Date: 9/5/80

Project Title: Studies on Fatty ACYL COA Dehydrogenase and ETF

Project No: G-33-K03

Project Director: Dr. Carole L. Hall

Sponsor: DHEW/PHS/NIH - National Institute of General Medical Sciences; Bethesda, MD 20014

Agreement Period: From 7/1/80 Until 6/30/81 (03 year)

Type Agreement: Grant No. 5 RO1 GM25494-03

Amount: $64,011 PHS Funds (G-33-K02)
     3,682 GIT Contribution (G-33-324)
   $67,693 Total

Reports Required: Annual Progress Reports with Continuation Applications; Terminal Progress Report upon Grant Expiration

Sponsor Contact Person (s):

Technical Matters	Contractual Matters (thru OCA)

Program Official
Arthur E. Heming, Ph.D.
Associate Director for Program Activities
National Institute of General Medical Sciences
Bethesda, MD 20014
	Grants Management Official
	Ms. Evelyn W. Carlin
	Grants Management Officer
	Office of Associate Director for
	  Program Activities
	National Institute of General
	  Medical Sciences
	Bethesda, MD 20014

Program Administrator: Dr. Marvin Cassman	
Phone: 301/496-7463	Grants Management Specialist:

NOTE: FOLLOW-ON PROJECT TO G-33-K02 (02 YEAR) Ms. Ruth C. Monaghan/Linda V. Glen

Defense Priority Rating: None

301-496-7746

Assigned to: Chemistry   (School/Laboratory)

COPIES TO:

Project Director
Division Chief (EES)
School/Laboratory Director
Dean/Director—EES
Accounting Office
Procurement Office
Security Coordinator (OCA)
Reports Coordinator (OCA)

Library, Technical Reports Section
EES Information Office
EES Reports & Procedures

Project Code (GTRI)
Other   C. E. Smith

SPONSORED PROJECT TERMINATION

Date: 4/3/81

Project Title: Studies on Fatty ACYL COA Dehydrogenase and ETF

Project No: G-33-K03

Project Director: Dr. Carole L. Hall

Sponsor: DHEW/PHS/NIH - National Institute of General Medical Sciences
5 R01 GM25494-03

Effective Termination Date: 6/30/81 (end of 03 year)

Clearance of Accounting Charges: ____

Grant/Contract Closeout Actions Remaining:

- _ Final Invoice and Closing Documents
- _ Final Fiscal Report
- _ Final Report of Inventions
- _ Govt. Property Inventory & Related Certificate
- _ Classified Material Certificate
- x Other Report of Expenditures due by 9/30/81

NOTE: Continued by G-33-K04

Assigned to: Chemistry (School/Laboratory)

COPIES TO:

Administrative Coordinator
Research Property Management
Accounting Office
Procurement Office
Research Security Services
Reports Coordinator (OCA)

Legal Services (OCA)
Library, Technical Reports
EES Research Public Relations (2)
Project File (OCA)
Other: ____

Hall, Carole L.

303-42-9705

transfer nature of these ligands. No evidence was seen for electron transfer from acetoacetyl CoA to G-AD (Benecky et al,1979). Frerman et al (1980) reinvestigated the subunit structure of G-AD; end-group analysis and peptide mapping were consistent with a structure of 4 identical subunits. Immunochemical and isoelectric focussing studies indicated only minor structural differences between oxidized enzyme and enzyme-substrate complexes of both C_4CoA and acetoacetyl CoA. Modification of carboxyl residues of G-AD supported the hypothesis that coulombic forces between the highly acidic G-AD and the highly basic ETF are important in protein-protein interaction and electron transfer. (Frerman et al,1980).

A general acyl CoA dehydrogenase has also been recently isolated by Thorpe et al (1979) from hog kidney using a different procedure. This G-AD showed very similar characteristics to the G-AD isolated from pig liver. An extinction at 560 nm for the neutral semiquinone of 5900 M^{-1} cm^{-1} was reported.

Although in the early literature it was asserted that acyl CoA derived from amino acid metabolism such as isovaleryl CoA were dehydrogenated by the green short chain enzyme (SC-AD) (Beinert 1963 A) evidence has accumulated suggesting a separate enzyme for that substrate (Tanaka et al, 1971, 1976; Rhead & Tanaka 1980). Recently a previously unidentified yellow flavoprotein which could be separated from the green and yellow fatty acyl CoA dehdyrogenases was found to interact with isovaleryl CoA while highly purified SC-AD, G-AD and LC-AD did not(Hall, 1979). An isovaleryl CoA dehydrogenase has also been isolated from rat liver by Tanaka's group. Low levels of this enzyme are implicated in some organic acidemias and acidurias (Rhead & Tanaka, 1980). The isovaleryl CoA dehydrogenase from pig liver is structurally different than the fatty acyl CoA dehydrogenases but catalytically similar. The enzyme does not interact with isobutyryl or glutaryl CoA (See progress report and Appendix 4).

The spectrum of SC-AD shows a broad long wavelength band, centered around 710 nm. (Beinert 1963 A; Steyn-Parvé & Beinert 1958 B) not present in the other acyl CoA dehydrogenases. A similar enzyme has been found in a bacterium, M. elsdenii (Engel and Massey,1971 A & B). Both enzymes can be converted to a yellow form by treatment with various materials. However, "degreening" by substrate is 10^4 slower than turnover. The 710 nm absorption of the green enzyme from *M. elsdenii* appears to be due to a charge transfer interaction of oxidized flavin with a CoA derivative (Engel and Massey,1971 A & B). The identity of the CoA derivative is not known. As noted by Engel and Massey, the identification of the acyl CoA derivative bound to the native green enzyme remains an important question, and the presence of such a complex may be important in fatty acid metabolism since such a tightly bound compound must be a potent inhibitor. Recently Engel has proposed that the bound acyl CoA ligand responsible for the green color might be due to a reversible equilibrium between thiolester and lactone (of β-OH- or β-keto acyl CoA) plus thiol while both moieties are bound to the enzyme. (Engel, 1980).

C. Progress Report for IR01 BM 25494
    For Period July 1,1978 to June 30, 1981

Professional Personnel:	Percent Effort
Hall, Carole L., Research Scientist II, Principal Investigator, July 1,1981	70-95%*
DeTar, Teresa M., Research Technician II, Aug 15, 1978 to Nov 9, 1979	100%
Juberg, Eric N., Research Technician II, Nov 12, 1979 –	100%

*Effort was reduced to 70% for July 1, 1978 to June 30, 1979 and compensation made for teaching duties. Effort was reduced to 95% for July 1, 1979, to June 30, 1980 and compensation made for teaching duties. Effort is planned to be reduced to 95% for July 1, 1980 to June 30, 1981 and compensation made for teaching duties.

Progress Report (Continued)

The specific aims of the previous proposal were:

1) To study the mechanism of electron transfers between substrate-dehydrogenase complex and ETF, and from ETF to acceptors, either natural or artificial; and to determine the fate of the product.

2) To probe the nature of the tight complexes between acyl CoA and these flavoproteins.

3) To compare kinetic and structural properties of SC-AD, G-AD and LC-AD.

4) To determine if possible the composition of the protein fraction which stimulated DCPIP reduction at low ETF concentrations.

5) To develop a nonambiguous assay for acyl CoA dehydrogenases and ETF.

6) To try to identify the "greening" ligand of SC-AD and study the effects of hypoglycemic agents on the enzyme.

1. (See Appendix #1) Changes in the spectra of both ETF and G-AD during slow turnover of relatively high concentrations of one flavoprotein in the presence of catalytic levels of the other flavoprotein permitted observation of spectral changes of both flavoproteins independently during reductive approach to steady state, during steady state, and in oxidative escape from steady state. The data suggested catalytic significance for spectral changes previously observed and shed light on the breakdown of the ternary complex, the fate of acyl CoA and the oxidation-reduction state of G-AD in the G-AD--acyl CoA complex.

Spectra of electron transfer flavoprotein observed during turnover with catalytic amounts of general acyl CoA dehydrogenase and 3-5 fold molar excesses of C_8CoA per electron transfer flavoprotein (ETF) flavin showed changes in the UV and near UV which could be correlated with visible absorbance changes known to reflect oxidation-reduction states of electron transfer flavoprotein flavin. All of the electron transfer flavoprotein was converted to the anionic semiquinone and fully reduced forms with rate constants virtually identical to those observed previously when both enzymes were present in "equal" concentrations. Absorption at 320 nm increased with a rate constant similar to that for formation of fully reduced ETF flavin, and decreased only as the flavin absorption at 440 nm returned. Increased absorbance at 280 nm due to enoyl CoA appeared only as the 320 nm absorbance decreased. Changes at 280 nm showed that 75-90% of the substrate was dehydrogenated. The data suggest that G-AD--C_8CoA complex interacts with ETF to transfer one electron to the ETF flavin and then a new complex (represented by absorbance at 320 nm) is formed followed by slower transfer of the second electron to ETF. This complex can not include G-AD in these experiments but seems to involve reduced ETF and acyl CoA since the enoyl CoA is not released until the ETF is reoxidized. Similar results were found when G-AD was being observed in the presence of catalytic amounts of ETF. Experiments with coupled β-oxidation enzyme systems also supported the view that release of enoyl CoA is linked to reoxidation of ETF rather than to transfer of the second electron to ETF. The data suggest an active role of ETF in the dehydrogenation of acyl CoA in addition to the electron transfer function already well described.
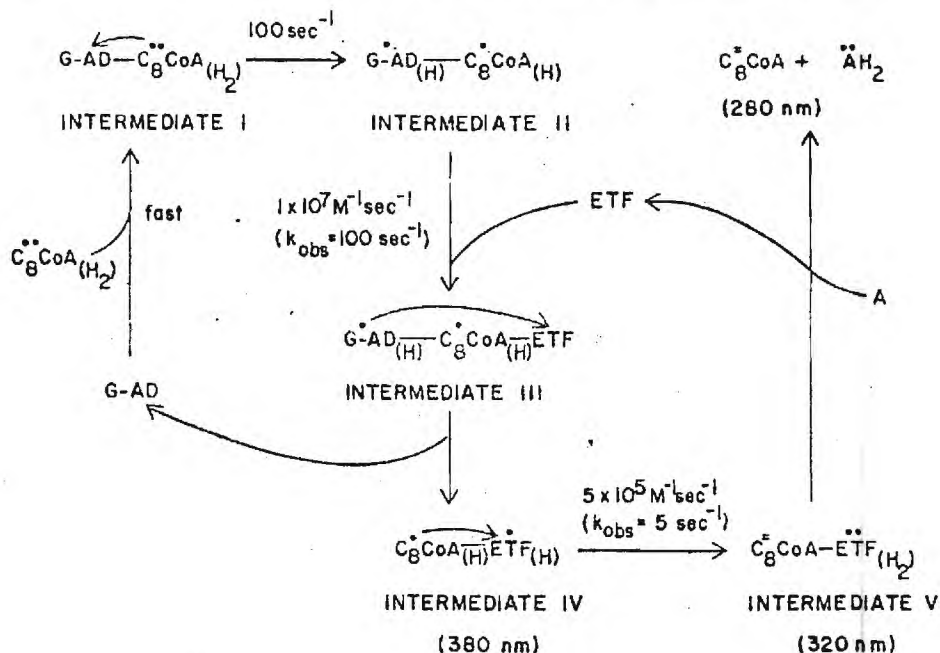
SCHEME I

The results can be described by Scheme I. In the scheme, dots indicate electrons, long dashes indicate complex formation, and $C_8^=$ CoA indicates presence of the α,β double bond. The reduction of G-AD flavin by C_8- and C_{10}CoA (Intermediate II) was shown to be on the order of 100 sec^{-1} and independent of substrate concentration (Hall et al 1979) implying a faster second-order association of acyl CoA with G-AD prior to electron flow (Intermediate I).

When G-AD--C_8CoA complex (Intermediate II), was rapidly mixed with ETF a rapid increase in absorbance at 380 nm, associated with anionic flavosemiquinone formation was observed (Intermediate IV). Intermediate III is inferred from Intermediate IV and possibly seen in fluorescence stopped flow measurements (Hall and Lambeth, 1980 A & B) The subsequent transfer of the second electron to ETF was observed on a much slower time scale (Intermediate V)(Hall and Lambeth, 1980 A & B). The formation of Intermediate V is also seen when G-AD was present in catalytic amounts (disappearance of 380 nm absorbance) with the same second order rate constants as in Hall and Lambeth, 1980 A&B. The agreement of rate constants for formation and decay of anionic semiquinone shows that the same molecular events are being recorded in this catalytic system as when the components were in equimolar amounts (Hall and Lambeth 1980, A & B). The experiments described seem to require the specified locations of the electrons.

Intermediate IV can not be a ternary complex of G-AD, C_8CoA and ETF, because it appeared even when G-AD was present only in catalytic amounts. ETF was converted probably quantitatively to anionic semiquinone (380 nm) with a high second order rate constant prior to formation of the 320 nm intermediate. The 320 nm intermediate appears with the same second order rate constant as fully reduced ETF (Hall and Lambeth 1980, A & B). The agreement of the rate constants for the appearance of ETF A_{320} (Intermediate V) and for the transfer of the second electron (decrease in 380 nm absorbance, conversion of Intermediate IV to Intermediate V) suggests the ETF 320 nm species is representative of reduced ETF. Since G-AD was present only in catalytic amounts in the experiments presented neither the species absorbing at 380 nm (ETF semiquinone) nor the species absorbing at 320 nm (formed after the semiquinone) could be ternary complexes. Approximately ten turnovers of G-AD are occurring in 5 sec to produce all the ETF as semiquinone. Thus G-AD must cycle back to the dissociated,

13

oxidized state after transfer of one electron to ETF.

If the enoyl CoA had dissociated from the dehydrogenase concomitantly with transfer of the first or second electron to ETF, absorbance at 280 nm due to free enoyl CoA should increase concomitantly with the increase at 380 (semiquinone) or at 320 (fully reduced ETF) nm. However, the opposite was observed; increase in 280 nm absorbance is correlated with the slow disappearance of the 320 nm absorbance. The experiments show that appearance of absorption at 280 nm due to free enoyl CoA exhibits a long lag followed by a rapid increase when either G-AD or ETF is present in reagent quantities and the other flavoprotein is present in catalytic amounts. This pattern is repeated in absorbance changes in ETF at 320 nm; after a rapid increase, absorbance at 320 nm decreased slowly at first and then more rapidly (G-AD was present in catalytic amounts). This pattern is repeated in absorbance changes in ETF at 320 nm; after a rapid increase, absorbance at 320 nm decreased slowly at first and then more rapidly (G-AD was present in catalytic amounts). These changes showed nearly the same kinetics as the changes at 280 nm but are opposite in direction. The increased ETF absorption at 320 nm appears with a second order rate constant virtually identical to that described for formation of fully reduced ETF (~5 X 10^5 M^{-1} sec^{-1} Hall and Lambeth, 1980)

The inverse correlation of 320 with 280 nm absorbance in these experiments suggested the reduced form of ETF represented by A_{320} is also complexed with the enoyl CoA which was released only upon reoxidation of ETF. Addition of submitochondrial particles increased the overall rate of both reduction and reoxidation but did not change the relationship between changes at 320 and 280 nm. Conversely, if the experiments were performed anaerobically, no increase in 280 nm or decrease in 320 nm absorbance was observed.

Implications for Electron Transfer to Dyes or to the Electron Transport Chain Acceptor. Binding of G-AD and fatty acyl CoA of six to fourteen carbons in the acyl chain occurs rapidly and with an apparently low K_D (Hall et al, 1979). Electron flow from substrate to dehydrogenase flavin is also rapid and probably not rate limiting in the DCPIP assay (Hall et al 1979), at least for substrates of six to twelve carbons in the acyl chain. Transfer of one electron from dehydrogenase substrate complex to ETF is rapid, while transfer of the second electron to form fully reduced ETF occurs more slowly (Hall and Lambeth 1980 A & B). The turnover of G-AD in the DCPIP assay with saturating C_8CoA (extrapolated to saturating ETF) is 24 sec^{-1} (Hall et al 1979). Using this value as k_{cat} for the DCPIP assay and using "K_{mETF}" of 5 X 10^{-7} M (Hall et al 1979), values for k_{cat}/"K_{mETF}' of about 1 X 10^7 $M^{-1}sec^{-1}$ may be calculated. The close correspondence between this value and the second order rate constant for transfer of the first electron (Hall and Lambeth 1980 A & B) suggests that the interaction of G-AD--C_8CoA complex with ETF and transfer of the first electron is the rate determining step in the DCPIP assay. However, the k_{obs} for the transfer of the second electron to ETF in the stopped flow spectrophotometer (Hall and Lambeth 1980 A & B) is lower than k_{cat} for the DCPIP assay (Hall et al 1979) and is possibly not involved in the reaction with DCPIP. Reduction of DCPIP by photoreduced ETF semiquinone is about 40 sec^{-1} (preliminary results of C.L.H.). These observations suggest that DCPIP interacts with ETF semiquinone prior to transfer of the second electron. In view of the proposed mechanism described in Scheme 1, reoxidation of ETF semiquinone prior to transfer of the second electron might result in a one-electron-reduced ETF-enoyl CoA complex, which might be a dead-end complex and account for the nonlinear double reciprocal plots of 1/v vs. 1/ETF [F] consistently seen with these enzymes (Hall and Kamin, 1975 Hall et al 1976, Hall, unpublished).

The above suggests that the physiological electron acceptor for ETF should accept electrons only from the fully reduced form, since otherwise the dehydrogenation of the acyl CoA substrate would seem to be hindered and might even result in inhibition (Hall et al, 1976). No information is currently available as to whether the proposed ETF dehydrogenase (Ruzicka & Beinert 1977) accepts electrons from semiquinone or fully reduced ETF. The data suggest that the dehydrogenation of acyl CoA

is carried out via a diradical pair mechanism and that the role of ETF in dehydrogenation of acyl CoA substrates involves much more than reoxidation of the dehydrogenase. Perhaps ETF should be viewed as a "co-dehydrogenase."

2. (See Appendix #2) Preliminary studies have been made on the reoxidation of G-AD--C_8CoA complex by PMS and pyocyanine. When G-AD [F] and PMS were in approximately equimolar concentrations and 1-5 equivalents of acyl CoA were added, the bimolecular rate constants for reappearance of 448 nm absorbance were ~1 X 10^3 $M^{-1}sec^{-1}$ while the bimolecular rate constants for disappearance of A_{600} were about 5 X 10^2 $M^{-1}sec^{-1}$. When pyocyanine was used instead of PMS the bimolecular rate constants were about 50 M^{-1} sec^{-1} at both wavelengths. The data support the observation that rates of G-AD--C_8CoA reoxidation by PMS are slower than with ETF (Hauge, 1956, Hall, 1980 B) which has a bimolecular rate constant of ~1 X 10^7 M^{-1} sec^{-1}.

(Hall and Lambeth, 1980 A and B). The difference in rate constants for flavin reoxidation (A_{448}) and disappearance of long wavelength absorption (A_{600}) is not large. It was thought that if the acy CoA dehydrogenase-acyl CoA complex is a diradical pair and the long wavelength absorbance were due to the postulated acyl radical, reoxidation by PMS [which is known to be reduced in two one-electron steps (Beinert and Sands, 1961)] might reveal the presence of either flavin semiquinone or acyl CoA radical after transfer of one electron. These aerobic experiments so far do not permit this conclusion. Anaerobic experiments might show some differences. Addition of C_8CoA to approximately equimolar mixtures of G-AD and PMS and freezing within 6-12 sec. in an EPR tube resulted in samples showing large EPR signals at g=2 (linewidth = 20 gauss). The signal amplitude varied with concentration of dehydrogenase and with time of freezing after mixing. However, freshly prepared PMS solutions of the same concentration show some of the same EPR signal. Amounts of signal must be quantitated to determine whether the signal is due to neutral flavosemiquinone, PMS radical or stoichiometric amounts of each.

Diradical pairs exhibit the magnetic characteristics of organic triplets and absorb at $\Delta M = 2$ in the EPR spectrometer (Wasserman et al 1964). Preliminary observations of G-AD--C_8CoA complex are suggestive of a signal which shows the expected power saturation behavior of a diradical pair. (EPR experiments are being done in collaboration with Mr. Mitchell Gould, and Drs. B.Yamanashi and S. Lerman of Emory University Medical Center.)

Since PMS appears to contain unknown amounts of radical and significant 450 nm absorption, its usefulness as a redox titration dye would be limited. Pyocyanine has little or no absorbance at 450 nm and indeed was used to determine the redox potential of the C_4CoA-C_4-enoyl CoA couple as mediated by G-AD (Hauge, 1956). The dehydrogenase potential was not investigated. Study of the redox potential of the dehydrogenases with different substrates might shed light on the differences in reactivity to different substrates and with ETF.

In a related preliminary study, when ETF and pyocyanine were mixed aerobically in equimolar concentrations ETF was reduced by NADH (via lipoyl dehydrogenase, or more slowly without lipoyl dehydrogenase; no reduction of ETF by NADH in the absence of pyocyanine is observed). This reduction of ETF by catalytically reduced pyocyanine is the first instance of ETF being reduced by any artificial system, and is potentially useful for further study. Substrate-reduced ETF did not reduce pyocyanine, suggesting its redox potential is higher than that of pyocyanine ($E'_o = -0.034V$).

3. (See Appendix # 9A). The relative kinetic properties of SC-AD, G-AD LC-AD and IV-D (isovaleryl CoA Dehydrogenase, see #7 below) with PMS and ETF were studied. Hauge (1956) had made a qualitative report that for all the acyl CoA dehydrogenases except SC-AD rates with ETF were greater than with PMS. (See above, #2). When rates of DCPIP reduction in the presence of 3 μM ETF [F] were compared to rates when PMS was 200 μM, SC-AD was 2.5 times as active with PMS as with ETF, IV-D was about equally active, G-AD was one-tenth as active, and LC-AD was 200 times less active with PMS than with ETF. These striking differences in reactivity with PMS bear further study.

4. The "factor" fraction obtained during purification of ETF (see Hall and Kamin, 1975 and Hall et al 1976) which stimulates DCPIP reduction at low ETF concentrations contains hydratase, β-OH-acyl CoA dehydrogenase activities and possibly β-keto-thiolase. Repeated chromatography on CM cellulose does not change the relative ratios of the first two activities. β-OH-acyl CoA dehydrogenase activity is typically 100 times as great as hydratase activity in these samples. It is not known if this reflects differences in amounts of enzymes. Chromatography on G-200 results in at least two protein bands which again contain the same relative activities of hydratase and OH-acyl CoA dehydrogenase. The smaller band eluted at $M_r \cong 100,000$ while the greater amount of protein eluted at $M_r = 50-60,000$. Disc PAGE of the two bands revealed striking differences. The $M_r = 100,000$ fraction showed a broad, diffuse band when stained with Coomassie blue which extended from the top of the gel to $R_f \cong 0.15$. The $M_r = 50-60,000$ band showed none of this band, and three discrete bands with R_f's of 0.05, 0.11 and 0.18 respectively were observed. These bands have been observed to bind bromphenol blue, which is used as the tracking dye. Since bovine serum albumin also binds bromphenol blue, the binding probably indicates the presence of hydrophobic binding sites on these proteins. Addition of bromphenol blue to assay mixtures seems to result in no loss of

activity. Thus bromphenol blue can be used to "label" these proteins to permit visual monitoring of separation on preparative electrophoresis (See Hall,1978 and Hall,1980,B). The $M_r \cong 100,00$ protein band containing the same ratios of activities as the 50-60,000 dalton band is suggestive of a β-oxidase complex similar to that seen in E. coli. (Binstock et al,1977; Gallen and Waterson, 1979).

5. (See Appendix #3) The disappearance of ETF fluorescence caused by addition of acyl CoA in the presence of acyl CoA dehydrogenase (Hall and Kamin 1976 Hall et al 1979, Hall and Lambeth,1980 A and B) was utilized to devise an assay of ETF content in crude mixtures. Using a ratio of $E_{ETF}/E_{Riboflavin} = 0.38$ (CF. Hall and Kamin 1975 and Siegel, 1978) estimates of ETF in sonic supernatants of pig liver mitochondria and various fractions obtained during purification were found to be in good agreement with amounts of ETF recovered and with total acyl CoA-reducible flavin absorbance. These results suggested this assay should be potentially useful where the DCPIP assay might lead to ambiguous results and when large amounts of purified acyl CoA dehydrogenases are not available. The presence of different acyl CoA dehydrogenases can be qualitatively assessed by use of different substrates.

6. Green SC-AD was heated to denature the protein in the hope of releasing the "greening" ligand intact. The heat supernatant was concentrated by evaporation or lyophilization and chromatographed on TLC. Treatment of the chromatogram with nitroprusside followed by methanolic KOH revealed a faint pink spot, suggesting the presence of a short-chain thiolester. However, in some cases the pink spot did not appear immediately but required several days at room temperature to develop. The reasons for this behavior are not understood and since large amounts of enzyme were required this approach was abandoned. C_{16}CoA could "degreen" SC-AD but not at stoichiometric levels. Denaturation of the enzyme followed after several hours. This apparent detergent effect was not duplicated by lysophosphatidyl CoA at concentrations above its CMC.

7. (See Appendix #4). A flavoprotein which exhibited different spectral characteristics upon addition of C_{16}CoA from those of G-AD, LC-AD or SC-AD was separated from the fatty acyl CoA dehydrogenases by preparative electrophoresis. The use of C_{16}CoA complex spectra has been described as a useful diagnostic for identifying these flavoproteins (Hall, 1978). When tested with isovaleryl CoA, changes in the spectrum indicative of complex formation were seen (CF. Hall et al,1979). Similar changes in the spectrum of G-AD have been shown to be correlated with activity in the catalytic DCPIP reduction assay as well as with rate constants for reduction of the flavin (Hall et al 1979). The extent of flavin reduction and long-wavelength absorption caused by addition of a substrate is a good measure of effectiveness of dehydrogenation of that substrate. Therefore the finding that the new flavoprotein was significantly complexed by isovaleryl CoA suggested the function of the enzyme was involved in branched chain amino acid catabolism. The study of this enzyme represents a new

direction in this project.

Since the new flavoprotein migrates very close to G-AD on the preparative electrophoresis, the possibility of inadvertant contamination of G-AD by isovaleryl CoA dehydrogenase (IV-D) was checked. Different lots of G-AD were found to vary in ability to form complex spectra with and show catalytic activity from IVCoA, but the best samples of G-AD showed less than 10% activity or complexation with IVCoA as compared to C_8CoA. The new flavoprotein shows evidence of complexation and activity with IVCoA, C_8CoA and C_6CoA. Evidence that this complexation with fatty acyl CoA's is not due to contamination with G-AD is the following: 1). the UV and visible absorption peaks are different; 2) the chain length specificity profile is different; 3) The native molecular weight and subunit structure are different. Evidence that IV-D is not significantly contaminated with degreened SC-AD is the following: 1) Spectra are somewhat different; 2) different migration on disc-PAGE and on preparative electrophoresis; 3) low activity and complexation with C_4CoA; 4) Different native molecular weight and subunit structure. Although in the older literature statements had been made that branched-chain acyl CoA's are dehydrogenated by SC-AD or G-AD, neither pure SC-AD or G-AD are significantly complexed with or show activity with IVCoA. Thus although IVCoA is perhaps not the "best" substrate for the new flavoprotein it is not a substrate for G-AD, SC-AD or LC-AD. The spectrum of the new enzyme and its low activity with C_{16}CoA also rule out LC-AD as a major contaminant of the new enzyme.

Complexes of G-AD with substrates of chain length C_6 to C_{14} are tight, stoichiometric and air-stable (Hall. et al, 1979). IV-D was titrated aerobically with IVCoA and also shows breaks in the plots of absorbance changes at 440 and 590 nm at about 1 mole IVCoA/mole IV-D flavin. Thus the K_D must be less than 10^{-7} M (Cf. Hall et al 1979).

The turnover number at saturating ETF (extrapolated) is 217 nmoles/min for C_6CoA and 174 nmoles/min for IVCoA. "$K_{M\ ETF}$" is about 2µM for both substrates, about 10x higher than for the fatty acyl CoA dehydrogenases. (Hall et al 1979). Breaks are also seen in the 1/v vs 1/ETF [F] plots as for the other enzymes. (Hall and Kamin, 1975, Hall et al,1977). The mode of interaction with ETF appears to be similar but less efficacious, at least aerobically.

IV-D shows dependence upon concentration of substrate for both C_6CoA and IVCoA, but inhibition at high substrate concentrations is observed. These results are similar to those observed for fatty acyl CoA dehydrogenases. Although K_M is not really meaningful for such tightly bound substrates, the 1/2 maximal concentrations are about 5 µM for both IVCoA and C_6CoA in the DCPIP assay. Since ETF is required (PMS may substitute), this "K_M" might reflect the interaction with ETF. "K_M's for the fatty acyl CoA dehydrogenases are all in the range of 1-10 µM (except G-AD for C_4CoA).

The native molecular weight of IV-D as measured by gel filtration on Sephadex G-200 is ~ 100,000 daltons. SDS gel electrophoresis indicates a subunit mwt of about 52,500 daltons. The minimal mwt based on flavin content is about 45,000 daltons. The flavin is at least 90% FAD (measured by comparison of fluorescence at pH 2 vs pH 7.5, see Siegel, 1978). The native enzyme shows less than 10% the fluorescence of the detached flavin at pH 7. Absorbance of the detached flavin is about 4% greater than native. The A_{265}/A_{440} ratio for the best samples is 6.8. Thus the enzyme appears to be a dimer of ~52,000 dalton subunits each containing one FAD, and turnover numbers/ mole enzyme are thus 350 for IV CoA and 450 for C_6CoA.

Neither the fatty acyl CoA dehydrogenases nor the new enzyme show any activity toward or complexation with isobutyryl CoA, glutaryl CoA or methylmalonyl CoA. Evidence has recently been presented for the existence of a separate isovaleryl CoA dehydrogenase in rat liver mitochondria (Rhead and Tanaka, 1979; Rhead and Tanaka 1980).

D. Methods of Procedure

1. Since flavins show characteristic absorption and fluorescence changes in different binding and redox states or in complexed states, they are especially amenable to study by spectral techniques using reagent rather than catalytic amounts of enzymes.

Hall, Carole L.

303-42-9705

These methods provide more useful data than can be gained by studies on dye-coupled catalysis alone for such a complicated system. The stability of the enzyme-acyl CoA complexes also facilitates such studies, since individual steps can be isolated more readily than in systems where the normal substrate is proceeding to product. Even though the purification of these enzymes is tedious they can be prepared and stored frozen, and it is possible to accumulate the large quantities required for such experiments. The investigator is extremely experienced in handling these enzymes, and has written 3 articles in Methods in Enzymology describing these techniques. (Hall, 1978; Hall, 1980 B,C). Observation of changes in the spectrum of ETF during reduction and reoxidation by SC-AD, LC-AD and IV-D as was done for G-AD (see Appendix #1) should show if these dehydrogenases interact with ETF in the same way as G-AD. Observation of the dehydrogenases during catalytic reoxidation by ETF plus submitochondrial particles, PMS or pyocyanine will provide further information on these interactions. Preliminary evidence suggests the IV-D interacts with ETF differently than does G-AD. The other dehydrogenases have not yet been investigated. Observation of SC-AD under similar turnover conditions might shed light on the effect of long-wavelength-absorbing ligand on catalysis.

The observation of Steyn-Parve and Beinert (1958 A) that bound acyl CoA could be displaced by free acyl CoA merits further study. These authors reported the exchange was rapid. In preliminary experiments the displacement of stoichiometric C_{16}CoA (one mole C_{16}CoA/mole enzyme flavin) by stoichiometric C_8CoA was shown by observing the change from the characteristic more resolved spectrum of G-AD--C_{16}CoA complex to the extensive reduction and complexation of the G-AD--C_8CoA complex (Hall, 1978 Hall et al 1979). This change occurred within the time of mixing in the cuvette. Stopped flow studies using substrates which markedly reduce the flavin to displace substrates which do not or vice versa (Cf. Hall et al 1979) can show whether such displacements are important catalytically. The question of whether the rate of displacement of same or similar chain length substrates is of any catalytic significance could be assessed by use of fluorescent-labelled acyl CoA and fluorescence stopped flow. It is possible that fluorescent-labelled substrates could be sensitive probes for presence of the α,β-bond, for binding to proteins, and for measurement of critical micelle concentrations. The fluorescent label should greatly facilitate purification of the thiolesters. Etheno-adenosyl-CoA is available commercially and the syntheses and purification of acyl CoA derivatives are well worked out (Stadtman, 1957, Al Arif and Blecher, 1971; Pullman 1973). Even if no fluorescence changes occurred upon dehydrogenation and/or binding, the presence of the label would greatly facilitate observation of the postulated reduced ETF enoyl CoA complex (See Appendix #1). Of course the possibility of altered binding due to the change in the CoA moiety must be investigated before any of the above experiments are begun. The presence of a fluorescent label on the CoA moiety might also interact with the fluorescent ETF flavin to provide mechanistic information. Fluorescent labelled substrates would also be useful for binding studies.

If the postulated diradical pair nature of the G-AD--C_8CoA complex can be confirmed by EPR techniques, (absorption at Δ_m = 2) (Wasserman, 1964), this would be another way to assess the substrate specificity of the dehydrogenases. The possibility that a similar diradical pair on ETF appears transiently as postulated (see Progress Report part 1 and Appendix #1) could be tested using rapid freeze EPR techniques. Rapid freeze EPR could also be used to test the suggestion that no G-AD semiquinone appears during turnover (Hall and Lambeth, 1980).

The redox potentials of acyl CoA dehydrogenase-substrate complexes can probably be assessed by titration with substrate in the presence of pyocyanine. Direct potentiometric titration of the photochemically reduced (via 5-deaza flavin) dehydrogenase and ETF may be possible (Stankovitch and Massey, 1978).

The suggestion that to ensure dehydrogenation of the acyl CoA,ETF should be reoxidized from the fully reduced state rather than the semiquinone (See Progress Report Part 1 and Appendix #1) would require that the natural acceptor in the electron transport chain either recognized only the fully reduced ETF or that it is an obligate $2\bar{e}$ acceptor. A preliminary observation of a rapid increase in fluorescence in

RIVILEGED COMMUNICATION

sonicated mitochondria upon aerobic addition of acyl CoA suggests this fluorescent material is the natural electron acceptor for ETF. This rapid aerobic response is in contrast to the requirement of anaerobic conditions (or KCN) to see heme reduction by acyl CoA via acyl CoA dehydrogenase and ETF (Beinert,1963 A;and C.L.H. unpublished observations). If this fluorescent material can be isolated and characterized it might serve as a model for choosing or synthesizing artificial oxidants for ETF. The ETF dehydrogenase described by Ruzicka and Beinert (1977) does contain FAD. It was not reported if it showed fluorescence under conditions of catalysis.

2. The efficacy of the "factor" fraction in stimulating the dye coupled assay at low levels of ETF suggests it is an enzymatic function, and the presence of activities of enoyl hydratase, β-OH acyl CoA dehydrogenase, and probably β-ketothiolase lends support to the idea (See Progress Report Part 2). However, there also appear to be proteins with hydrophobic binding sites, and surface active lipids present in the fraction also. Some attempts to resolve the mixture have been made, but further work is needed. Since the dye bromphenol blue (BPB) binds to three of the proteins, it would be easy to separate them on preparative electrophoresis. The presence of BPB in the mixture is also achieved upon gel filtration. When the "effector" is isolated it should be possible to determine if it facilitates reduction of DCPIP by $ETFH_{(2)} \sim P$ or reoxidation of ETF; or if it permits product dissociation before reoxidation.

Preliminary studies show that purified bovine liver hydratase does not enhance the reoxidation of catalytically reduced ETF (see Appendix of Appendix # 1). It has not been tested for stimulation of DCPIP reduction. The possibility of some physical association of enzymes with a "β-oxidase complex" if it exists, needs to be investigated.

3. The kinetic and structural properties of isovaleryl CoA dehydrogenase from pig liver will be studied along the lines already utilized or proposed for G-AD, SC-AD and LC-AD. Relative rates of reduction of IV-D by branched and straight chain substrates needs to be investigated in the stopped flow spectrophotometer.

4. Protein structural studies such as amino acid analysis, peptide mapping and immunological studies may help to elucidate how these proteins recognize each other, and what structural relationship the substrate-level dehydrogenases have to each other. New techniques for peptide mapping such as those described by Cleveland et al (1977) are especially attractive and should provide information on the extent of homology of the different acyl dehydrogenases. Frerman et al have reported a peptide map for G-AD (1980).

5. The early studies on the flavoproteins of β-oxidation were done on aqueous extracts of acetone powders. New isolation techniques use sonic oscillation for disruption of mitochondria. It is possible that the low yield of LC-AD from pig liver currently observed (Hall, 1978; Hall, 1980 A) is related to this difference. The acetone treatment might tend to extract LC-AD from mitochondrial membranes. Extraction of the submitochondrial particles by acetone and buffer, detergents or chaotropic salts might result in a better yield of LC-AD so that further kinetic and structural studies may be made.

6. The fluorescence techniques for assay of ETF and ETF-linked dehydrogenases described in the progress report and Appendix will be applied to mitochondria from other tissues. Dr. S. I. Goodman has requested tests for ETF in the liver of a victim of an as-yet uncharacterized organic acidemia. The possibility of testing for these enzymes from other sources of material and in small amount such as from fibroblasts, needs to be developed.

7. As described in #1 above, observation of SC-AD during catalytic reduction and reoxidation by ETF might shed light on the catalytic capabilities of the green enzyme. Resonance Raman spectroscopy of the green enzyme, especially in conjunction with Resonance Raman study of other acyl CoA complexes of acyl CoA dehydrogenases might suggest some possible structures of the presumed acyl CoA ligand. Resonance Raman spectra of acetoacetyl CoA and crotonyl CoA complexes of G-AD have been reported (Benecky et al 1979) but the spectra of substrate complexes have not been shown.

8. The kinetic and structural properties of the new acyl CoA dehydrogenase from *Ps. oleovorans* will be studied by the techniques already used or proposed for the mammalian acyl CoA dehydrogenases. An ETF or ETF-like flavoprotein has not yet been observed, but the fact that this enzyme interacts with mammalian ETF suggests there probably is one. The potential for isolating mutants in this gene suggest the bacterial system might be a good model for study of organic acidemias in humans.

E. Facilities Available

The proposed experiments call for reagent amounts of high purity enzymes. It has been demonstrated that these enzymes can be obtained in such quantities and stored until needed (Hall 1978, Hall 1980 B & C). Dr. Sheldon May has granted me space in his laboratory. Instrumentation necessary for such preparation is available in either the School of Chemistry or the School of Biology at Georgia Tech, including a high speed evacuated centrifuge. Equipment for rapid reaction absorbance measurements and for anaerobic titration is available within Dr. May's lab. However, as noted under Section II of this application, heavy use of Dr. May's Beckman Acta spectrophotometer by members of his research group hampers progress on this project. Since the study of these enzymes heavily utilizes subtle spectral changes, a microprocessor controlled spectrophotometer with baseline correction, high sensitivity, low signal to noise, and derivative functions would greatly improve data collection, processing and interpretation.

Scintillation counting equipment is available in the School of Chemistry (Dr. J. Powers laboratory) or in the School of Biology. Fluorescence stopped flow equipment is available to me at the University of Georgia in Athens. Dr. May has equipment for microbiology including a 14 liter fermentor NMR, mass spectrometry, CD/ORD, EPR and Laser Raman spectroscopy is available in the School of Chemistry, and rapid-freeze EPR techniques can probably be set up using existing rapid mix equipment.

F. Collaborative Arrangements

While collaboration with other scientists is not required to carry out this project, Drs. R. Felton and N. T. Yu in the School of Chemistry are expert Raman spectroscopists and collaborative arrangements can be made. Collaboration on EPR studies is already established with Drs. B. Yamanashi and S. Lerman from Emory University. Possible collaborations with Drs. Byron Rubin, Robert Waterson and J. D. Lambeth (also of Emory University) in areas of mutual interest could be fruitful. Contact with members of the Department of Biochemistry at the University of Georgia at Athens has been made and use of their equipment can be arranged. In addition, the facilities and expertise of a number of scientists in a variety of areas (such as amino acid analysis) at the CDC, Coca Cola Research Center, Emory University are expected to be available.

Some collaboration on the study of the *Ps. oleovorans* acyl CoA dehydrogenase with Dr. Sheldon May is possible since it was discovered in the course of purification of enzymes from this organism by members of his research group. Dr. Dwight Hall in the School of Biology is available for consultation and possible collaboration in genetic manipulations of this organism.

G. Principal Investigator Assurance

The undersigned agrees to accept responsibility for the scientific and technical conduct of the research project and for provision of required progress reports if a grant is awarded as the result of this application.

Date *May 23, 1980*   *Carole L. Hall*

PRIVILEGED COMMUNICATION

REFERENCES

Al-Arif, A. T. Blecher, M. (1971) Biochem.Biophys. Acta, 248 416

Beinert, H. (1963 A), The Enzymes, Boyer, P. D., Lardy, H. and Myrback, K. (eds.) 2nd Ed., Vol. 7, p. 447 - 466, A.P., N.Y.

Beinert, H. (1963 B) in The Enzymes, Boyer, P. D., Lardy, H. and Myrback, K. (eds.) 2nd Ed., Vol. 7, p. 467 - 473, A.P., N.Y.

Beinert, H. and Crane, F. L. (1956) Inorganic Nitrogen Metabolism, W. D. McElroy and B. Glass (eds.), Baltimore, 601-627.

Beinert, H. and Page, E. (1957) J. Biol. Chem., 225, 479 - 497.

Beinert, H. and Sands, R. H. (1961) "Free Radicals in Biological Systems, Blois, M. S., Brown, H. W., Lemmon, R. M., Lindblom, R. O. and Weissbluth, M., (eds.) A. P., N. Y., p. 17.

Beinert, H., and Frisell, W. R. (1962) J. Biol. Chem. 237 2988-2990

Benecky, M; Li, T.Y.; Schmidt, J., Frerman, F., Watters, K. L.; and McFarland, J. (1979) Biochemistry 18 3471 - 3476.

Cleveland, D.W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252 1102-1106.

Crane, F. L. Hauge, J. G. and Beinert, H. (1955), Biochim Biophys. Acta, 17, 293 - 295.

Engel,P. C. (1980) Flav. & Flav. Prot.   423-430. 6th Int. Symp. Yagi, T. & Yamano,Eds, Japan Scientific Societies Press, Tokyo .
Engel,P. C. and Massey, V. (1971) Biochem. J., 125, 879 - 887.

Engel,P.C. and Massey, V. (1971) Biochem. J.,125, 889 - 902.

Frerman, F. E., Mielke, D. & Huhta, K. (1980) J. Biol. Chem. 255 2199 - 2202.

Hall, C. L. (1973) Fed. Proc., 32, 596 abs.

Hall, C. L. (1974) Fed. Proc., 33, 1338 abs.

Hall, C. L. (1976) Fed. Proc., 35, 1435 abs.

Hall, C. L. (1978) Methods Enzymol., 53 502 - 518., 53 502 - 518. (Appendix #5).

Hall, C. L. 1979 XIth International Congress of Biochemistry (Toronto) Abstracts p. 434 (Appendix # 11).

Hall, C. L. (1980 A) Fed. Proc. 39 Abs. 1640 (Appendix # 11)

Hall, C. L. (1980 B + C) Methods in Enzymology, in press. (Appendix #9A & 9B).

Hall, C. L. and Kamin, H. (1975)J. Biol. Chem., 250, 3476 - 3486.

Hall, C. L. and Kamin. H.   (1976) Flavins and Flavoproteins, T. P. Singer, (ed.) ASP, Amsterdam, 679 - 687.

Hall, C. L., Heijkenskjold, L., Bartfai, T., Ernster, L., and Kamin, H. (1976) Arch. Biochem. Biophys., 177, 402 - 414.

Hall, C. L., Lambeth, J. D. and Kamin, H. (1979) J. Biol. Chem. 254 2023 - 2031.

Hall, C. L., and Lambeth J. D. (1980 A) Flavins & Flavoproteins, 6th International Symposium, Yagi, K. and Yamanno, T. (Eds) Japan Scientific Societies Press, Tokyo p. 657-667.

Hall, C. L. and Lambeth, J. D. (1980B) J. Biol. Chem. 255 3591 - 3595.

Hauge, J. G. (1956) J. A. C. S., 78, 5266 - 5272.

Holland, P. C., Senior, A. E. and Sherratt, H. S. A. (1973) Biochem J., 173 - 184.

Hoskins, D.D.; & Bjur, R.A.; (1965) JBC 240 2201 - 2208.

McKean, M.C., Frerman, F. E. & Mielke, D. M. (1979) JBC 254 2730 - 2735.

Osmundson, H., Billington, D. and Sherratt, H.S.A., (1974) Biochemical Society Transaction 1286 - 1288.

Pullman, M.E., (1973) Anal. Biochem. 54 188 - 198.

Rhead, W. and Tanaka, K. (1979) Fed. Proc. 38 648 Abs.

Rhead, W. J. and Tanaka, K. (1980) Proc. Nat. Acad. Sci. USA 77 580, 583.

Ruzicka, F. J. and Beinert, H. (1977) J. Biol. Chem. 252, 8440 - 8445.

Siegel, L. M. (1978) Methods. Enzymol. 53 419 - 429.

Singer, T.P., Steenkamp, D. J., Kenney, W. C. and Beinert, H. (1980) Flavins and Flavoproteins, 6th International Symposium, Yagi, K. & Yamano, T. (Eds) 277 - 287.

Stadtman, E. R. (1957) Methods in Enzymology 3, 931 - 941.

Stankovitch, M. T. and Massey, V. (1978) Biochem. Biophys. Acta 327 306 - 312.

Steyn-Parve, E. P. and Beinert, H. (1958)A J. Biol. Chem., 233, 843 - 852.

Steyn-Parve, E. P. and Beinert, H. (1958)B J. Biol. Chem., 233, 853 - 861.

Tanaka, K., Miller, E. M., and Isselbacker, K. J. (1971) Proc. Nat. Acad. Sci., 68, p. 20 - 24.

Tanaka, K., Kean, E. A. and Johnson, B. (1976) New England Jour. Med., 295, p. 461 - 467

Thorpe, C. Mathews, R. G. and Williams, C. H. Jr.; (1979) Biochemistry 18, 331 - 337.

Wasserman, E., Snyder, L. C. and Yager, W. A. (1964) J. Chem. Phys. 41 1763 - 1772.