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REFERENCES

1 J. GERGELY, *Ann. N.Y. Acad. Sci.*, 72 (1959) 538.
2 J. GERGELY, F. N. BRIGGS AND G. KALDOR, *4th Intern. Congr. Biochem., Vienna, 1958, Abstr. Communs.*, p. 83.
3 J. R. BENDALL, *Nature*, 172 (1953) 586.
4 J. R. BENDALL, *Proc. Roy. Soc. (London)*, *B*, 142 (1954) 409.
5 L. LORAND, J. MOLNAR AND C. MOOS, *Conference on the Chemistry of Muscular Contraction, Tokyo, 1957*, Igaku Shoin Ltd., p. 85.
6 E. BOZLER AND J. T. PRINCE, *J. Gen. Physiol.*, 37 (1954) 53.
7 F. N. BRIGGS, G. KALDOR AND J. GERGELY, *Biochim. Biophys. Acta*, 34 (1959) 211.
8 J. GERGELY, G. KALDOR AND F. N. BRIGGS, *Biochim. Biophys. Acta*, 34 (1959) 218.
9 H. PORTZEHL, *Z. Naturforsch.*, 7b (1952) 1; also cited by H. H. WEBER, *Z. Elektrochem.*, 55 (1951) 511.

# STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS

## VI. SPECTROPHOTOMETRIC ASSAY AND STOICHIOMETRY OF FATTY ACID SYNTHESIS

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SUMMARY

A spectrophotometric assay for the synthesis of long-chain fatty acids from acetyl CoA which depends upon the oxidation of TPNH has been described. The oxidation of TPNH requires all the components of the system and correlates very well with the incorporation of radioactivity from [1-$^{14}$C]acetyl CoA to palmitate.

The stoichiometry of the fatty acid synthesis has been studied and the results for the synthesis of palmitic acid may be expressed by the following equation:

$$8\ \text{AcCoA} + 16\ \text{ATP} + 16\ \text{TPNH} \xrightarrow[\text{HCO}_3^-,\ \text{Mn}^{++}]{\text{R}_{1g}\text{ and }\text{R}_{2g}} \text{palmitic acid} + 16\ \text{ADP} + 16\ \text{P}_i + 16\ \text{TPN} + 8\ \text{CoA}$$

The following abbreviations were used: AcCoA, acetyl coenzyme A; CoA, coenzyme A; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide; DPN and DPNH, oxidized and reduced diphosphopyridine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; $P_i$, orthophosphate; and $R_{1g}$ and $R_{2g}$, the two enzyme preparations referred to in ref. 4.

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## INTRODUCTION

In previous communications of this series[1-5] we have reported a synthesis of long-chain fatty acids from acetyl CoA which is catalyzed by two highly purified enzyme fractions from avian livers ($R_{1g}$ and $R_{2g}$), and which requires the presence of ATP, $Mn^{++}$, $HCO_3^-$ and TPNH. There is very little or no synthesis in the absence of any one of these cofactors. Radio-labelled bicarbonate is not incorporated into the fatty acids and a catalytic role for this compound was therefore postulated[3,4]. Evidence was also presented which indicates that the synthesis of fatty acids in this system does not require the enzymes of the fatty acid oxidation sequence in contrast to the system reported recently by SEUBERT *et al.*[6].

In order to understand the mechanism of the synthesis in this system, it is essential to know the stoichiometric relationships between the various reactants and products of the reactions. The availability of highly purified enzyme fractions made it possible to obtain such information. Furthermore, since TPNH is the sole electron donor for the reduction of the carbonyl group during the synthesis of the fatty acids, the disappearance of TPNH as measured at 340 m$\mu$ provides an exact and convenient measure of the synthesis of long-chain acids[5]. Thus the present communication deals with the description of a spectrophotometric assay method for fatty acid synthesis and with the chemical stoichiometry for the over-all synthesis of long-chain acids from acetyl CoA.

## METHODS AND MATERIALS

The enzyme fractions ($R_{1g}$ and $R_{2g}$) were prepared as described previously[1,4]. Extraction of the fatty acids, identification of palmitate, degradation and radioactivity measurements were carried out by methods described in our early communications of this series[1,2,4,7].

The adenine nucleotides were analyzed by ion-exchange chromatography on Dowex-1 Cl by a modified procedure of COHN AND CARTER[8]. Orthophosphate was assayed by the LOWRY-LOPEZ procedure[9]. Free CoA was determined by an unpublished procedure of WAKIL AND HÜBSCHER.

## RESULTS

### *Spectrophotometric assay of fatty acid synthesis*

The conversion of acetyl CoA to the long-chain fatty acids requires in addition to the two enzyme fractions ($R_{1g}$ and $R_{2g}$) the presence of ATP, $Mn^{++}$, $HCO_3^-$ and TPNH. The disappearance of TPNH during fatty acid synthesis provides an exact measure of fatty acid synthesis and can be conveniently followed by spectrophotometric measurement at 340 m$\mu$. The assay system contains: 25 $\mu$moles of potassium phosphate buffer of pH 6.5; 1 $\mu$mole of ATP; 0.3 $\mu$moles of $Mn^{++}$; 0.03 to 0.1 $\mu$moles of AcCoA; 0.03 to 0.06 $\mu$moles of TPNH; and $H_2O$ to a final volume of 0.5 ml. The reaction is usually started by the addition of the two enzyme fractions (0.5 to 2.0 mg total protein). Fluoride ion (2.0 $\mu$moles of KF per assay mixture) may be added to the reaction mixture to minimize the destruction of ATP by phosphatases.

Fig. 1 shows a typical result that can be obtained by plotting the reduction

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in absorption at 340 mμ versus time. This result was obtained only when all the components were added[4,5], while very little or no change in absorption at 340 mμ takes place in the absence of any one of the required cofactors (*cf.* Fig. 1). The rate of oxidation of TPNH runs parallel with the rate of incorporation of [1-$^{14}$C]AcCoA into the long-chain fatty acids as determined by the pentane extraction of the fatty acids from the reaction mixture as reported earlier[1,3].

The rate of oxidation of TPNH as a function of the concentration of $R_{1g}$ and $R_{2g}$ is shown in Figs. 2 and 3. No oxidation of TPNH takes place in the absence of either one of the two enzyme fractions. The results also show that the initial rate of oxidation of TPNH is proportional to the concentration of whichever enzyme fraction is rate limiting. This rate of oxidation of TPNH is used as a measure for the enzymic activity of each fraction.

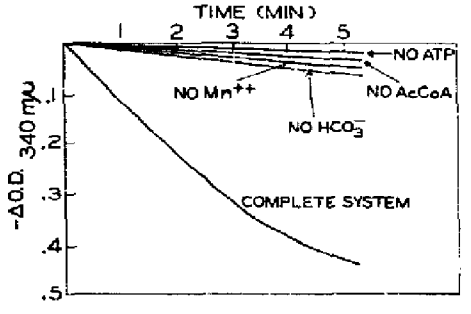


Fig. 1. Oxidation of TPNH. Each cuvette contained the following reagents (except when omitted as indicated): 25 μmoles of potassium phosphate buffer (pH 6.5); 1.0 μmole of ATP; 0.3 μmole of $MnCl_2$; 4.0 μmoles of $KHCO_3$; 0.05 μmole of [1-$^{13}$C]acetyl CoA; and 0.08 μmole of TPNH in a final volume of 0.50 ml. The reaction was started by the addition of 0.7 mg of $R_{1g}$ and 0.4 mg of $R_{2g}$. The temperature was maintained at 38°. At the end of 5 min 0.032 μmole of acetyl CoA was incorporated into fatty acids.

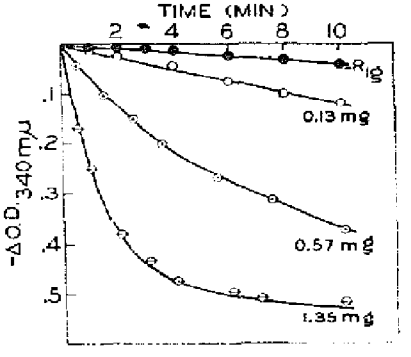


Fig. 2. Each cuvette contains the same reaction mixture as in Fig. 1 except that 0.6 mg of $R_{2g}$ was used and variable amounts of $R_{1g}$ were added as indicated.

Evidence has been presented earlier[4] to show that the two enzyme fractions $R_{1g}$ and $R_{2g}$ are free from acetic thiokinase[10,11]. This can be again demonstrated in the spectrophotometric assay. When AcCoA was hydrolyzed by alkali, there was no oxidation of TPNH (*cf.* Fig. 4). However, on the addition of acetic thiokinase[10] TPNH is oxidized as shown in Fig. 4 with a corresponding amount of fatty acid synthesized as measured by the amount of [1-$^{14}$C]AcCoA converted to fatty acid. This experiment demonstrates conclusively that AcCoA is the substrate of or starting point for fatty acid synthesis.

As was stated previously[2,4], DPNH can substitute for TPNH in fatty acid synthesis. This can be demonstrated in the spectrophotometric assay (*cf.* Fig. 5), although the rate of oxidation of DPNH is slower than that of TPNH. This could not be interpreted to be due to the presence of DPNH-TPN transhydrogenase[12], because under such conditions we could not assay for transhydrogenase in the two enzyme preparations. This lack of specificity distinguishes the avian liver system from the mammary gland system of HELE AND POPJÁK[13], in which only DPNH is required for fatty acid synthesis, and TPNH cannot substitute for DPNH, and also

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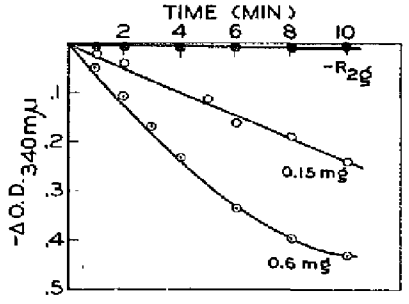


Fig. 3. The same conditions were used as in Fig. 1 except that 0.57 mg of $R_{1g}$ was used and variable amounts of $R_{2g}$ as indicated.

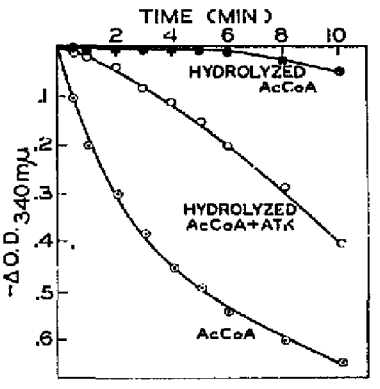


Fig. 4. Absence of acetic thiokinase in the $R_{1g}$ and $R_{2g}$ fraction. The curve represented by solid points (–●—●–) was obtained by mixing acetyl CoA (0.1 $\mu$mole) with alkali to pH 13 and incubating for 10 min at 38°. The mixture was neutralized to pH 6.5. The same treatment was made on the experiment represented by the open points (–○—○–). The acetyl CoA in the experiment represented by (–⊙—⊙–) curve was not hydrolyzed. To the above cuvettes the following were added: 25 $\mu$moles potassium phosphate (pH 6.5), 1.0 $\mu$mole ATP, 0.3 $\mu$mole $Mn^{++}$, 4.0 $\mu$moles $KHCO_3$ and 0.1 $\mu$mole of TPNH. The reaction was started by the addition of 0.7 mg of $R_{1g}$ and 0.45 mg of $R_{2g}$. 0.2 mg of acetate-activating enzyme (ATK) was added where indicated. The final volume was 0.5 ml and the temperature was maintained at 38°.

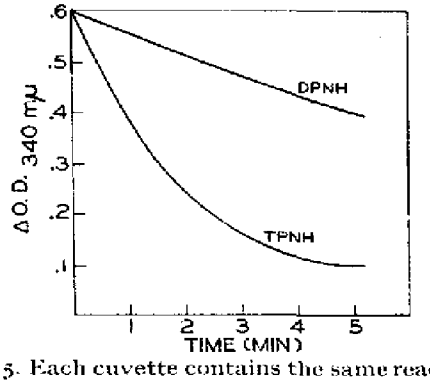


Fig. 5. Each cuvette contains the same reaction mixture as in Fig. 1 except that 0.08 $\mu$mole of DPNH was substituted for TPNH.

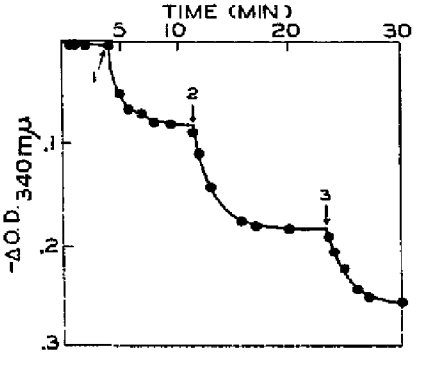


Fig. 6. The cuvette contained the following reagents: 50 $\mu$moles potassium phosphate (pH 6.5), 2.0 $\mu$moles ATP, 0.6 $\mu$mole $MnCl_2$, 8.0 $\mu$moles of $KHCO_3$, 0.08 $\mu$mole of TPNH, 1.0 mg of $R_{1g}$ and 0.6 mg of $R_{2g}$. At Arrow 1, 7.0 m$\mu$moles of [1-$^{14}$C]AcCoA (12,000 counts/min) were added, and the reaction was followed by measuring the optical density at 340 m$\mu$. At Arrow 2, 0.2 ml of the reaction mixture was withdrawn for analysis of the long-chain fatty acids, and again 7.0 m$\mu$moles of [1-$^{14}$C]AcCoA were added. 0.2 ml of the reaction mixture was withdrawn at Arrow 3 as above and again 7.0 m$\mu$moles of acetyl CoA were added. (See text for the results.)

from the mitochondrial system described recently by SEUBERT *et al.*[6] in which both TPNH and DPNH are required for the synthesis of fatty acids from AcCoA and hexanoyl CoA. The relationship between our system and the above two systems will be discussed in a later section.

*Stoichiometry of the fatty acid synthesis*

In the presence of the aforementioned component of the fatty acid synthesizing system, the quantitative conversion of limited amounts of acetyl CoA to fatty acids

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TABLE I

STOICHIOMETRY OF FATTY ACID SYNTHESIS FROM AcCoA

The following reaction mixture was used in each of the above experiments: 100 $\mu$moles of histidine buffer (pH 6.5); 4 $\mu$moles of ATP, 0.12 $\mu$mole of $MnCl_2$; 0.62 $\mu$mole of [1-$^{14}$C]acetyl CoA (120,000 counts/min); 8 $\mu$moles of KF; 4.0 mg of $R_{1g}$ and 3.0 mg of $R_{2g}$. In the first experiment 1.0 $\mu$mole of TPNH was added, and in the second experiment 10 $\mu$moles of isocitrate and 0.1 $\mu$mole of TPN were added. The final volume was 2.0 ml. The following controls were run simultaneously: without ATP, without TPNH (or without isocitrate and $TPN^+$), without AcCoA and without incubation. The reaction was started by the addition of the enzymes, and all the tubes were incubated for 30 min. at 38°. At the end of this time, 0.3 ml of the reaction mixture was withdrawn for the determination of long-chain fatty acids[1], and to the remainder was added 0.04 ml of 60% $HClO_4$. The mixture was centrifuged in the cold, and the clear supernatant was saved and reneutralized to pH 7.0 with KOH. Samples of the resulting solution were taken for the various analytical procedures. All values were corrected for the blank without AcCoA.

| *Experiment number* | *AcCoA** | *TPNH*** | *ATP* | *ADP* | *Pi* | *CoA* | *TPNH / AcCoA* | *ATP / AcCoA* |
|---|---|---|---|---|---|---|---|---|
| 1 | —0.410 | —0.867 | —0.890 | +0.98 | +0.90 | +0.38 | 2.10 | 2.17 |
| 2 | —0.462 | | —0.848 | +0.891 | +0.91 | +0.50 | | 1.84 |

* AcCoA was determined by the amount of radioactivity incorporated into the higher fatty acids[1].

** TPNH was measured spectrophotometrically at 340 m$\mu$.

is achieved (*cf.* Fig. 6). As demonstrated in Fig. 6, the addition of 7.0 m$\mu$moles of [1-$^{14}$C]acetyl CoA at Arrow 1 results in an immediate oxidation of TPNH. Aliquots were withdrawn at Arrow 2 and were analyzed for the fatty acids. Almost a quantitative conversion of the acetyl CoA to the long-chain fatty acids appears to take place. 14.5 m$\mu$moles of TPNH and 7.0 m$\mu$moles of acetyl CoA disappeared during this synthesis. The experiment was repeated by the addition of the same amount of acetyl CoA at Arrow 2. Again, in the presence of excess cofactors the complete conversion of the added acetyl CoA to the fatty acids took place. This quantitative conversion is consistent with the fact that it is difficult to identify any intermediates of shorter chain acids during the synthesis.

With the aid of this reconstructed system it was possible to ascertain the overall stoichiometry of the synthesis of long-chain acids from acetyl CoA. The results in Table I show that with the incorporation of one mole of acetyl CoA into palmitate (palmitate is estimated in these experiments to represent about 80% of the total fatty acids) there is a phosphorolysis of two moles of ATP to ADP and inorganic phosphate with a concomitant oxidation of two moles of TPNH and the formation of 1 mole of free CoA. Thus, the synthesis of fatty acids (palmitate) from acetyl CoA may be represented by the following equation:*

$$8\,\text{AcCoA} + 16\,\text{TPNH} + 16\,\text{ATP} \xrightarrow[R_{1g}\text{ and }R_{2g}]{Mn^{++},\,HCO_3^-} \text{palmitate} + 16\,TPN^+ + 16\,\text{ADP} + 16\,P_i + 8\,\text{CoA}$$

The exact role of ATP and the nature of the intermediates in the overall synthesis are not known. ATP is not used in this system for the regeneration of AcCoA as shown by the absence of acetic thiokinase in the $R_{1g}$ and $R_{2g}$ fractions[4] (*cf.* Fig. 4).

* Theoretically there should be 14 moles of TPNH consumed per 8 moles of AcCoA since the last carbonyl group of AcCoA, which constitute the carboxyl group of palmitic acid is not reduced. In the case of ATP the exact proportion is not as yet clear, but for the overall expression the above equation is preferred.

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It is reasonable, therefore, to postulate a role for ATP in some process other than formation of AcCoA, possibly the activation of $HCO_3^-$ (or one of its equilibrium species, *e.g.*, $CO_2$ or $H_2CO_3$) or the activation of a component on the enzyme. It is of interest to mention here that one of the protein fractions ($R_{1E}$) contains a significant amount of biotin[5]. The possible relationship between ATP, $HCO_3^-$ and protein bound biotin has to await further information.

The phosphorolysis of ATP appears to be dependent upon the presence of all the components of the system. In the absence of any one of the key components of the system (AcCoA, $HCO_3^-$ or TPNH) no significant amounts of ADP are formed as compared to the complete system. The same results are obtained when TPNH was replaced by isocitrate, catalytic amounts of $TPN^+$ and isocitric dehydrogenase. Thus it is important to have the complete system turning over in order to accumulate significant and measurable amounts of ADP in the reaction mixture. The limits of the present analytical methods used prevent us from measuring minute amounts of ADP which may be formed in the incomplete system and which may be stoichiometric with an enzyme-bound moiety.

## DISCUSSION

BRADY AND GURIN[11,15] studied the breakdown and synthesis of fatty acids first in normal and diabetic liver slices and later in cell-free enzymes and concluded that one or more separate and distinct chemical reactions distinguish fatty acid synthesis from fatty acid oxidation. This hypothesis was set aside during the period when the mechanism of fatty acid oxidation was being elucidated[16,17]. Since all the reactions of the fatty acid oxidation sequence are reversible, the assumption was immediately made that synthesis is accomplished by the reversal of the reactions of $\beta$-oxidation. This hypothesis received a wide and early acceptance. It was not until recently that experimental evidence bearing on the requirements for the synthesis of the various long-chain acids from acetyl CoA and shorter acyl CoA became available. LANGDON[18] discovered an enzyme which catalyzes the reduction of crotonyl CoA to butyryl CoA by TPNH. SEUBERT *et al.*[6] studied this reaction extensively, purified the enzyme from pig liver mitochondria and showed that it has a wide range of specificity for the $\alpha,\beta$-unsaturated acyl derivatives ($C_4$ to $C_{18}$) of CoA. A combination of this enzyme, thiolase, $\beta$-hydroxyacyl dehydrogenase and enoyl hydrase in presence of TPNH and DPNH was used by SEUBERT *et al.* to demonstrate the formation of longer-chain fatty acids from a mixture of acetyl CoA and hexanoyl CoA. The various enzymes of the SEUBERT system are derived from mitochondria[10–19], in contrast to the enzymes of the present avian liver system, which is non-mitochondrial in origin[20].

Our own studies[1–5] on the mechanism of fatty acid synthesis in the avian liver system have not given any encouragement to the view that synthesis is a reversal of the $\beta$-oxidation enzyme sequence. The system described above shows cofactor requirements completely different from the expected requirements for the reversal of $\beta$-oxidation and clearly must entail some new mechanism for the multiple condensations of acetyl units. The minimum requirement seems to be two enzyme fractions ($R_{1E}$ and $R_{2E}$) and AcCoA, ATP, $Mn^{++}$, $HCO_3^-$ and TPNH. Recently we have presented evidence for the presence and participation of biotin in the synthesis of fatty acids by this system. Altogether the data presented above and elsewhere, namely,

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(1) the phosphorolysis of ATP, (2) the requirement for bicarbonate, (3) the presence and participation of biotin and (4) the absence of the key enzymes of the $\beta$-oxidation sequence[4] (thiolase and TPNH-$\alpha,\beta$-unsaturated acyl CoA reducing enzymes[6]) indicate that our system is a different and new system for fatty acid synthesis. Furthermore, during the synthesis there is no evidence for the accumulation of short-chain fatty acids such as $C_4$, $C_6$, etc., and compounds as butyryl CoA, hexanoyl CoA or octanoyl CoA are not incorporated into the long-chain fatty acids during active synthesis from AcCoA nor do these compounds trap any radioactivity from [1-$^{14}$C]acetyl CoA. Thus the active acyl intermediates which we presume are formed during the synthesis of long-chain fatty acids cannot be fatty acyl COA derivatives. Recently WAKIL[21] has presented evidence to show that the first step in fatty acid synthesis is the carboxylation of acetyl CoA to malonyl CoA catalyzed by the biotin-containing $R_{1g}$ fraction in presence of ATP and $Mn^{++}$. The subsequent successive condensation and reductive steps of malonyl CoA are catalyzed by $R_{2g}$ in presence of TPNH.

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REFERENCES

1 S. J. WAKIL, J. W. PORTER AND D. M. GIBSON, *Biochim. Biophys. Acta*, 24 (1957) 453.
2 J. W. PORTER, S. J. WAKIL, M. I. JACOB, A. TIETZ AND D. M. GIBSON, *Biochim. Biophys. Acta*, 24 (1957) 35.
3 D. M. GIBSON, E. B. TITCHENER AND S. J. WAKIL, *J. Am. Chem. Soc.*, 80 (1958) 2908.
4 D. M. GIBSON, E. B. TITCHENER AND S. J. WAKIL, *Biochim. Biophys. Acta*, 30 (1958) 376.
5 S. J. WAKIL, E. B. TITCHENER AND D. M. GIBSON, *Biochim. Biophys. Acta*, 29 (1958) 225.
6 W. SEUBERT, F. FREULL AND F. LYNEN, *Angew. Chem.*, 69 (1957) 359.
7 J. W. PORTER AND A. TIETZ, *Biochim. Biophys. Acta*, 25 (1957) 41.
8 W. E. COHN AND C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 4273.
9 H. O. LOWRY AND J. A. LOPEZ, *J. Biol. Chem.*, 162 (1946) 421.
10 P. HELE, *J. Biol. Chem.*, 206 (1954) 671.
11 M. E. JONES, S. BLACK, R. M. FLYNN AND F. LIPMANN, *Biochim. Biophys. Acta*, 12 (1953) 141.
12 N. O. KAPLAN, S. P. COLOWICK AND E. F. NEUFELD, *J. Biol. Chem.*, 205 (1953) 1.
13 P. HELE, G. POPJAK AND M. LAURYSSENS, *Biochem. J.*, 65 (1957) 348.
14 R. O. BRADY AND S. GURIN, *J. Biol. Chem.*, 199 (1952) 421.
15 J. VAN BAALEN AND S. GURIN, *J. Biol. Chem.*, 205 (1953) 303.
16 D. E. GREEN, *Biol. Revs. Cambridge Phil. Soc.*, 29 (1954) 330.
17 F. LYNEN AND S. OCHOA, *Biochim. Biophys. Acta*, 12 (1953) 299.
18 R. G. LANGDON, *J. Biol. Chem.*, 226 (1957) 615.
19 H. R. MAHLER, S. J. WAKIL AND R. M. BOCK, *J. Biol. Chem.*, 204 (1953) 453.
20 S. J. WAKIL AND D. M. GIBSON, in preparation.
21 S. J. WAKIL, *J. Am. Chem. Soc.*, 80 (1958) 6465.