Using the FOL3 Mutant Yeast to Determine the Bioactivity of [6R]-5-Formyl-Tetrahydrofolate

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Abstract

The purpose of this research was to test certain aspects of Dr. Joseph E. Baggott's claim that [6R]-5-formyl-tetrahydrofolate (5-HCO-THF) is bioactive in humans. In order to investigate the possible bioactivity of this particular isomer, the *fol3* mutant yeast was used. This yeast was grown on YPD rich media plates in the presence of a racemic mixture of folinic acid ([6RS]-5-HCO-THF) and also the individual [6R] and [6S] isomers. The qualitative results obtained demonstrate that the [6R] isomer is not bioactive in yeast; this result brings into question the first step of Baggott's proposed pathway and therefore calls for significant further investigation.

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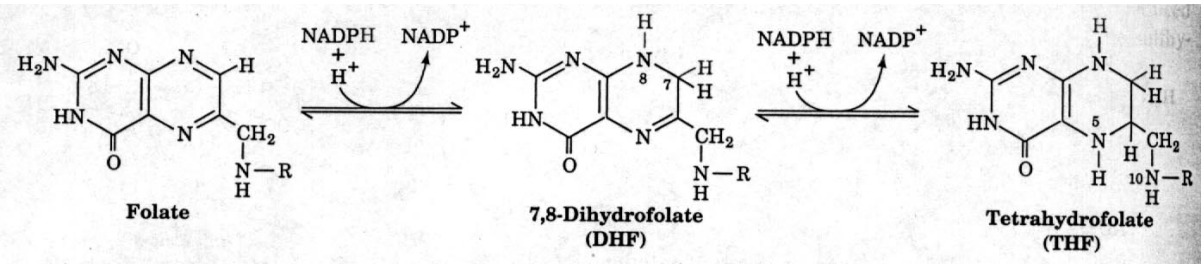
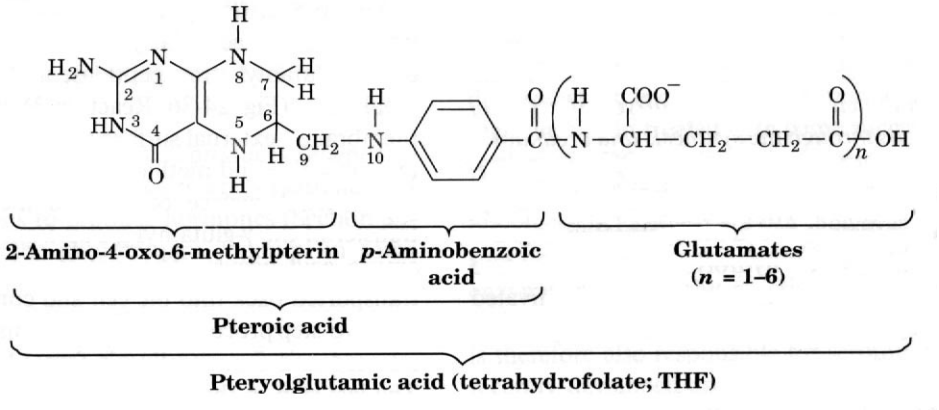
Introduction

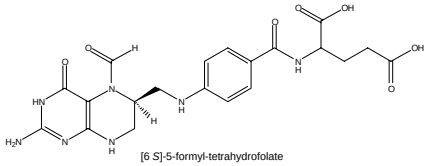
Folic acid, an essential vitamin in the mammalian diet, can be doubly reduced to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR).

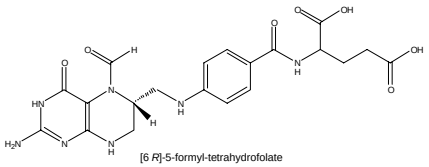


THF actively transports C_1 units which are vital for both DNA and protein synthesis. C_1 can bind to either the N5 or N10 position or both.



5-formyl-tetrahydrofolate (5-HCO-THF) is a chiral molecule and comes in either a [6S] or [6R] form.

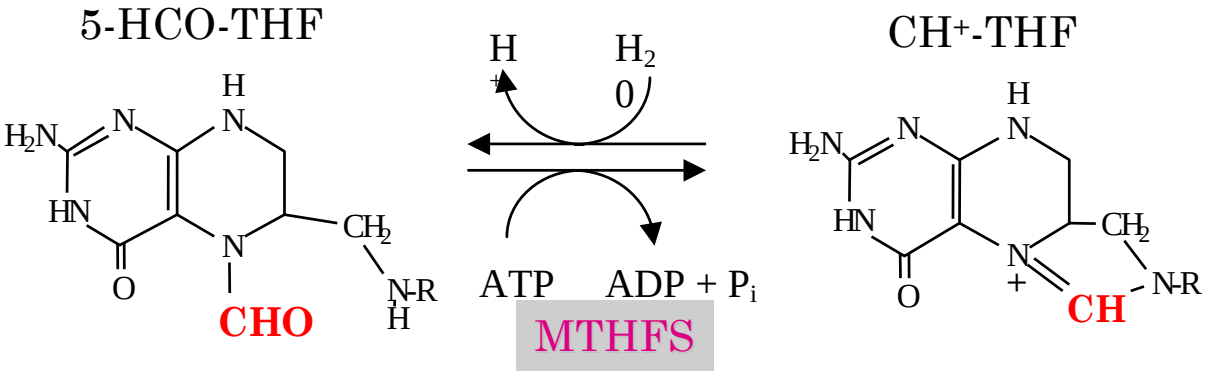

[6 *S*]-5-formyl-tetrahydrofolate


[6 *R*]-5-formyl-tetrahydrofolate

This chiral quality affects which isomer can bind to the enzyme methenyltetrahydrofolate synthetase (MTHFS). MTHFS initiates the first of several reactions that synthesize THF, and only the [6S] isomer is known to bind with MTHFS. [6S]-5-HCO-THF converts to 5,10-methenyl-THF.

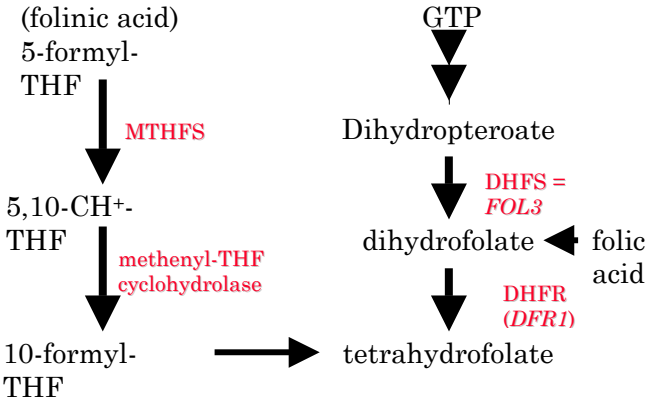
Dr. Joseph Baggott of the University of Alabama at Birmingham, however, claims that the [6R] isomer is bioactive in humans.

Because enzymes are so specific, one cannot say that MTHFS binds to [6R]-HCO-THF; instead, Baggott suggests that the reaction that takes places is chemical rather than enzymatic. Below are the chemical (non enzymatic) reaction and the enzymatic reaction involving ATP that take place.



Because Baggott's proposal deviates from a common and well-established fact in this field, it was necessary to probe the matter only this time using the *fol3* mutant yeast.

The *fol3* mutant yeast is used because the *FOL3* gene, which encodes for dihydrofolate synthase, is removed from this yeast. With this enzyme lacking, the synthesis of THF cannot be completed. This then makes the yeast dependent on folinic acid (FA; 5-HCO-THF) for growth. In a sense, the yeast takes an alternate route to synthesize THF.



Procedure

Chemicals: *Ingredients for YPD rich media plates* - Bacto-peptone, bacto-agar, and yeast extract were obtained from Difco Laboratories of Detriot, Michigan, U.S.A. Racemic mixture ([6RS]-5-HCO-THF) of folinic acid calcium salt ($C_{20}H_{21}N_7O_7Ca$) along with glucose ($C_6H_{12}O_6$) were obtained from Sigma of St. Louis, Missouri, U.S.A.

100mg of [6R]-5-HCO-tetrahydrofolic acid disodium salt and 100mg of the [6S] isomer were obtained from Eprova of Switzerland.

Concentrated hydrochloric acid and KH_2PO_4 were used for later experiments.

Preparation of YPD Plates (General): Add 1g yeast extract, 2g peptone, and 2g agar to 95mL double-distilled (DD) water in a 500mL Erlenmeyer flask. Cover the flask with foil and a small piece of autoclave tape. Autoclave the flask with the solution in the 20 minute liquid cycle (20'L). After autoclaving, add 5mL 40% glucose solution (for a final 2% glucose) to YPD solution. Pour ~20mL of solution into 5 Petri-dishes. Use a Bunsen burner to flame away any air bubbles in the solution. Cool the plates for 10 minutes by slightly uncovering the tops of the dishes. Cover again and let stand for ~30mintues before moving plates to own bench. Let the plates sit overnight. When the plates are streaked with yeast, let them incubate at 30 °C for 2-3 days.

Preparation of 100x Folinic Acid Stock (10mg/mL): Weigh out 57mg of folinic acid (racemic) and dissolve completely in 5.7mL of DD water. Filter-sterilize (f.s.) the stock through a 0.22μm filter. When making five YPD plates with this racemic mixture, add 1mL of the stock into the liquefied solution before pouring the plates.

Preparation of 5mg/mL Stock for Individual Isomers: Weigh out 5mg of each individual isomer ([6R] and [6S]) and completely dissolve each in a neutral (ph 7) f.s. buffer. KH_2PO_4 is neutral so it was used as a buffer.

Spectra Analysis of Stocks to Determine Exact Concentration: All stocks were diluted with neutral KPO_7 by a factor of 600 so that the spectrometer would more accurately determine absorbance. 500μL KH_2PO_4 was placed in the cuevette to first measure a blank. After the dilution was read, the cursor function on the computer program was used to clearly read the OD at maximum absorbance. "Results and Conclusions" will show the measured concentrations of the three different solutions.

Racemic Folinic Acid: 990 μL KH_2PO_4 was dissolved with 10 μL [6RS]-5-HCO-THF for 100x dilution. 100 μL of that solution was dissolved with 500 μL KH_2PO_4 for a final dilution of 600x. After reading the blank, 500μL of the 600x dilution was placed in the cuevette and read with the spectrometer.

Individual Isomers: 3μL of each isomer was dissolved in 297μL KH_2PO_4 for 100x dilution. 100μL of that dilution was dissolved in 500μL KH_2PO_4 for a total of 600x dilution. After reading the blank, 500μL of the 600x dilution was placed in the cuevette and read with the spectrometer.

Important Pre-Data for Calculations: Molar Mass of calcium salt of [6RS] mixture= 511.5g/mol; Molar Mass of disodium salts of [6R] or [6S] isomers= 517.40g/mol; folinic acid (either racemic or individual isomers) at ph 7 has A_{287} and

ε_{287}= 31.5mM^{-1}; 10mg/mL of racemic folinic acid is 19.6mM/L; 5mg/mL of [6S] or [6R] folinic acid is 9.66mM/L

Testing the Bioactivity of [6RS]-5-HCO-THF: One YPD plate, with an addition of the racemic folinic acid, were streaked with the *fol3* mutant yeast. After a few unsatisfactory trials which included slow growth and mold contaminants, 1.2mL of folinic acid was added to the YPD solution instead of the usual 1mL and much better growth resulted. This was because the concentration as reported by the spectrometer was lower than 10mg/mL. See "Results and Conclusions" for data and calculations on this matter.

Testing the Bioactivity of [6R] and [6S]-5-HCO-THF: Five YPD plates were streaked with the mutant yeast. However, each plate had a different addition. Each plate had one of the following: no folinic acid, 0.1mM [6RS], 0.2mM [6RS], 0.1mM [6R], and 0.1mM [6S] folinic acid. If a plate needed folinic acid in it, the FA was added to the plate right before the YPD solution was poured. See "Results and Conclusions" for pictures of the results on the plates.

Using the same method, a second experiment was performed only this time using 0.15mM and 0.2mM for both individual isomers.

Please note that the above method was a third attempt to obtain clear results. Two other different methods were used, but both methods did not give clear results.

Acid Dependent Conversion of 5-HCO-THF to 5,10-methenyl-THF: As mentioned in the introduction, Baggott proposed that [6R]-5-HCO-THF becomes active due to a chemical reaction. An experiment was performed in the lab to demonstrate that at least the reaction takes place *in vitro*. This reaction is the hypothesized first step that turns the [6R] isomer bioactive. The spectrometer was used to observe the reaction as it took place.

Racemic folinic acid was diluted by ~600 fold by dissolving 8.3 μL FA in 4991.7μL KH_2PO_4. The buffer blank was first read with 500μL in the cuevette. The 600x dilution was read next. 10μL of concentrated hydrochloric acid was then added to the diluted solution to instigate the reaction. Spectra readings were taken 0, 3, 6, 11, 16, 21, 26, 31, 36, 41, 46, and 51 minutes after the HCl was added.

The computer program used to read this data recorded a new graph each time. One graph with overlaying line curves was desired. To do so, the data was exported to an Excel spreadsheet. The data was selected and converted to an "X-Y Scatter Graph" with curved lines and no data markers. This produced the desired graph.

Results and Conclusions

Spectra Analysis of Stocks to Determine Exact Concentration:

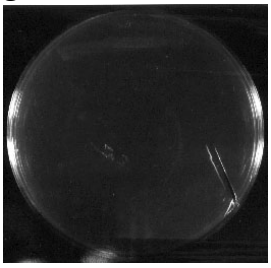
Racemic Folinic Acid: The OD at wavelength 287nm was read at 0.854. The following calculations are used to determine concentration: 0.854*600=512/31.5=**16.5mM** *(16.*5mmol/L) X (511.5mg/mmol) X (1L/1000mL)=**8.34mg/mL**

Individual Isomers: The [6R] isomer at wavelength 287nm has an OD reading of 0.537. That translates to **10.23mM** and **5.29mg/mL**. The [6S] isomer at 287nm has an OD of 0.5203 which translates to **9.91mM** and **5.13mg/mL**.

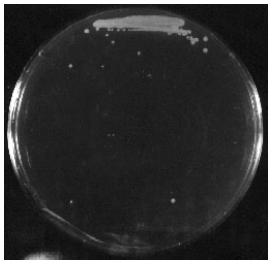
Testing the Bioactivity of [6RS]-5-HCO-THF: 1.2mL of FCA were added to the YPD solution because since the stock was not quite 10mg/mL, the calculation 10/8.34=1.2mL was used to determine an amount that would sufficiently support the mutant yeast's growth.

After a few days, there was significant growth and clearly defined individual colonies. It was obvious that the racemic mixture supports growth. The next experiment was done to determine which isomer supports growth

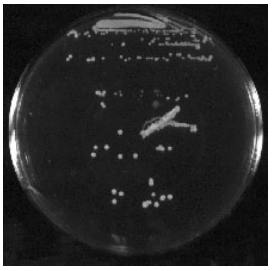
Testing the Bioactivity of [6R] and [6S]-5-HCO-THF: The following pictures show the growth results on each plate after 3 days at 30°:



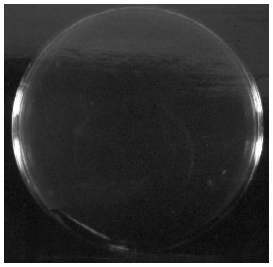
No FA



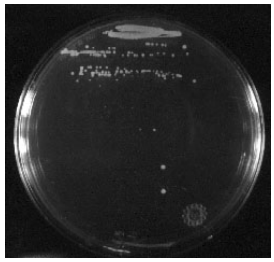
0.1 mM [6S] FA



0.2 mM [6RS] FA



0.1mM [6R] FA

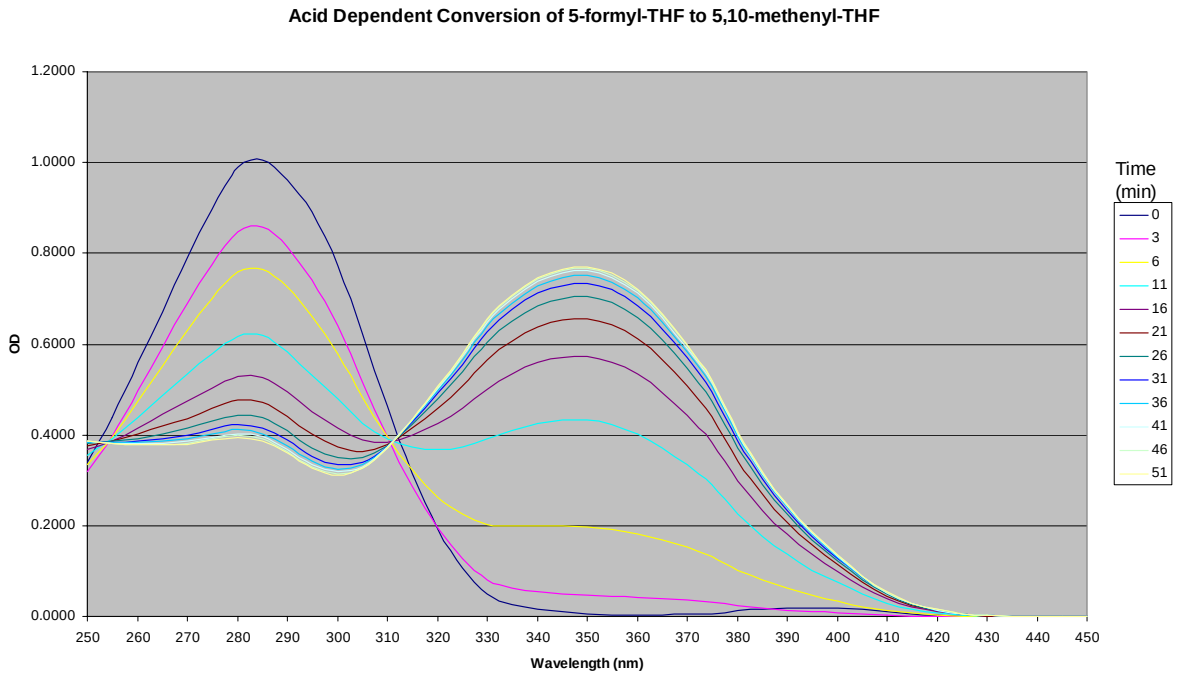


0.1mM [6RS] FA

Even when the [6R] dose was increased to 0.15mM and 0.2mM, there was still no growth response (data not shown). Obviously, the [6R] proves to be not bioactive in yeast and the [6S] isomer does.

Acid Dependent Conversion of 5-HCO-THF to 5,10-methenyl-THF: The following chart is the data that the computer program sent to Excel. The graph is what the data converts to.

Wavelength(nm)	0	3	6	11	16	21	26	31	36	41	46	51
250	0.3405	0.3199	0.3345	0.3560	0.3681	0.3761	0.3812	0.3836	0.3846	0.3854	0.3854	0.3858
255	0.4376	0.3967	0.3942	0.3906	0.3871	0.3855	0.3849	0.3838	0.3827	0.3821	0.3816	0.3811
260	0.5609	0.4985	0.4744	0.4377	0.4140	0.4005	0.3920	0.3863	0.3826	0.3802	0.3786	0.3772
265	0.6725	0.5940	0.5529	0.4869	0.4446	0.4186	0.4029	0.3925	0.3859	0.3818	0.3793	0.3773
270	0.7916	0.6904	0.6299	0.5340	0.4736	0.4365	0.4141	0.3997	0.3908	0.3854	0.3818	0.3794
275	0.8956	0.7745	0.6987	0.5790	0.5038	0.4576	0.4297	0.4120	0.4013	0.3945	0.3902	0.3875
280	0.9895	0.8484	0.7585	0.6179	0.5299	0.4760	0.4436	0.4233	0.4108	0.4030	0.3982	0.3952
285	1.0062	0.8583	0.7655	0.6186	0.5265	0.4705	0.4368	0.4159	0.4029	0.3953	0.3902	0.3869
290	0.9623	0.8134	0.7252	0.5842	0.4952	0.4414	0.4087	0.3885	0.3760	0.3687	0.3636	0.3605
295	0.8904	0.7419	0.6613	0.5321	0.4509	0.4017	0.3715	0.3529	0.3414	0.3343	0.3298	0.3268
300	0.7735	0.6409	0.5791	0.4783	0.4134	0.3734	0.3490	0.3336	0.3241	0.3182	0.3145	0.3120
305	0.6212	0.5163	0.4826	0.4260	0.3880	0.3637	0.3486	0.3391	0.3332	0.3298	0.3272	0.3258
310	0.4646	0.3965	0.3957	0.3912	0.3848	0.3796	0.3765	0.3740	0.3729	0.3723	0.3714	0.3709
315	0.3171	0.2884	0.3236	0.3740	0.4004	0.4148	0.4233	0.4277	0.4306	0.4331	0.4332	0.4337
320	0.1913	0.1943	0.2628	0.3668	0.4260	0.4598	0.4804	0.4914	0.4983	0.5032	0.5049	0.5067
325	0.1065	0.1281	0.2246	0.3729	0.4583	0.5086	0.5387	0.5554	0.5653	0.5722	0.5756	0.5775
330	0.0489	0.0813	0.2032	0.3908	0.5010	0.5659	0.6046	0.6265	0.6399	0.6481	0.6521	0.6550
335	0.0257	0.0630	0.1990	0.4092	0.5336	0.6070	0.6504	0.6759	0.6907	0.6993	0.7043	0.7071
340	0.0144	0.0537	0.1992	0.4247	0.5586	0.6379	0.6847	0.7125	0.7288	0.7383	0.7438	0.7466
345	0.0095	0.0503	0.1999	0.4322	0.5703	0.6522	0.7005	0.7293	0.7460	0.7562	0.7616	0.7653
350	0.0060	0.0473	0.1981	0.4324	0.5724	0.6555	0.7047	0.7337	0.7512	0.7613	0.7670	0.7705
355	0.0035	0.0444	0.1922	0.4221	0.5601	0.6422	0.6911	0.7195	0.7369	0.7469	0.7526	0.7562
360	0.0030	0.0419	0.1827	0.4016	0.5331	0.6115	0.6583	0.6855	0.7020	0.7120	0.7169	0.7200
365	0.0038	0.0391	0.1692	0.3715	0.4926	0.5646	0.6083	0.6326	0.6486	0.6575	0.6625	0.6653
370	0.0050	0.0351	0.1526	0.3341	0.4427	0.5080	0.5471	0.5691	0.5842	0.5914	0.5957	0.5982
375	0.0063	0.0302	0.1320	0.2897	0.3841	0.4406	0.4753	0.4940	0.5076	0.5145	0.5174	0.5205
380	0.0121	0.0230	0.1019	0.2247	0.2987	0.3433	0.3700	0.3866	0.3968	0.4015	0.4039	0.4065
385	0.0154	0.0179	0.0797	0.1761	0.2337	0.2686	0.2897	0.3025	0.3112	0.3145	0.3168	0.3184
390	0.0187	0.0135	0.0615	0.1362	0.1809	0.2080	0.2242	0.2335	0.2402	0.2433	0.2451	0.2461
395	0.0192	0.0098	0.0460	0.1023	0.1364	0.1569	0.1689	0.1763	0.1808	0.1836	0.1854	0.1860
400	0.0175	0.0069	0.0332	0.0739	0.0988	0.1134	0.1223	0.1277	0.1308	0.1326	0.1342	0.1345
405	0.0143	0.0040	0.0216	0.0487	0.0653	0.0750	0.0810	0.0845	0.0863	0.0879	0.0891	0.0891
410	0.0091	0.0018	0.0127	0.0286	0.0388	0.0446	0.0484	0.0507	0.0517	0.0523	0.0531	0.0534
415	0.0057	0.0006	0.0067	0.0155	0.0210	0.0242	0.0263	0.0276	0.0281	0.0284	0.0289	0.0291
420	0.0024	0.0000	0.0028	0.0075	0.0103	0.0119	0.0131	0.0138	0.0141	0.0142	0.0143	0.0145
425	0.0011	0.0000	0.0007	0.0029	0.0041	0.0049	0.0055	0.0059	0.0061	0.0061	0.0059	0.0062
430	0.0002	0.0000	0.0000	0.0007	0.0010	0.0012	0.0015	0.0017	0.0019	0.0019	0.0017	0.0017
435	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0003	0.0004	0.0002	0.0002	0.0001
440	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
445	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
450	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000



Discussion: In short, the experiments explained above show that the [6S]-5-HCO-THF is bioactive in yeast, while the [6R] isomer is not. The chemical conversion of folinic acid to 5,10-methenyl-THF is demonstrated *in vitro*.

A possible explanation for Baggott's results that the [6R] isomer is bioactive in humans is that an oral administration of folinic acid would come into contact with hydrochloric acid in the stomach.

A few weeks are not enough to investigate this problem. More experiments must be administered. One possible experiment that could be conducted in the future is attempting to grow yeast in a highly acidic environment; although, this may prove arduous to complete since the yeast may die soon after coming into contact with the high acidity.

Works Cited

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hirae, and cytochrome *c* oxidation of 10-formyl-tetrahydrofolate to 10-formyl-dihydrofolate."

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I'd like to also express my heartfelt appreciation to the Welch Foundation and all those involved in the program: Dr. J.J Lagowski and Ms. Anna Bergstrom specifically. This has truly been a worthwhile and memorable experience, and I do hope the program continues to give other students the same opportunity I have been given.

I cannot forget my chemistry teacher back at Dallas Jesuit, Mr. Tom Brock. He has given me the two most unique and fun-filled years of chemistry, and I also appreciate his nominating me for the program. He is a great friend, and I will always seek out his advice and will keep in touch with him for many years.

Last but not least, I have to give a big shout out to all my friends from the WSSP Class of 2002. I don't think anyone could ask for a better group. We all got along great, and I hope to keep in touch with all of you as we finish high school and enter college.