was a real reflection of the desaturation activity.

Involvement of Cytochrome b_5 in NADH-dependent Desaturation—Demonstration of the involvement of cytochrome b_5 in the NADH-dependent desaturation by observing the

spectrophotometric effect of stearyl CoA was more difficult than the case of NADPH as electron donor, because the reduction of microsomal bound cytochrome b_5 by NADH is very fast. Since this reduction proceeds several hundred times faster than the autoxidation of cytochrome b_5, the addition of NADH to aerobic liver microsomes brought the cytochrome to the practically fully reduced state almost instantaneously. The addition of stearyl CoA to such a NADH-treated suspension of microsomes resulted in no change in the reduction level of cytochrome b_5 (Fig. 2). The same was true even in the presence of 0.032 mM $HgCl_2$ which inhibited the NADH-cytochrome b_5 reductase activity considerably (Fig. 3), suggesting that the remaining NADH-cytochrome b_5 reductase activity was still much faster than the utilization of reducing equivalents for desaturation. The use of higher concentrations of $HgCl_2$ was impractical because of alteration of turbidity and the possible inhibition of the terminal region of the desaturation system.

After preliminary search, we found that 0.032 mM *p*-chloromercuribenzenesulfonate (PCMS) could inhibit microsomal NADH-cytochrome b_5 reductase, measured with external cytochrome *c* as acceptor, by about 99% and thus made the rate of cytochrome b_5 reduction by NADH comparable to that of overall desaturation. In the presence of 0.032 mM PCMS, it was possible to demonstrate clearly the stearyl CoA-induced decrease in the steady state in NADH-treated microsomes (Fig. 7). It was also noticed that the reduction level of cytochrome b_5 induced by NADH under these conditions was about 90% of full reduction, indicating that the cytochrome b_5 reduction by NADH in the presence of PCMS was still faster than the autoxidation of the cytochrome. It is of interest to note that the stearyl CoA-induced change in the NADPH-supported reduction level in the presence of

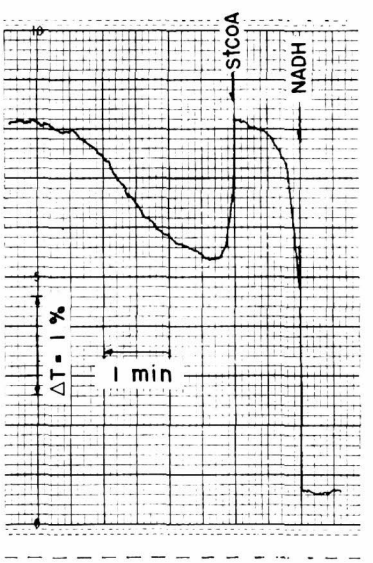

Fig. 7. Effect of stearyl CoA on NADH-supported reduction level of cytochrom b_5 in the presence of PCMS. The reaction conditions were the same as described in Fig. 2, except that 0.032 mM PCMS was included in the reaction mixture and 272 nmoles of NADH was added as indicated.

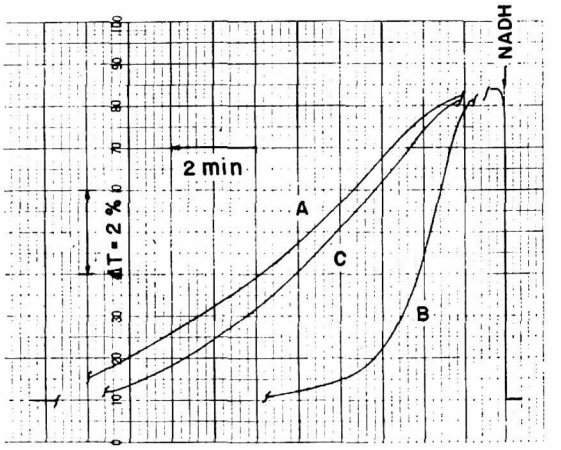

Fig. 8. Stimulation of cytochrome b_5 reoxidation by stearyl CoA and its inhibition by cyanide. The reaction mixture contained, in a final volume of 3.0 ml, liver microsomes from "induced" rats (0.6 mg of protein per ml), 0.1 M Tris-HCl buffer (pH 7.2), 0.2 mM freshly dissolved Na_2S, and if necessary, 0.5 mM KCN. After preincubation at 30°C for 4 min, NADH (final concentration, 0.7 μM) or NADH plus 7 μM stearyl CoA was added. The reoxidation phase after rapid reduction of cytochrome b_5 was followed by recording the change in transmittance increment between 424 and 409 nm, using an Aminco-Chance dual wavelength spectrophotometer. Upward deflection of the trace represents the reduction of cytochrome b_5. *Curve A*, autoxidation in the absence of both stearyl CoA and cyanide; *Curve B*, reoxidation in the presence of stearyl CoA; *Curve C*, reoxidation in the presence of both stearyl CoA and cyanide.

0.032 mM PCMS was not so pronounced as in the NADH-treated system under comparable conditions. This would indicate that NADH-cytochrome b_5 reductase is more sensitive to PCMS than the NADPH-specific flavoprotein.

Direct Demonstration of Stimulation of Cytochrome b_5 Oxidation by Stearyl CoA—So far, the stimulation of cytochrome b_5 reoxidation by stearyl CoA has been suggested based on the shift of the steady state reduction level of the cytochrome. However, it was also possible to demonstrate the stimulation in a more direct way by following the oxidation of the cytochrome upon complete consumption of the added electron donor. As mentioned above, the addition of a small amount of NADH to aerobic microsomes (from "induced" rats) resulted in almost complete reduction of cytochrome b_5. When all the NADH added was oxidized, the cytochrome underwent slow autoxidation according to the first-order kinetics. As shown in Fig. 8, this oxidation was accelerated greatly if the system contained a sufficient concentration of stearyl CoA. Furthermore, 0.5 mM KCN could prevent this acceleration almost completely. In these experiments, 0.2 mM Na_2S was included in the system to inhibit the cytochrome oxidase activity of contaminating mitochondria; this concentration of sulfide had no effect on the NADH-dependent desaturation and on the autoxidation of cytochrome b_5. Similar phenomena could also be observed with NADPH as electron donor, but in this case the inhibition of the NADPH-specific flavoprotein by $NADP^+$ accumulated (*19*) resulted in a somewhat peculiar kinetics of cytochrome b_5 oxidation. The γ-globulin fraction of rabbit antiserum against rat NADH-cytochrome b_5 reductase (*28*) was found to inhibit the NADH-dependent desaturation activity.

The results described in this and previous sections were consistent with the view that the reducing equivalents fed to cytochrome b_5 from NADH (*via* NADH-cytochrome b_5 reductase) was transferred to the cyanide-sensitive factor to be finally utilized for the desaturation process.

Involvement of Cytochrome b_5 in Ascorbate-dependent Desaturation—Since ascorbate can reduce microsomal bound cytochrome b_5 partially (*20*), it seemed likely that the cytochrome is also involved in the weak ascorbate-dependent desaturation activity. However, the very low reduction level of the cytochrome attained by ascorbate made it rather difficult to demonstrate this involvement spectrophotometrically. The reduction levels of cytochrome b_5 in the steady states established in aerobic microsomes (pH 7.2) in the presence of 1 mM NADH, 1 mM NADPH and 10 mM ascorbate were 100, 75–80 and 15–25%, respectively. These values correlated well with the relative specific activities for stearyl CoA desaturation by the same microsomes under comparable conditions, namely 100, 80 and 20 for the NADH-, NADPH- and ascorbate-dependent reactions, respectively. It was also found that the steady-state reduction level of cytochrome b_5 in the presence of 10 mM ascorbate increased from 15 to 40%

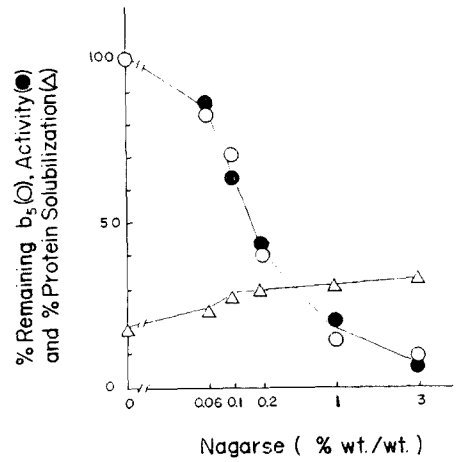

Fig. 9. Correlation between ascorbate-dependent desaturation and concentration of remaining cytochrome b_5 after proteolytic digestion of liver microsomes. Microsomes from "fed" rats were digested with indicated amount of Nagarse as described in "EXPERIMENTAL PROCEDURE." The stearyl CoA desaturation activity with 10 mM ascorbate as electron donor (30°C, 4 min) (○) and the content of cytochrome b_5 (●) in the Nagarse-treated pellet as well as the percent solubilization of the total microsomal protein (△) were measured. The NADH- and NADPH- and ascorbate-dependent stearyl CoA desaturation activities of the microsomes used were 3.8, 3.7 and 0.5 nmoles of oleate formed per min per mg of protein, respectively, at 30°C.

when the pH was raised from 7.0 to 8.0, because of the decrease in the velocity of cytochrome b_5 autoxidation. Keeping pace with this, the ascorbate-dependent desaturation activity increased as the pH was raised and reached maximum at about pH 7.8. In contrast, both the NADH- and NADPH-dependent activities showed a broad pH optimum around 7.0 (*4*).

To confirm further the involvement of cytochrome b_5 in the ascorbate-dependent desaturation, an attempt was made to correlate the desaturation activity with the amount of cytochrome b_5 remaining after mild proteolytic digestion of microsomes. It has been shown that anaerobic treatment of liver microsomes with increasing concentrations of *B. subtilis* protease (Nagarse) caused increasing solubilization of the cytochrome (*29*). Using various microsomal preparations from which cytochrome b_5 had been removed to varying degrees, it was possible to show that the ascorbate-dependent desaturation of stearyl CoA was strictly proportional to the amount of remaining cytochrome b_5 (Fig. 9). The protease treatment also solubilized the NADPH-specific flavoprotein up to more than 90%, though most of the NADH-cytochrome b_5 reductase activity remained attached to the membrane (*29*). It was unexpectedly found that the NADH- and NADPH-dependent desaturation activities retained 60 to 70% of the original values even after proteolytic removal of more than 90% of both cytochrome b_5 and the NADPH-specific flavoprotein. It is possible that less than 10% of the NADPH-flavoprotein remaining in these microsomes could still provide sufficient reducing equivalents from NADPH to the desaturation site, but the lack of parallelism between these activities and the content of remaining cytochrome b_5 is puzzling. At any rate, proteolytic attack under these conditions did not seem to have impaired the terminal desaturation site significantly.

Behavior of Cytochrome b_5 in Desaturation by Adipose Tissue Microsomes—The stearyl CoA desaturation activity has been detected not only in liver micsosomes but also in adipose tissue microsomes (*3, 6*). As shown in Table I, microsomes prepared from rat epididymal adipose tissue contained a significant amount of cytochrome b_5 as well as NADH- and NADPH-cytochrome *c* reductase activities. Cytochrome b_5 was reducible by both NADH and NADPH. However, neither cytochrome P-450 nor aniline hydroxylation activity could be detected. High activities of stearyl CoA desaturation were demonstrated using both NADH and NADPH as electron donors. It is interesting to note that feeding of the high-carbohydrate diet also induced the desaturation activity of adipose tissue microsomes. Spectrophotometric studies indicated that adipose tissue cytochrome b_5 behaved similarly to the hepatic counterpart in connection to the desaturation activity. As shown in Fig. 10, for example, the addition of a small amount of NADPH to adipose tissue microsomes resulted in the establishment of a steady-state reduction level of cytochrome b_5. Further addition of stearyl CoA caused an immediate shift of the steady state in favor of more oxidation, followed by gradual resto-

TABLE I. Cytochrome b_5 content and some enzymatic activities in epididymal adipose tissue microsomes prepared from "fed" and "induced" rats. The content of cytochrome b_5 is expressed in terms of nmoles per mg of microsomal protein. NADH- and NADPH-cytochrome *c* reductase activities are nmoles of cytochrome *c* reduced per min per mg of microsomal protein. Stearyl CoA desaturation activity was measured with NADH as electron donor and is expressed as nmoles of oleate formed per min per mg of protein.

Type of microsomes	Cytochrome b_5	NADH-cyt. *c* reductase	NADPH-cyt. *c* reductase	Stearyl CoA desaturation
"Fed"	0.07	541	28	2.3
"Induced"	0.13	1675	71	7.9

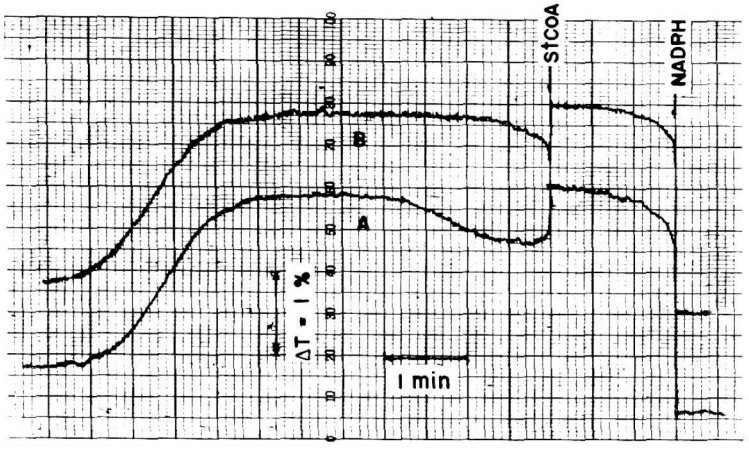

Fig. 10. Effect of stearyl CoA on NADPH-supported reduction level of cytochrome b_5 in adipose tissue microsomes and its inhibition by cyanide. The microsomes used were prepared from adipose tissues of "induced" rats. The reaction mixture contained, in a final volume of 3.0 ml, microsomes (0.5 mg of protein per ml), 0.1 M Tris-HCl buffer (pH 7.2), 0.2 mM Na_2S, and, if necessary, 1 mM KCN. After preincubation for 4 min at 30°C, 100 nmoles of NADPH and 13 nmoles of stearyl CoA were added in this order at indicated time points. The redox behavior of cytochrome b_5 was followed in the Amino-Chance spectrophotometer as in Fig. 8. *Experiment A*, without cyanide; *Experiment B*, with cyanide.

ration of the original reduction level. These responses were quite similar to those observed with liver microsomes (Fig. 2). Furthermore, the presence of 1 mM KCN in the system caused much faster restoration of the original level also as in the case of liver microsomes (Fig. 5). It was thus concluded that cytochrome b_5 is playing a role as an electron carrier and a cyanide-sensitive factor is involved also in the reaction by adipose tissue microsomes.

DISCUSSION

We have previously shown that the supply of electrons from NADH, NADPH or ascorbate to a cyanide-sensitive factor is essential for oxidative desaturation of stearyl CoA by rat liver microsomes (*4*). The results described here indicate that stearyl CoA stimulates the reoxidation of microsomal bound cytochrome b_5 concomitant with the desaturation of the stearate derivative. This stimulation of cytochrome b_5 reoxidation is interfered with by cyanide in a characteristic way. These findings, together with the good correlation between the desaturation activity and the extent of stearyl CoA-induced stimulation, strongly suggest that cytochrome b_5 is involved in the desaturation process as an electron carrier, which passes electrons from the donors to the cyanide-sensitive factor to be utilized there for the desaturation reaction *per se*. The electron transport from NADH and NADPH to cytochrome b_5 is mediated by their respective flavoproteins, whereas ascorbate reduces the cytochrome directly. If this is really the case, the flow of electrons in the desaturation system may be formulated as shown in Fig. 1. The results reported by Jones *et al.* (*5*) on the lipid requirement for microsomal stearyl CoA desaturase can also be readily interpreted by assuming this mechanism.

Although most of the results reported in this paper are consistent with the above conclusion, a puzzling observation is that the NADH- and NADPH-dependent desaturation activities, but not the ascorbate-dependent

activity, of liver microsomes retained 60 to 70% of the original values even after proteolytic removal of 95% of cytochrome b_5. However, it seems likely that the proteolytic treatment has caused considerable alterations in the membrane conformation and thus created artificial routes of electrons, by which the two flavoproteins can interact directly with the cyanide-sensitive factor. Alternately, the change in the membrane structure may have enabled the small amount of cytochrome b_5 to act more effectively as electron carrier than in intact microsomes.

The role of cytochrome b_5 in fatty acid desaturation is not confined to liver microsomes, but can also be demonstrated in adipose tissue microsomes. In this connection, it is of interest to note that cytochrome b_5 occurs also in microsomes from intestinal mucosa (*30, 31*) and milk (*32*), both of which have been reported to carry out the same type of desaturation (*7, 8*). Oxidative desaturation of fatty acyl CoA's also takes place in particulate fractions of yeast cells (*33*), which contains a pigment called "cytochrome b_1" (*34*). This hemoprotein is spectrally almost identical with cytochrome b_5 (*35*). Essential requirements of a flavin and ferrous iron have been shown for a particulate enzyme system from *Mycobacterium phlei* (*36*). It would be of interest to look for the presence of a *b*-type cytochrome in this system.

Euglena gracilis grown autotrophically in the light has a quite different system for oxidative fatty acid desaturation (*37, 38*). This system is soluble and utilizes acyl carrier protein derivatives of fatty acids as substrates. It consists of three components, *i.e.* a NADPH-specific flavoprotein, a ferredoxin, and a terminal "desaturase," but the last-mentioned component has not yet been characterized. When grown heterotrophically in the dark, this organism acquires a particulate system for oxidative desaturation; this system requires fatty acyl CoA's as substrates (*37*). The various reports mentioned above suggest that the mechanism of oxidative fatty acid desaturation is multifold and that the membrane-bound system involving a *b*-type cytochrome is most highly developed.

Little is as yet known of the nature and mode of action of the cyanide-sensitive factor which is the key enzyme of the microsomal desaturation system. Its sensitivity to cyanide suggests that it contains a heavy metal, which in the reduced form activates oxygen for the desaturation process. However, direct evidence for this is as yet lacking. The data of Fig. 5 suggest that the factor can react with cyanide at a relatively slow rate only when the substrate (stearyl CoA) is supplied to the system. It seems likely that the factor is embedded in a hydrophobic region of membrane, to which cyanide is not accessible. The reaction of cyanide with the factor probably takes place when stearyl CoA causes certain disturbances in the hydrophobic region because of its detergent action.

The rate of cytochrome b_5 reduction by NADH in rat liver microsomes is of the order of 1,000 nmoles per min per mg of protein, as estimated from the NADH-cytochrome *c* reductase activity, and the rate constant for cytochrome b_5 reduction by NADPH has been reported to be about 1 sec^{-1} (*27*). In contrast, the activity to desaturate stearyl CoA is at most less than 10 nmoles of oleate formed per min per mg of protein even in highly induced microsomes. Thus, the capacity of the microsomal system to supply reducing equivalents to cytochrome b_5 is in much surplus compared with the need for the desaturation of saturated fatty acyl CoA's. This suggests that the reducing equivalents pooled in cytochrome b_5 can also be utilized for other metabolic reactions. The most likely candidate for such a reaction is the oxidative formation of polyunsaturated fatty acids. The system responsible for this reaction also resides in liver microsomes (*39, 40*), but has been shown to differ from that catalyzing the formation of monounsaturated acids (*41, 42*). The two systems are, however, similar to each other in the requirements of both oxygen and NADH or NADPH (*39*). Moreover, both reactions are sensitive to cyanide and azide (*40*).

Recently, Miller *et al.* (*43*) have shown that oxidative demethylation of lanosterol by liver microsomes, a step in cholesterol biosynthesis, requires both oxygen and NADH or

NADPH. Furthermore, Gaylor and Mason (*44*) have reported that this reaction is sensitive to cyanide and does not involve P-450. Despite the suggestion of these authors against the participation of cytochrome b_5, it appears possible that lanosterol demethylation also involves both cytochrome b_5 and a cyanide-sensitive factor. It is interesting in this connection that proteolytic removal of cytochrome b_5 does not lower the demethylase activity significantly as in the case of fatty acid desaturation.

This work was supported in part by a grant-in-aid from the Toyo Rayon Science Foundation, for which the authors' gratitude is due. The authors are also indebted to Dr. B. Hagihara for his kindness in permitting us to use the triple wavelength spectrophotometer and for his help in operation of the instrument.

REFERENCES

1. J.B. Marsh and A.T. James, *Biochim. Biophys. Acta*, **60**, 320 (1962).
2. R.W. Holloway, R. Peluffo and S.J. Wakil, *Biochem. Biophys. Res. Commun.*, **12**, 300 (1963).
3. A. Gellhorn and W. Benjamin, *Biochim. Biophys. Acta*, **84**, 167 (1964).
4. N. Oshino, Y. Imai and R. Sato, *Biochim. Biophys. Acta*, **128**, 13 (1966).
5. P.D. Jones, P.W. Holloway, R.O. Peluffo and S.J. Wakil, *J. Biol. Chem.*, **244**, 744 (1969).
6. G.M. Kellerman and D.J. Jollow, *Biochim. Biophys. Acta*, **84**, 478 (1964).
7. R.D. McCarthy, F.L.A. Ghiardi and S. Patton, *Biochim. Biophys. Acta*, **98**, 216 (1965).
8. P. Boucrot and J. Clement, *Arch. Sci. Physiol.*, **19**, 181 (1965).
9. Y. Imai, *J. Biochem.*, **49**, 642 (1961).
10. W. Benjamin and A. Gellhorn, *J. Biol. Chem.*, **239**, 64 (1964).
11. A. Gellhorn and W. Benjamin, *Science*, **146**, 1166 (1964).
12. J. Elvson, *Biochim. Biophys. Acta*, **106**, 291 (1965).
13. P.H.W. Butterworth, R.B. Guchhait, H. Baum, E.B. Olson, S.A. Margolis and J.W. Porter, *Arch. Biochem. Biophys.*, **116**, 453 (1966).
14. M. Uchiyama, M. Nakagawa and S. Okui, *J. Biochem.*, **62**, 1 (1967).
15. A. Gellhorn and W. Benjamin, *Biochim. Biophys. Acta*, **116**, 460 (1966).
16. A. Gellhorn and W. Benjamin, *Ann. N.Y. Acad. Sci.*, **131**, 344 (1965).
17. D. Gompertz and A.L. Greenbaum, *Biochim. Biophys. Acta*, **116**, 441 (1966).
18. C.H. Williams and H. Kamin, *J. Biol. Chem.*, **237**, 587 (1962).
19. A.P. Phillips and R.G. Langdon, *J. Biol. Chem.*, **237**, 2652 (1962).
20. T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2370 (1964).
21. T. Omura, R. Sato, D.Y. Cooper, O. Rosenthal and R.W. Estabrook, *Federation Proc.*, **124**, 1181 (1965).
22. D.Y. Cooper, S. Levin, S. Narasimhulu, O. Rosenthal and R.W. Estabrook, *Science*, **147**, 400 (1965).
23. S. Orrenius, J. Ericsson and L. Ernster, *J. Cell Biol.*, **25**, 627 (1965).
24. N. Oshino, Y. Imai and R. Sato, *Abstracts, 7th Intern. Congr. Biochem. Tokyo*, p. 725 (1967).
25. G. Dallner, *Acta Pathol. Microbiol. Scand.*, Suppl. 166 (1963).
26. B. Hagihara, *Tampakushitsu Kakusan Koso*, **13**, 370 (1968) (in Japanese).
27. T. Omura, *Seikagaku*, **39**, 756 (1967) (in Japanese).
28. S. Takesue and T. Omura, *J. Biochem.*, **67**, 267 (1970).
29. A. Ito and R. Sato, *J. Cell Biol.*, **40**, 179 (1969).
30. M. Bailis and R.K. Morton, *Nature*, **175**, 749 (1955).
31. Y. Takesue and R. Sato, *J. Biochem.*, **64**, 873 (1968).
32. M. Bailis and R.K. Morton, *Biochem. J.*, **69**, 35 (1958).
33. D.K. Bloomfield and K. Bloch, *J. Biol. Chem.*, **235**, 337 (1960).
34. K. Ishidate, K. Kawaguchi, K. Tagawa and B. Hagihara, *J. Biochem.*, **65**, 375 (1969).
35. Y. Yoshida and H. Kumaoka, *Biochim. Biophys. Acta*, **189**, 461 (1969).
36. A.J. Fulco and K. Bloch, *J. Biol. Chem.*, **239**, 994 (1964).
37. J. Nagai and K. Bloch, *J. Biol. Chem.*, **241**, 1925 (1966).
38. J. Nagai and K. Bloch, *J. Biol. Chem.*, **243**, 4626 (1968).
39. D.H. Nugteren, *Biochim. Biophys. Acta*, **60**, 656 (1962).
40. W. Stoffel and H.-G. Schiefer, *Z. physiol. Chem.*, **345**, 41 (1966).
41. R.R. Brenner and P.O. Peluffo, *J. Biol. Chem.*, **241**, 5213 (1966).
42. C.A. Inkpen, R.H. Harris and F.W. Quackenbush, *J. Lipid Res.*, **10**, 277 (1969).
43. W.L. Miller, M.E. Kalfer, J.L. Gaylor and C.V. Delwiche, *Biochemistry*, **6**, 2673 (1967).
44. J.L. Gaylor and H.S. Mason, *J. Biol. Chem.*, **243**, 4966 (1968).