MASTER

FINAL TECHNICAL REPORT - COO-1750-03

November 1, 1969 - January 31, 1971

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Prepared by Donald K. Dougall

The Technical Progress Reports No. COO-1750-01 for November 1, 1967 to June 30, 1968 and No. COO-1750-02 for July 1, 1968 to June 30, 1969 are integral parts of this Final Technical Report and will be considered together with the progress made from July 1, 1969 to January 31, 1971.

EFFORT (July 1, 1969 to January 31, 1971):

Persons who have participated in the investigation described in this report are Dr. Pei Hsing Wu (1 month at 80%), Sheila Long (14 months at 71%), Harry Sommer M.S. (17 months at 51%) and Dr. D. K. Dougall (17 months at 22%).

SUMMARY

Extensive attempts to demonstrate the presence of acyl-CoA synthetase, acyl-CoA:glutamate acyl-transferase and acetyl-glutamate kinase in extracts of Paul's Scarlet Rose and other tissues have not been successful. Experiments in which 1-¹⁴C-glutamic acid was fed to tissue in attempts to show that externally supplied glutamic acid is a significant source of ornithine and arginine have led to the conclusion that the major fate of externally supplied glutamic acid is decarboxylation and that some of the CO₂ produced is re-incorporated into the ureido-group of arginine.

Attempts to trace the synthesis of arginine from glutamic acid using radioactive glutamic acid or by verification of predicted enzymes seems to be unprofitable. More profitable would seem to be the purification of acetylornithine transaminase and use of it as a generator of acetyl glutamic semialdehyde to search for the preceding step in extracts of tissue.

The data obtained indicate that the intermediates in ornithine biosynthesis are acetylated.

The attempts to isolate acetyl-glutamate kinase from *E. coli* to provide a system for the generation of acetyl-glutamate phosphate for the substrate to search for acetyl-glutamic semialdehyde dehydrogenase were not successful. The data obtained indicate quite clearly that the enzyme obtained by published procedures is acetate kinase and not acetyl-glutamate kinase.

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INVESTIGATIONS PERFORMED (July 1, 1969 to January 31, 1971)

Acyl CoA Synthetases.

Using the method of Steer and Gibbs, 1969 (Plant Physiology, 44:775-780), small amounts of succinyl-CoA synthetase could be detected in one of three extracts of Black Valentine Beans. Small activity of acetyl-CoA synthetase could also be detected in one such experiment. Crude extracts from Paul's Scarlet Rose tissue prepared and assayed for succinyl-CoA synthetase by Steer and Gibbs methods and assayed for acetyl-CoA synthetase by replacing succinate with acetate gave no indication of the desired activity in two experiments.

Acetyl-CoA:Glutamate Acetyl-Transferase.

Crude extracts were prepared from seedlings of bean, sunflower, corn, castor bean, radish and pea seedlings by grinding in a mortar using 0.1% bovine serum albumin, 0.05% cysteine and 1mM EDTA at pH 7.2. The material precipitating at 70% saturated ammonium sulphate was redissolved in 1/10 volume of buffer containing 2×10^{-3} M potassium phosphate and 10^{-4} M dithiothreitol at pH 7.2 and dialysed overnight against the same buffer.

These preparations were assayed for acetyl-transferase activity using 1-C¹⁴-glutamic acid as described in Technical Progress Report COO-1750-02. Results are shown in Table I.

TABLE I

Assay of Acetyl-CoA:Glutamate Acetyl-Transferase in extracts of Plant Tissues.

Plant	Initial cpm.	cpm. not absorbed	
		+Acetyl CoA	-Acetyl CoA
Bean	138,000	1270, 1110	1490, 1080
Sunflower	138,000	2210, 2780	1710, 2400
Corn	138,000	1400, 943	1380, 1380
Pea	75,000	2490, 2460	1590, 1570
Bean	75,000	1640, 1630	740, 780
Radish	68,000	3730, 3620	2490, 2460

Acetyl-Glutamate Kinase.

a. From Paul's Scarlet Rose Tissue. The effects of buffers (HEPES, BICINE, TRIS), sucrose, polyclar AT and 2-mercaptoethanol or dithiothreitol on the activity of acetyl-glutamate kinase in crude extracts of Paul's Scarlet Rose were examined. The best combination seemed to be the use of 1 ml/g.F.wt. of 0.1 M BICINE pH 7.4 and 0.1 g/g.F. wt of polyclar AT. Results with this extraction buffer are shown in Table II.

TABLE II

Acetyl-Glutamate Kinase from Paul's Scarlet Rose.

	OD ₅₄₀ x 1000 0 hrs.	1 hr.	ΔOD ₅₄₀ x 1000
Crude Extracts			
Complete Assay	78	120	42
-Acetyl-Glutamate	51	109	58
After Sephadex G-50 Treatment			
Complete Assay	26	61	35
-Acetyl-Glutamate	49	60	21

Assayed by hydroxamate method as described in Tech. Prog. Rep. COO-1750-02. Sephadex Treatment in 5mM BICINE pH 7.2.

An attempt was made to detect acetyl-glutamate kinase in extracts of peas and beans. Seedlings were ground in 0.1 M BICINE pH 7.4 and centrifuged. The material in the supernatant which was not soluble in 70% saturation ammonium sulphate was redissolved in 2.5 mM phosphate + 10^{-3} dithiothreitol at pH 7.2, dialysed overnight against the same buffer and assayed. The results are in Table III.

TABLE III

Acetyl-Glutamate Kinase in Peas and Beans.

	Mean OD ₅₄₀ x 1000
Pea + Acetyl-Glutamic Acid	31
+ Acetyl-Glutamine	26
None.	27
Beans + Acetyl-Glutamic Acid	57
+ Acetyl-Glutamine	56
None	53

Hydroxamate assays as in Tech. Prog. Rept. COO-1750-02.

Acetyl-glutamine was tested here because of the possibility that acetyl-glutamate kinase by analogy with glutamine synthetase may catalyse several reactions or use different substrates for the synthesis of gamma N-acetyl-glutamyl phosphate.

Following the parallel between glutamine synthesis and acetyl-glutamate kinase, crude extracts of Paul's Scarlet Rose tissue which are known to have glutamine synthetase activity (unpublished) were prepared and the ability of these extracts to catalyse hydroxamate formation with different substrates was examined. The results are shown in Table IV.

TABLE IV

Substrate Specificity of Glutamine Synthetase
from Paul's Scarlet Rose Tissue.

Substrate	Mean OD ₅₄₀ x 10 ³	
	1 hr.	0 hrs.
None	92	50
Glutamic Acid	171	
Acetyl-Glutamic Acid	91	
Formyl-Glutamic Acid	93	
Pyroglutamic Acid	102	

B. From E. coli. Dialysed crude extracts from E. coli gave insignificant changes in optical density in the hydroxamate assay (Baich and Vogel, 1962. Biochem. Biophys. Res. Comm. 7: 491-496) for acetyl-glutamate kinase either with increasing substrate or increasing time of incubation. The effect of increasing acetate in place of acetyl-glutamate is shown in Table V.

TABLE V

Acetate Kinase in dialysed crude extracts of
E. coli - substrate dependence.

mls. 0.15M Na Acetate	Mean OD ₅₄₀ x 10 ³
0	17
0.05	428
0.10	593
0.15	737
0.25	858
0.35	939

Attempts were made to separate the acetate kinase from any acetyl-glutamate kinase in extracts of E. coli K12 by ammonium sulphate fractionation. The results are shown in Table VI.

TABLE VI

Attempted separation of Acetate and Acetyl-Glutamate
Kinase of E. coli by Ammonium Sulphate

Fraction	Δ OD ₅₄₀ x 10 ³	
	Acetate	Acetyl-Glutamate
Crude extract	688	39
0-20% sat.	387	17
20-40% sat.	682	37
40-60% sat.	753	44
60-100% sat.	276	14

When an extract of E. coli was prepared and fractionated by ammonium sulphate and chromatography on DEAE-cellulose as described by Baich and Vogel (1962), only very low acetyl-glutamate kinase could be detected. The active fractions were combined, precipitated with ammonium sulphate and dialysed overnight. This material was used to test for the presence of inhibitors in extracts of Paul's Scarlet Rose tissue. The results are shown in Table VII.

TABLE VII

Inhibition of E. coli kinases by extracts of Paul's Scarlet Rose tissue

Source	Mean $\Delta OD_{540} \times 10^3$	
	+Acetyl-glutamate	+Acetate
<u>E. coli</u>	17	337
" +PSR crude	6	175
PSR crude	0	0
<u>E. coli</u>	17	323
" +PSR (sephadex treated)	12	254
PSR (sephadex treated)	0	0
<u>E. coli</u>	12	308
" +PSR (0-50% sat. $(NH_4)_2SO_4$)	12	281
PSR (0-50% sat. $(NH_4)_2SO_4$)	0	0
<u>E. coli</u>	10	350
" +PSR (50-100% sat. $(NH_4)_2SO_4$)	20	304
PSR (50-100% sat. $(NH_4)_2SO_4$)	0	0

The data in Table VII support the following contentions

- i) The E. coli enzyme is an acetate kinase
- ii) The crude extracts of Paul's Scarlet Rose inhibit the E. coli
- iii) Sephadex treatment or ammonium sulphate fractionation and dialysis remove the inhibitor
- iv) Neither acetate nor acetyl-glutamate kinase can be detected in extracts of Paul's Scarlet Rose.

N-Acetyl Substituted Intermediates

The specificity of the arginine synthesizing enzymes for acetyl substituted intermediates was examined using the reversible reaction catalysed by acetyl-ornithine:glutamate transacetylase and measuring the disappearance of ornithine. Crude extracts of Paul's Scarlet Rose prepared by breaking the cells in $10^{-2}M$ TRIS- PO_4 pH 7.5 and $10^{-4}M$ EDTA (1 ml/g f. wt) with 10% w/w polyclar AT and glass beads. The assay mixture contained in a final volume of 0.5 ml, 0.05 M TRIS- PO_4 pH 7.5, $4 \times 10^{-4}M$ ornithine, 0.02M N-acetyl-glutamate and 0.1 ml crude extract. The results are shown in Table VIII.

TABLE VIII

Specificity of Acetyl-Ornithine:glutamate Transacetylase.

	Mean OD x 10 ³		Δ OD
	0 hrs.	1 hr.	
Acetyl-glutamate + ornithine	711	550	-161
Acetyl-glutamate	220	180	-40
Formyl-glutamate + ornithine	740	680	-60
Formyl-glutamate	280	220	-60
Pyroglutamic acid + ornithine	720	650	-70
Pyroglutamic acid	220	175	-45
*Acetyl-ornithine + glutamate	290	515	+225
*Acetyl-ornithine	240	185	-55

*Assay in the reverse direction as in Tech. Prog. Rept. COO-1750-02.

The data in Table VIII indicate that acetyl-ornithine:glutamate transacetylase is relatively specific for acetyl substituents and implies that the intermediates in ornithine biosynthesis are acetylated.

The presence of acetyl-ornithine:glutamate transacetylase and acetyl-ornithine:glutamate transaminase in extracts of Paul's Scarlet Rose tissue allowed the design of another type of experiment to test for acetyl-glutamic semialdehyde dehydrogenase and acetyl-glutamic kinase in the extracts. These enzymes acting in reverse should in suitable conditions oxidize ornithine-C¹⁴ to acetyl-glutamic acid-C¹⁴. Ornithine-C¹⁴ will be retained by Dowex-50 at pH 7 while acetyl-glutamic acid would not, thus providing a method for estimating the extent of the reaction. The incubation mixture (1 ml) contained L-ornithine-1-C¹⁴ (100,000 cpm), sodium arsenate (10⁻³M), NAD (1mg), NADP (1mg), acetyl-glutamic acid (10⁻³M), 25 mM TRIS-PO₄⁻⁻⁻ pH 7.2 and 0.2 ml crude extract prepared as described in Tech. Prog. Rept. COO-1750-02 "Acetyl-ornithine:glutamate acetyltransferase" p. 9. The reaction was terminated by boiling and the supernatant passed through Dowex-50 and washed with water. The results obtained were:

Time of Incubation	0 hrs.	1 hr
cpm not absorbed on Dowex-50	430	420
cpm eluted with 2N NH ₄ OH	66,400	62,200

This data provides no evidence for the production of acetyl-glutamic acid by the extract.

Feeding Experiments with Radioactive Glutamic Acid

In further work on the labeling of free amino acids by 1-C¹⁴;glutamic acid, Dr. Wu showed that citrulline and arginine but not ornithine became labeled. The radioactive arginine was

recovered from thin layer chromatograms, diluted with carrier, and degraded by treatment with urease. Both ornithine and urea were detected and only the urea was radioactive. This result suggests that C^{14} from glutamate is being incorporated into arginine via carbon dioxide. Decarboxylation of glutamic acid occurs rapidly in the cultures. It is clear that ornithine is unlabeled in these cultures. However, it is not clear whether a large pool of ornithine is present at the beginning of the experiments or if it is synthesized from endogenous but unlabeled glutamic acid or from a compound other than glutamic acid.

A feeding experiment using $l\text{-}C^{14}$ and $U\text{-}C^{14}$ -glutamic acids was performed over longer periods of time to see if substantial radioactivity would be incorporated into either free basic-amino acid or basic-amino acids in protein. Paul's Scarlet Rose Tissue was incubated with either $l\text{-}C^{14}$ or $U\text{-}C^{14}$ -glutamic acid, samples taken at intervals, fractionated, and counted. The outline of the fractionation is as shown

SCHEME OF TISSUE FRACTIONATION

The medium and the TCA extract were each further fractionated as below:

The results are shown in Table IX for I-C¹⁴-glutamic acid and in Table X for U-C¹⁴-glutamic acid. The conclusions to be drawn from this data is that in both cases:

- 1) Very little of the externally supplied glutamic acid reaches protein either as glutamic acid or as other compounds.
- 2) The glutamic acids are rapidly converted to amides intracellularly.
- 3) By far the largest amount of radioactivity is lost from the cultures presumably as CO₂.
- 4) Because so little radioactivity appeared in either neutral compounds or neutral and basic amino acids, the externally supplied compounds are not entering the general metabolism of the cells.

TABLE IX

Distribution of Radioactivity From 1-C¹⁴-Glutamate
In Paul's Scarlet Rose Cell Cultures.

Hours Incubation	0	3	7-1/2	17-1/2	25-1/2
<u>Culture Medium</u> (Total)	324,450	152,000	25,600	10,300	13,100
Acids	175,200	101,600	18,500	7,500	6,800
Amide	24,400	7,300	400	-	700
Neutral & Basic Amino Acids	15,000	7,900	400	500	400
Neutral	2,600	1,400	200	240	200
<u>TCA extract</u> (Total)	5,700	65,300	98,600	46,950	21,150
Acids	300	2,300	5,900	4,200	1,700
Amides	-	42,300	56,200	27,300	11,000
Neutral & Basic Amino Acids	-	-	160	-	240
Neutral	1,600	960	3,480	920	480
<u>Residue</u> (Total)	325	1,300	4,075	5,500	5,675
Acids	150	300	1,350	1,650	1,500
Neutral & Basic Amino Acids	-	240	1,920	2,080	2,400
Neutral	50	350	50	-	25

TABLE X

Distribution of Radioactivity from U-C¹⁴-Glutamate
In Paul's Scarlet Rose Cell Cultures

Hours Incubation	0	3	7-1/2	17-1/2	25-1/2
<u>Culture Medium</u> (Total)	254,800	192,000	65,400	27,750	34,200
Acids	190,400	124,500	53,600	20,600	23,500
Amides	4,700	9,100	1,300	-	1,600
Neutral & Basic Amino Acids	11,000	7,600	300	600	1,500
Neutral	2,280	1,600	160	200	1,100
<u>TCA extract</u> (Total)	8,850	72,350	136,750	116,650	73,300
Acids	800	6,400	16,700	20,000	10,300
Amides	3,600	42,500	79,300	62,900	39,100
Neutral and Basic Amino Acids	240	80	160	480	480
Neutral	360	1,880	1,880	2,220	1,720
<u>Residue (Total)</u>	-	1,100	4,150	8,225	8,675
Acids	50	300	1,450	2,000	2,200
Basic & Neutral Amino Acids	-	80	1,120	2,000	2,200
Neutral	0	80	950	3,340	4,240

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These preparations were assayed for acetyl-transferase activity using 1-C¹⁴-glutamic acid as described in Technical Progress Report COO-1750-02. Results are shown in Table I.

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Assay of Acetyl-CoA:Glutamate Acetyl-Transferase in extracts of Plant Tissues.

Plant	Initial cpm.	cpm. not absorbed on Dowex -50	
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Acetyl-Glutamate Kinase.

a. From Paul's Scarlet Rose Tissue. The effects of buffers (HEPES, BICINE, TRIS), sucrose, polyclar AT and 2-mercaptoethanol or dithiothreitol on the activity of acetyl-glutamate kinase in crude extracts of Paul's Scarlet Rose were examined. The best combination seemed to be the use of 1 ml/g.F.wt. of 0.1 M BICINL pH 7.4 and 0.1 g/g.F. wt of polyclar AT. Results with this extraction buffer are shown in Table II.

TABLE II

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	OD ₅₄₀ x 1000		ΔOD ₅₄₀ x 1000
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Assayed by hydroxamate method as described in Tech. Prog. Rep. COO-1750-02. Sephadex Treatment in 5mM BICINE pH 7.2.

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Hydroxamate assays as in Tech. Prog. Rept. COO-1750-02.

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TABLE IV

Substrate Specificity of Glutamine Synthetase
from Paul's Scarlet Rose Tissue.

Substrate	Mean OD ₅₄₀ x 10 ³	
	1 hr.	8 hrs.
None	92	50
Glutamic Acid	171	
Acetyl-Glutamic Acid	91	
Formyl-Glutamic Acid	93	
Pyroglutamic Acid	102	

B. From E. coli. Dialysed crude extracts from E. coli gave insignificant changes in optical density in the hydroxamate assay (Baich and Vogel, 1962. Biochem. Biophys. Res. Comm. 7: 491-496) for acetyl-glutamate kinase either with increasing substrate or increasing time of incubation. The effect of increasing acetate in place of acetyl-glutamate is shown in Table V.

TABLE V

Acetate Kinase in dialysed crude extracts of
E. coli - substrate dependence.

mls. 0.15M Na Acetate	Mean OD ₅₄₀ x 10 ³
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0.05	428
0.10	593
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Attempts were made to separate the acetate kinase from any acetyl-glutamate kinase in extracts of E. coli K12 by ammonium sulphate fractionation. The results are shown in Table VI.

TABLE VI

Attempted separation of Acetate and Acetyl-Glutamate
Kinase of E. coli by Ammonium Sulphate

Fraction	Δ OD ₅₄₀ x 10 ³	
	Acetate	Acetyl-Glutamate
Crude extract	688	39
0-20% sat.	387	17
20-40% sat.	682	37
40-60% sat.	753	44
60-100% sat.	276	14

When an extract of *E. coli* was prepared and fractionated by ammonium sulphate and chromatography on DEAE-cellulose as described by Baich and Vogel (1962), only very low acetyl-glutamate kinase could be detected. The active fractions were combined, precipitated with ammonium sulphate and dialysed overnight. This material was used to test for the presence of inhibitors in extracts of Paul's Scarlet Rose tissue. The results are shown in Table VII.

TABLE VII

Inhibition of *E. coli* kinases by extracts of Paul's Scarlet Rose tissue

Source	Mean $\Delta OD_{540} \times 10^3$	
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The data in Table VII support the following contentions

- i) The *E. coli* enzyme is an acetate kinase
- ii) The crude extracts of Paul's Scarlet Rose inhibit the *E. coli*
- iii) Sephadex treatment or ammonium sulphate fractionation and dialysis remove the inhibitor
- iv) Neither acetate nor acetyl-glutamate kinase can be detected in extracts of Paul's Scarlet Rose.

N-Acetyl Substituted Intermediates

The specificity of the arginine synthesizing enzymes for acetyl substituted intermediates was examined using the reversible reaction catalysed by acetyl-ornithine:glutamate transacetylase and measuring the disappearance of ornithine. Crude extracts of Paul's Scarlet Rose prepared by breaking the cells in $10^{-2}M$ TRIS- PO_4 pH 7.5 and $10^{-4}M$ EDTA (1 ml/g f. wt) with 10% w/w polyclar AT and glass beads. The assay mixture contained in a final volume of 0.5 ml, 0.05 M TRIS- PO_4 pH 7.5, $4 \times 10^{-4}M$ ornithine, 0.02M N-acetyl-glutamate and 0.1 ml crude extract. The results are shown in Table VIII.

TABLE VIII

Specificity of Acetyl-Ornithine:glutamate Transacetylase.

	Mean OD x 10 ⁵		Δ OD
	0 hrs.	1 hr.	
Acetyl-glutamate + ornithine	711	550	-161
Acetyl-glutamate	220	180	-40
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The data in Table VIII indicate that acetyl-ornithine:glutamate transacetylase is relatively specific for acetyl substituents and implies that the intermediates in ornithine biosynthesis are acetylated.

The presence of acetyl-ornithine:glutamate transacetylase and acetyl-ornithine:glutamate transaminase in extracts of Paul's Scarlet Rose tissue allowed the design of another type of experiment to test for acetyl-glutamic semialdehyde dehydrogenase and acetyl-glutamic kinase in the extracts. These enzymes acting in reverse should in suitable conditions oxidize ornithine-C¹⁴ to acetyl-glutamic acid-C¹⁴. Ornithine-C¹⁴ will be retained by Dowex-50 at pH 7 while acetyl-glutamic acid would not, thus providing a method for estimating the extent of the reaction. The incubation mixture (1 ml) contained L-ornithine-1-C¹⁴ (100,000 cpm), sodium arsenate (10⁻³M), NAD (1mg), NADP (1mg), acetyl-glutamic acid (10⁻³M), 25 mM TRIS-PO₄⁻⁻⁻ pH 7.2 and 0.2 ml crude extract prepared as described in Tech. Prog. Rept. COO-1750-02 "Acetyl-ornithine:glutamate acetyltransferase" p. 9. The reaction was terminated by boiling and the supernatant passed through Dowex-50 and washed with water. The results obtained were:

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A feeding experiment using $1-C^{14}$ and $U-C^{14}$ -glutamic acids was performed over longer periods of time to see if substantial radioactivity would be incorporated into either free basic-amino acid or basic-amino acids in protein. Paul's Scarlet Rose Tissue was incubated with either $1-C^{14}$ or $U-C^{14}$ -glutamic acid, samples taken at intervals, fractionated, and counted. The outline of the fractionation is as shown

SCHEME OF TISSUE FRACTIONATION

The medium and the TCA extract were each further fractionated as below:

The results are shown in Table IX for I-C¹⁴-glutamic acid and in Table X for U-C¹⁴-glutamic acid. The conclusions to be drawn from this data is that in both cases:

- 1) Very little of the externally supplied glutamic acid reaches protein either as glutamic acid or as other compounds.
- 2) The glutamic acids are rapidly converted to amides intracellularly.
- 3) By far the largest amount of radioactivity is lost from the cultures presumably as CO₂.
- 4) Because so little radioactivity appeared in either neutral compounds or neutral and basic amino acids, the externally supplied compounds are not entering the general metabolism of the cells.

TABLE IX

Distribution of Radioactivity From 1-C¹⁴-Glutamate
In Paul's Scarlet Rose Cell Cultures.

Hours Incubation	0	3	7-1/2	17-1/2	25-1/2
<u>Culture Medium</u> (Total)	324,450	152,000	25,600	10,300	13,100
Acids	175,200	101,600	18,500	7,500	6,800
Amide	24,400	7,300	400	-	700
Neutral & Basic Amino Acids	15,000	7,900	400	500	400
Neutral	2,600	1,400	200	240	200
<u>TCA extract</u> (Total)	5,700	65,300	98,600	46,950	21,150
Acids	300	2,300	5,900	4,200	1,700
Amides	-	42,300	56,200	27,300	11,000
Neutral & Basic Amino Acids	-	-	160	-	240
Neutral	1,600	960	3,480	920	480
<u>Residue</u> (Total)	325	1,300	4,075	5,500	5,675
Acids	150	300	1,350	1,650	1,500
Neutral & Basic Amino Acids	-	240	1,920	2,080	2,400
Neutral	50	350	50	-	25

TABLE X
Distribution of Radioactivity from U-C¹⁴-Glutamate
In Paul's Scarlet Rose Cell Cultures

Hours Incubation	0	3	7-1/2	17-1/2	25-1/2
<u>Culture Medium</u> (Total)	254,800	192,000	65,400	27,750	34,200
Acids	190,400	124,500	53,600	20,600	23,500
Amides	4,700	9,100	1,300	-	1,600
Neutral & Basic Amino Acids	11,000	7,600	300	600	1,500
Neutral	2,280	1,600	160	200	1,100
<u>TCA extract</u> (Total)	8,850	72,350	136,750	116,650	73,300
Acids	800	6,400	16,700	20,000	10,300
Amides	3,600	42,500	79,300	62,900	39,100
Neutral and Basic Amino Acids	240	80	160	480	480
Neutral	360	1,880	1,880	2,220	1,720
<u>Residue (Total)</u>	-	1,100	4,150	8,225	8,675
Acids	50	300	1,450	2,000	2,200
Basic & Neutral Amino Acids	-	80	1,120	2,000	2,200
Neutral	0	80	950	3,340	4,240

FINAL TECHNICAL REPORT - COO-1750-03

November 1, 1969 - January 31, 1971

Submitted to the U. S. Atomic Energy Commission
Contract number AT(11-1)-1750.

Prepared by Donald K. Dougall

The Technical Progress Reports No. COO-1750-01 for November 1, 1967 to June 30, 1968 and No. COO-1750-02 for July 1, 1968 to June 30, 1969 are integral parts of this Final Technical Report and will be considered together with the progress made from July 1, 1969 to January 31, 1971.

EFFORT (July 1, 1969 to January 31, 1971):

Persons who have participated in the investigation described in this report are Dr. Pei Hsing Wu (1 month at 80%), Sheila Long (14 months at 71%), Harry Sommer M.S. (17 months at 51%) and Dr. D. K. Dougall (17 months at 22%).

SUMMARY

Extensive attempts to demonstrate the presence of acyl-CoA synthetase, acyl-CoA:glutamate acyl-transferase and acetyl-glutamate kinase in extracts of Paul's Scarlet Rose and other tissues have not been successful. Experiments in which 1-¹⁴C-glutamic acid was fed to tissue in attempts to show that externally supplied glutamic acid is a significant source of ornithine and arginine have led to the conclusion that the major fate of externally supplied glutamic acid is decarboxylation and that some of the CO₂ produced is re-incorporated into the ureido-group of arginine.

Attempts to trace the synthesis of arginine from glutamic acid using radioactive glutamic acid or by verification of predicted enzymes seems to be unprofitable. More profitable would seem to be the purification of acetylornithine transaminase and use of it as a generator of acetyl glutamic semialdehyde to search for the preceding step in extracts of tissue.

The data obtained indicate that the intermediates in ornithine biosynthesis are acetylated.

The attempts to isolate acetyl-glutamate kinase from *E. coli* to provide a system for the generation of acetyl-glutamate phosphate for the substrate to search for acetyl-glutamic semialdehyde dehydrogenase were not successful. The data obtained indicate quite clearly that the enzyme obtained by published procedures is acetate kinase and not acetyl-glutamate kinase.

INVESTIGATIONS PERFORMED (July 1, 1969 to January 31, 1971)

Acyl CoA Synthetases.

Using the method of Steer and Gibbs, 1969 (Plant Physiology, 44:775-780), small amounts of succinyl-CoA synthetase could be detected in one of three extracts of Black Valentine Beans. Small activity of acetyl-CoA synthetase could also be detected in one such experiment. Crude extracts from Paul's Scarlet Rose tissue prepared and assayed for succinyl-CoA synthetase by Steer and Gibbs methods and assayed for acetyl-CoA synthetase by replacing succinate with acetate gave no indication of the desired activity in two experiments.

Acetyl-CoA:Glutamate Acetyl-Transferase.

Crude extracts were prepared from seedlings of bean, sunflower, corn, castor bean, radish and pea seedlings by grinding in a mortar using 0.1% bovine serum albumin, 0.05% cysteine and 1mM EDTA at pH 7.2. The material precipitating at 70% saturated ammonium sulphate was redissolved in 1/10 volume of buffer containing 2×10^{-3} M potassium phosphate and 10^{-4} M dithiothreitol at pH 7.2 and dialysed overnight against the same buffer.

These preparations were assayed for acetyl-transferase activity using 1-C¹⁴-glutamic acid as described in Technical Progress Report COO-1750-02. Results are shown in Table I.

TABLE I

Assay of Acetyl-CoA:Glutamate Acetyl-Transferase in extracts of Plant Tissues.

Plant	Initial cpm.	cpm. not absorbed	
		+Acetyl CoA	-Acetyl CoA
Bean	138,000	1270, 1110	1490, 1080
Sunflower	138,000	2210, 2780	1710, 2400
Corn	138,000	1400, 943	1380, 1380
Pea	75,000	2490, 2460	1590, 1570
Bean	75,000	1640, 1630	740, 780
Radish	68,000	3730, 3620	2490, 2460

Acetyl-Glutamate Kinase.

a. From Paul's Scarlet Rose Tissue. The effects of buffers (HEPES, BICINE, TRIS), sucrose, polyclar AT and 2-mercaptoethanol or dithiothreitol on the activity of acetyl-glutamate kinase in crude extracts of Paul's Scarlet Rose were examined. The best combination seemed to be the use of 1 ml/g.F.wt. of 0.1 M BICINE pH 7.4 and 0.1 g/g.F. wt of polyclar AT. Results with this extraction buffer are shown in Table II.

TABLE II

Acetyl-Glutamate Kinase from Paul's Scarlet Rose.

	OD ₅₄₀ x 1000		ΔOD ₅₄₀ x 1000
	0 hrs.	1 hr.	
Crude Extracts			
Complete Assay	78	120	42
-Acetyl-Glutamate	51	109	58
After Sephadex G-50 Treatment			
Complete Assay	26	61	35
-Acetyl-Glutamate	49	60	21

Assayed by hydroxamate method as described in Tech. Prog. Rep. COO-1750-02. Sephadex Treatment in 5mM BICINE pH 7.2.

An attempt was made to detect acetyl-glutamate kinase in extracts of peas and beans. Seedlings were ground in 0.1 M BICINE pH 7.4 and centrifuged. The material in the supernatant which was not soluble in 70% saturation ammonium sulphate was redissolved in 2.5 mM phosphate + 10^{-3} dithiothreitol at pH 7.2, dialysed overnight against the same buffer and assayed. The results are in Table III.

TABLE III

Acetyl-Glutamate Kinase in Peas and Beans.

	Mean OD ₅₄₀ x 1000
Pea + Acetyl-Glutamic Acid	31
+ Acetyl-Glutamine	26
None.	27
Beans + Acetyl-Glutamic Acid	57
+ Acetyl-Glutamine	56
None	53

Hydroxamate assays as in Tech. Prog. Rept. COO-1750-02.

Acetyl-glutamine was tested here because of the possibility that acetyl-glutamate kinase by analogy with glutamine synthetase may catalyse several reactions or use different substrates for the synthesis of gamma N-acetyl-glutamyl phosphate.

Following the parallel between glutamine synthesis and acetyl-glutamate kinase, crude extracts of Paul's Scarlet Rose tissue which are known to have glutamine synthetase activity (unpublished) were prepared and the ability of these extracts to catalyse hydroxamate formation with different substrates was examined. The results are shown in Table IV.

TABLE IV

Substrate Specificity of Glutamine Synthetase
from Paul's Scarlet Rose Tissue.

Substrate	Mean OD ₅₄₀ x 10 ³	
	1 hr.	0 hrs.
None	92	50
Glutamic Acid	171	
Acetyl-Glutamic Acid	91	
Formyl-Glutamic Acid	93	
Pyroglutamic Acid	102	

B. From E. coli. Dialysed crude extracts from E. coli gave insignificant changes in optical density in the hydroxamate assay (Baich and Vogel, 1962. Biochem. Biophys. Res. Comm. 7: 491-496) for acetyl-glutamate kinase either with increasing substrate or increasing time of incubation. The effect of increasing acetate in place of acetyl-glutamate is shown in Table V.

TABLE V

Acetate Kinase in dialysed crude extracts of
E. coli - substrate dependence.

mls. 0.15M Na Acetate	Mean OD ₅₄₀ x 10 ³
0	17
0.05	428
0.10	593
0.15	737
0.25	858
0.35	939

Attempts were made to separate the acetate kinase from any acetyl-glutamate kinase in extracts of E. coli K12 by ammonium sulphate fractionation. The results are shown in Table VI.

TABLE VI

Attempted separation of Acetate and Acetyl-Glutamate
Kinase of E. coli by Ammonium Sulphate

Fraction	Δ OD ₅₄₀ x 10 ³	
	Acetate	Acetyl-Glutamate
Crude extract	688	39
0-20% sat.	387	17
20-40% sat.	682	37
40-60% sat.	753	44
60-100% sat.	276	14

When an extract of E. coli was prepared and fractionated by ammonium sulphate and chromatography on DEAE-cellulose as described by Baich and Vogel (1962), only very low acetyl-glutamate kinase could be detected. The active fractions were combined, precipitated with ammonium sulphate and dialysed overnight. This material was used to test for the presence of inhibitors in extracts of Paul's Scarlet Rose tissue. The results are shown in Table VII.

TABLE VII

Inhibition of E. coli kinases by extracts of Paul's Scarlet Rose tissue

Source	Mean $\Delta OD_{540} \times 10^3$	
	+Acetyl-glutamate	+Acetate
<u>E. coli</u>	17	337
" +PSR crude	6	175
PSR crude	0	0
<u>E. coli</u>	17	323
" +PSR (sephadex treated)	12	254
PSR (sephadex treated)	0	0
<u>E. coli</u>	12	308
" +PSR (0-50% sat. $(NH_4)_2SO_4$)	12	281
PSR (0-50% sat. $(NH_4)_2SO_4$)	0	0
<u>E. coli</u>	10	350
" +PSR (50-100% sat. $(NH_4)_2SO_4$)	20	304
PSR (50-100% sat. $(NH_4)_2SO_4$)	0	0

The data in Table VII support the following contentions

- i) The E. coli enzyme is an acetate kinase
- ii) The crude extracts of Paul's Scarlet Rose inhibit the E. coli
- iii) Sephadex treatment or ammonium sulphate fractionation and dialysis remove the inhibitor
- iv) Neither acetate nor acetyl-glutamate kinase can be detected in extracts of Paul's Scarlet Rose.

N-Acetyl Substituted Intermediates

The specificity of the arginine synthesizing enzymes for acetyl substituted intermediates was examined using the reversible reaction catalysed by acetyl-ornithine:glutamate transacetylase and measuring the disappearance of ornithine. Crude extracts of Paul's Scarlet Rose prepared by breaking the cells in $10^{-2}M$ TRIS- PO_4 pH 7.5 and $10^{-4}M$ EDTA (1 ml/g f. wt) with 10% w/w polyclar AT and glass beads. The assay mixture contained in a final volume of 0.5 ml, 0.05 M TRIS- PO_4 pH 7.5, $4 \times 10^{-4}M$ ornithine, 0.02M N-acetyl-glutamate and 0.1 ml crude extract. The results are shown in Table VIII.

TABLE VIII

Specificity of Acetyl-Ornithine:glutamate Transacetylase.

	Mean OD x 10 ³		Δ OD
	0 hrs.	1 hr.	
Acetyl-glutamate + ornithine	711	550	-161
Acetyl-glutamate	220	180	-40
Formyl-glutamate + ornithine	740	680	-60
Formyl-glutamate	280	220	-60
Pyroglutamic acid + ornithine	720	650	-70
Pyroglutamic acid	220	175	-45
*Acetyl-ornithine + glutamate	290	515	+225
*Acetyl-ornithine	240	185	-55

*Assay in the reverse direction as in Tech. Prog. Rept. COO-1750-02.

The data in Table VIII indicate that acetyl-ornithine:glutamate transacetylase is relatively specific for acetyl substituents and implies that the intermediates in ornithine biosynthesis are acetylated.

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<u>Residue</u> (Total)	325	1,300	4,075	5,500	5,675
Acids	150	300	1,350	1,650	1,500
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Neutral	50	350	50	-	25

TABLE X

Distribution of Radioactivity from U-C¹⁴-Glutamate
In Paul's Scarlet Rose Cell Cultures

Hours Incubation	0	3	7-1/2	17-1/2	25-1/2
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Neutral	360	1,880	1,880	2,220	1,720
<u>Residue (Total)</u>	-	1,100	4,150	8,225	8,675
Acids	50	300	1,450	2,000	2,200
Basic & Neutral Amino Acids	-	80	1,120	2,000	2,200
Neutral	0	80	950	3,340	4,240