only extremely high levels of human EGF and may greatly underestimate their true values. We have not found immunoreactivity in the plasma of normal adult humans. Ances (*16*), using the same antiserum and EGF standard as we did in our assay but only one dilution of serum, has reported immunoreactive EGF levels of 0.5 to 6.0 ng/ml in serum from pregnant women. While these levels are well within the range of detectability of our assay, we have been unable to confirm these findings. We have, however, detected low levels of immunoreactivity (100 to 300 pg equivalents of mouse EGF per milliliter) in the plasma of newborn infants, suggesting that the levels in newborns may actually be quite high. In most of the adult urine specimens we have assayed, we were able to detect the equivalent of 50 to 400 pg/ml of immunoreactive mouse EGF. Human saliva and extracts of human submaxillary glands, prepared as described (*1*), did not contain detectable concentrations of immunoreactive EGF, suggesting that the salivary glands are probably not the source of the hormone in humans.

Thus, we have detected in human urine a material that is biologically and immunologically similar to EGF. Once a sensitive and specific radioimmunoassay for human EGF is developed, it should be possible to measure accurately minute amounts of EGF in human tissues and fluids to elucidate the role of this new human hormone in health and disease.

Addendum. Since this manuscript was submitted for publication, highly purified human EGF has been obtained from urine by means of an improved extraction technique, monitored with the aid of a new radioreceptor assay for EGF. The amino acid composition and estimated molecular weight of human EGF indicate that it is similar to, but biochemically distinct from, mouse EGF (*17*). On the basis of comparisons of radioimmunoassay and radioreceptor assay results, we have also confirmed that human EGF is about three orders of magnitude less reactive with antibodies raised to mouse EGF than is mouse EGF itself (*18*). Thus, the mouse EGF radioimmunoassay does greatly underestimate levels of human EGF.

RALPH H. STARKEY
STANLEY COHEN, DAVID N. ORTH
Departments of Medicine and
Biochemistry, Vanderbilt University
School of Medicine,
Nashville, Tennessee 37232

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Chemotaxis away from Uncouplers of Oxidative Phosphorylation in *Bacillus subtilis*

Abstract. *In a capillary assay, uncouplers of oxidative phosphorylation and inhibitors of electron transport are repellents for* Bacillus subtilis. *They also cause transient tumbling in naturally smooth swimming strains. Tumbling strains can be made to swim smoothly by addition of attractant and then immediately returned to tumbling by subsequent addition of repellent. Arsenate does not cause transient tumbling, suggesting that decrease in concentration of adenosine triphosphate does not cause tumbling and that adenosine triphosphate concentration does not govern tumbling frequency. Instead, the evidence suggests that diminution of the energized state of the membrane, or membrane potential, causes tumbling although the level of the energized state itself does not govern tumbling frequency.*

Chemotaxis is the process by which motile bacteria swim to higher concentrations of attractant or lower concentrations of repellent. *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis* normally swim smoothly but randomly change direction by tumbling (*1*). When headed to higher concentrations of attractants (for example, some amino acids and sugars) the bacteria tumble less often than in isotropic medium; when headed to lower concentrations, they show at most a slight tendency to tumble more often (*1, 2*). Behavioral changes can be observed directly by adding attractant or repellent to a bacterial suspension and observing transient decreases or increases in tumbling frequency (*1, 3*). Chemotaxis, then, is caused by modulation of tumbling frequency by these reagents; the question is, How do they act? We show here that, on the basis of capillary assays and on direct observation, uncouplers of oxidative phosphorylation and inhibitors of electron transport repel *Bacillus subtilis* and cause it to tumble temporarily, which suggests that diminution of the high energy state of the membrane or membrane potential (*4*) affects tumbling frequency but that the actual level of the high energy state itself does not govern it.

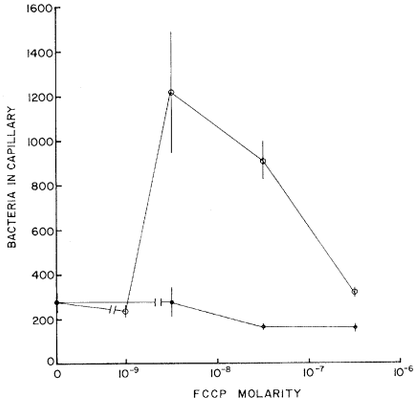

Fig. 1. Capillary assay of the repellent FCCP. Bars indicating standard error of each mean are shown. Symbols are as follows: ○, FCCP in pond and buffer in capillary; ●, FCCP in both pond and capillary.

Three related strains of *B. subtilis* were used in this study: (i) OI 1, which swims mostly, tumbles little; (ii) OI 8, which tumbles slightly more than OI 1; and (iii) OI 151, which swims little and tumbles mostly (*5*). The capillary assay, using OI 8, was performed by the "repellent in pond" method of Tso and Adler (*6*). The test substance in the bacterial suspension diffuses into a capillary containing buffer to form a gradient; bacteria flee down this gradient into the capillary. In the control experiment, the test substance is present in both capillary and pond from the beginning. If more bacteria collect in the capillary containing originally only buffer, the reagent is a repellent.

The response of OI 8 to different concentrations of FCCP (*7*) (Fig. 1) indicates that this uncoupler of oxidative phosphorylation repels *B. subtilis*. Other uncouplers of oxidative phosphorylation and some inhibitors of electron transport repel *B. subtilis* in the capillary assay (Table 1); significance of the results based on the *t*-test is also shown. Motility is always satisfactory at assay concentrations, based on comparisons of accumulations in the presence or absence of reagent in both capillary and pond (Table 1), and also on microscopic observation. High concentrations abolish motility.

Strain OI 1 swims smoothly in chemotaxis buffer (Fig. 2a) (*8*). Administration of repellent makes these bacteria tumble, then later resume smooth swimming shown in Fig. 2, b and c. Strain OI 151, by contrast, tumbles (Fig. 2d). However, as in the case of tumbling mutants of wild-type *E. coli* and *S. typhimurium* (*9*), administration of attractant causes smooth swimming (Fig. 2e). If repellent is now added, the bacteria immediately return to tumbling. By this method, OI 151 can be used to assay repellents.

Smooth swimming by OI 151 when given $10^{-4}M$ proline lasts 51 ± 8 seconds (mean and standard deviation for determinations over 10 days), but the uncoupler and repellent FCCP at $10^{-7}M$ given 25 seconds after proline restores tumbling at once. Prior incubation in $10^{-7}M$ FCCP for 30 seconds has no effect on duration of proline-induced swimming.

FCCP can promote transient tumbling in OI 1; hence the bacteria can adapt. The time required for adaptation is greater at higher FCCP concentrations. In one experiment the time for adaptation was 40 seconds at $10^{-7}M$, but it was 90 seconds at $6 \times 10^{-7}M$ FCCP. The bacteria were much slower at $10^{-6}M$ FCCP, but even for these cells the frequency of smooth swimmers compared with tumblers was not much different after about 8 minutes compared with untreated cells.

Other uncouplers of oxidative phosphorylation and inhibitors of electron transport were tested and found to be repellents in similar microscope experiments (Table 2). The threshold concentration is the minimum required to make OI 1 tumble or to make OI 151 return to tumbling when given 25 seconds after $10^{-4}M$ proline. The concentrations are generally similar to those that uncouple oxidative phosphorylation in mitochondria and similar to or lower than those used in bacteria. Table 2 includes references to work in mitochondria, bacteria, or artificial membranes for each reagent.

To investigate the possible role of bacterial ATP concentration in tumble regulation, we treated cells in tris [tris-(hydroxymethyl)aminoethylmethane] buffer with different levels of arsenate, and the effects on tumbling frequency and on ATP concentrations were observed. Sodium arsenate ($0.1M$) alone has no reproducible effect on behavior although it sometimes evokes a brief (15 seconds) swim from OI 151. Preliminary incubation of OI 151 for several minutes in $0.1M$ arsenate reduces cellular ATP by two-thirds (*10*), but does not shorten swims induced by subsequent addition of $10^{-4}M$ proline. Furthermore, FCCP below about $10^{-6}M$ does not reduce ATP (*10*), though $10^{-7}M$ is sufficient to cause tumbling and also to increase respiration, as measured with an oxygen monitor (Yellow Springs Instrument model 53) and recorded with a strip chart recorder (Varian G2000) (*10*). It should be noted that there was no sign of any "adaptation" (return to normal value found in absence of FCCP) in respiration rate or ATP levels after several minutes in any of the experiments in which these parameters changed (*10*). We conclude that the ATP concentration, when at least one-third that of normal, does not directly affect tumbling frequency.

In mitochondria, uncouplers of oxidative phosphorylation and inhibitors of electron transport reduce the high energy state of the membrane (*4*). Recent experiments in bacteria indicate that this energy state may consist in part of an electric potential across the plasma membrane. Maloney *et al.* (*11*) have shown that ATP may be synthesized in both *Streptococcus lactis*, a gram-positive organism like *B. subtilis*, and in *E. coli* from a membrane potential generated upon addition of valinomycin to cells high in K^+. Harold and Papineau (*12*) have measured this potential for *Streptococcus faecalis*, using the lipid-soluble cations DDA^+ and $TPMP^+$, and have found it to be collapsed by proton-conducting uncouplers like TCSA and CCCP, with only slight stimulation of glycolysis. Laris and Pershadsingh (*13*) have also measured the potential across the membrane of *S. faecalis* using the cyanine dye CC_6, whose fluorescence reflects membrane potential in squid axons and red cells. The electric potential was generated by adding glucose or

Table 1. Capillary assays of repellents. Reagents marked with superscript α are uncouplers; those marked β are inhibitors of electron transport. The level of significance P of the difference between experimental and control values was calculated by the *t*-test.

Compound (*7*)	Concentration	Number of cells in capillary			P
		Experimental*	Control†	Motility control‡	
FCCP$^{\alpha}$	$3 \times 10^{-9}M$	1221 ± 273	278 ± 69	277 ± 46	.02
CCCP$^{\alpha}$	$3 \times 10^{-7}M$	683 ± 182	171 ± 20	214 ± 18	.05
CCP$^{\alpha}$	$5 \times 10^{-7}M$	1892 ± 113	432 ± 94	288 ± 22	.01
PCP$^{\alpha}$	$10^{-6}M$	1594 ± 201	429 ± 102	496 ± 101	.001
TCSA$^{\alpha}$	$10^{-9}M$	624 ± 78	321 ± 80	396 ± 108	.02
DBP$^{\alpha}$	$3 \times 10^{-5}M$	4742 ± 484	1260 ± 267	1336 ± 275	.001
DNP$^{\alpha}$	$5 \times 10^{-5}M$	1116 ± 131	400 ± 60	604 ± 63	.001
Quinacrine$^{\alpha,\beta}$	$10^{-5}M$	7320 ± 540	1843 ± 755	1047 ± 243	.001
HOQNO$^{\beta}$	1 μg/ml	947 ± 182	321 ± 117	430 ± 98	.025

*Reagent in pond, buffer in capillary. †Reagent in pond and in capillary. ‡Buffer in pond and in capillary.

Table 2. Microscope assays of repellents. Reagents marked with superscript α are uncouplers; those marked β are inhibitors of electron transport. Column 4 indicates other organisms or systems in which these reagents have been used and gives references. Designations of letters a to i are as follows: a, mitochondria; b, *Micrococcus denitrificans*; c, *Streptococcus lactis*; d, artificial membranes; e, *Escherichia coli* membrane vesicles; f, *Thiobacillus novellus* cell-free extract; g, *Streptococcus faecalis*; h, chloroplasts; and i, *Bacillus subtilis* membrane vesicles.

Compound (*7*)	Threshold for OI 1	Threshold for OI 151	Organism or system
FCCP$^{\alpha}$	$1 \times 10^{-7}M$	$1 \times 10^{-7}M$	a (*16, 20*), b (*14, 20*), c (*11*), d (*21*)
CCCP$^{\alpha}$	$4 \times 10^{-7}M$	$2 \times 10^{-7}M$	a (*16, 20*), d (*20*), e (*22*), f (*23*), g (*12*)
CCP$^{\alpha}$	$7.5 \times 10^{-6}M$	$3 \times 10^{-6}M$	a (*16*)
PCP$^{\alpha}$	$3 \times 10^{-7}M$	$10^{-7}M$	a (*20, 24*), d (*20*), f (*23*)
TCSA$^{\alpha}$	$2.5 \times 10^{-8}M$	$2 \times 10^{-8}M$	e (*22*), g (*12*)
DBP$^{\alpha}$	$6.4 \times 10^{-6}M$	$1.6 \times 10^{-6}M$	f (*23*)
DNP$^{\alpha}$	$2 \times 10^{-4}M$	$10^{-4}M$	a (*20, 25*), d (*16, 20*), f (*23*)
Na azide$^{\alpha,\beta}$	$1.5 \times 10^{-2}M$	$1.5 \times 10^{-2}M$	a (*26, 27*)
Quinacrine$^{\alpha,\beta}$	$7.5 \times 10^{-6}M$	$1.5 \times 10^{-5}M$	a (*25*), h (*28*)
HOQNO$^{\beta}$	0.625 μg/ml	1.5 μg/ml	a (*29*), e (*30*), i (*31*)
NaCN$^{\beta}$	$3.3 \times 10^{-5}M$	$1.25 \times 10^{-4}M$	a (*29*), e (*30*), i (*31*)
o-Chlorophenol	$>2 \times 10^{-4}M$	$>2 \times 10^{-4}M$	
o-Nitrophenol	$> 10^{-4}M$	$> 10^{-4}M$	
p-COOH CCP	$>5 \times 10^{-4}M$	$>5 \times 10^{-4}M$	a (*16*)

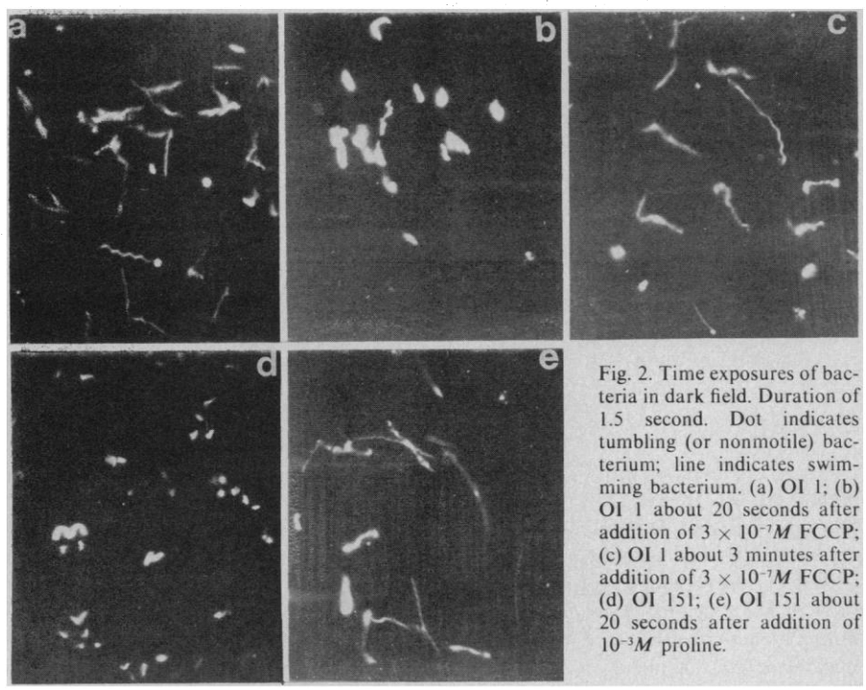

Fig. 2. Time exposures of bacteria in dark field. Duration of 1.5 second. Dot indicates tumbling (or nonmotile) bacterium; line indicates swimming bacterium. (a) OI 1; (b) OI 1 about 20 seconds after addition of $3 \times 10^{-7}M$ FCCP; (c) OI 1 about 3 minutes after addition of $3 \times 10^{-7}M$ FCCP; (d) OI 151; (e) OI 151 about 20 seconds after addition of $10^{-3}M$ proline.

valinomycin to K^+-loaded cells in K^+-free medium. Scholes and Mitchell (*14*) have found that incubation of *Micrococcus denitrificans* in $5 \times 10^{-7}M$ FCCP makes the membrane highly permeable to protons.

Hence, it seems likely that an electric potential lies across bacterial plasma membranes and that uncouplers cause its diminution by transfer of protons. The fact that FCCP at $10^{-7}M$ causes increase of respiration, analogous to release of respiratory control in mitochondria (*14*), and that HOQNO at 0.625 μg/ml and NaCN at $3.3 \times 10^{-5}M$ cause 21 and 28 percent decreases in respiration, respectively, indicates that in all probability *B. subtilis* is no different from other bacteria or mitochondria in basic energetics (Table 2 and references for FCCP, HOQNO, and cyanide).

We think that the uncouplers are not acting at orthodox chemoreceptors, such as the galactose binding protein (*15*), although proteins may be involved in the activity of uncouplers. The uncouplers are active as repellents at concentrations corresponding to those used to produce uncoupling in mitochondria. Furthermore, the series of decreasing strength—FCCP, CCCP, CCP, *p*COOH CCP, the last being ineffective—is found both for uncouplers in mitochondria and repellents in *B. subtilis* (*16*). It seems unlikely that chemoreceptors would by coincidence have those specificities for these reagents if the uncoupling property were unconnected with behavioral change.

An interpretation of our results is as follows: Since uncouplers and inhibitors of electron transport cause tumbling and also destroy the high energy state, de-energization (depolarization) of the cell membrane is assumed to cause tumbling. Membrane potential itself—or any cell property such as the "high energy intermediate of oxidative phosphorylation" if that is distinct—cannot directly determine tumbling frequency since the bacteria adapt to these reagents: after initial tumbling, OI 1 or OI 8 resume swimming normally and become indistinguishable from untreated bacteria, and OI 151 swims smoothly for the same period of time when treated with $10^{-4}M$ proline in presence or absence of uncoupler or inhibitor (*17*). However, the increase of respiration for uncouplers or decrease for inhibitors is persistent: bacteria do not return to the original rate.

Internal ATP concentration does not play any significant role since arsenate decreases ATP concentration without affecting tumbling frequency, and ATP concentration is not affected by concentrations of FCCP which strongly affect behavior (*10*). It is doubtful that rate of electron transport has any effect either since uncouplers increase it, while inhibitors decrease it although both cause temporary tumbling.

How may the behavioral adaptation be explained? When *Paramecium* strikes a barrier, its membrane depolarizes somewhat, triggering substantial influx of Ca^{2+}, which causes the cilia to reverse orientation. In time, after the membrane has returned close to normal potential, the Ca^{2+} is pumped back out, and *Paramecium* resumes forward motion (*18*). By analogy, one might suppose that the initial diminution of electric potential presumed to be caused by uncouplers or inhibitors is accompanied by flux of ions across the plasma membrane and that restoration of original internal ionic concentrations takes longer. One of these ions may interact with the switch that determines whether the flagella rotate counterclockwise for smooth swimming or clockwise for tumbling (*19*).

GEORGE W. ORDAL
DANIEL J. GOLDMAN
Department of Biochemistry and
School of Basic Medical Sciences
at Urbana-Champaign,
University of Illinois, Urbana 61801

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6. Bacteria for the capillary assay were prepared as follows. Spores in distilled water were inoculated into tryptone broth (1 percent tryptone, 0.5 percent NaCl), containing 0.14 m*M* $CaCl_2$, 0.20 m*M* $MgCl_2$, and 0.01 m*M* $MnCl_2$ [R. A. Carls and R. S. Hanson, *J. Bacteriol.* **106**, 848 (1971)] and allowed to grow (overnight) to the stationary phase. Bacteria were diluted 1 : 50 into mineral salts medium (0.05*M* potassium phosphate, *p*H 7, 0.12 m*M* $MgCl_2$, 1 m*M* $(NH_4)_2SO_4$, 0.14 m*M* $CaCl_2$, 0.01 m*M* $MnCl_2$) supplemented with 0.02*M* sorbitol and required amino acids (0.3 m*M*), grown at 37°C with shaking to Klett 32.5 (filter 66), and made 5 m*M* in sodium lactate and 0.05 percent in glycerol. After 15 minutes, bacteria were centrifuged and washed twice in chemotaxis buffer, and diluted to an $A_{600\,nm}$ of 0.01 unit. Chemotaxis buffer is 0.01*M* potassium phosphate, *p*H 7 (sometimes 0.01*M* tris, *p*H 7), 0.14 m*M* $CaCl_2$, 0.3 m*M* $(NH_4)_2SO_4$, 0.1 m*M* EDTA, 5 m*M* sodium lactate, and 0.05 percent glycerol (G. W. Ordal and K. Gibson, unpublished results). Capillary assays were performed by the method of Tso and Adler [W.-W. Tso and J. Adler, *J. Bacteriol.* **118**, 560 (1974)] using 3-μl microcapillaries.
7. Abbreviations used in this report are the following: FCCP, trifluoromethoxycarbonylcyanidephenylhydrazone; CCCP, *m*-chlorocarbonylcyanidephenylhydrazone; CCP, carbonylcyanidephenylhydrazone; *p*-COOH CCP, *p*-carboxycarbonylcyanidephenylhydrazone; PCP, pentachlorophenol; TCSA, 3,3′,4′,5-tetrachlorosalicylanilide; DBP, 2,6-dibromophenol; DNP, 2,4-dinitrophenol; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; DDA^+, dibenzyldimethyl ammonium ion; $TPMP^+$, triphenylmethyl phosphonium ion; CC_6, 3,3′-dihexyloxacarbocyanine iodide.
8. Bacteria were prepared as for capillary assays (*6*), except that bacteria were grown in nutrient broth to Klett 180 and were not supplemented with lactate and glycerol before harvesting. Bacteria were held at 30°C, and placed as a drop on a cleaned microscope slide at room temperature (about 23°C) with a Rainin adjustable micropipette. Thirty seconds later or at appropriate times reagent was administered using a disposable microcapillary. Observations on bacteria near the surface of the slide began about 5 seconds later. In a few cases, observations were made on bacteria which had been held at room temperature for 15 minutes or longer. OI 1 and OI 8 had a higher tumbling frequency and were somewhat slower then, but still mostly swam.
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Simplex Optimization of Reaction Yields

Abstract. *The sequential simplex algorithm, an efficient optimization strategy, rapidly improved reaction yield as a function of time and temperature in the synthesis of* π-$C_5H_5Mo(CO)_2CSN(CH_3)_2$. *The work demonstrates the feasibility and efficiency of the simplex design and suggests its application and usefulness in other syntheses.*

Recent awareness of the finite character of both material and energy resources has stimulated a renewed interest in the optimization of reaction yields. We report here the successful application of an efficient optimization strategy that could have broad applicability in the development of synthetic chemical methods.

The development of reliable, high-yield chemical syntheses requires three efforts: (i) producing some yield of the desired compound; (ii) improving the yield; and (iii) understanding the effects of the various experimental factors (variables) upon the yield. Synthetic chemists probably devote most of their time to the definition and discovery of synthetic routes that produce the desired compound in some yield. The systematic improvement and understanding of yields are less frequently investigated, although both are often important. For example, the overall yield of a multistep synthesis is a multiplicative function of the yields at each step; improving intermediate yields is usually necessary to produce an acceptable yield of final product. Understanding the effects of the various experimental factors upon the reaction yield allows the investigator to evaluate and specify tolerances (*1*) within which each factor must be held so that the stated yield can be reliably reproduced in other laboratories.

A common optimization strategy is the one-factor-at-a-time method (*2*) in which each factor is optimized in turn while all others are held constant. Although this method is still popular (*3*), it has been shown (*4, 5*) that by this means it is impossible to find an existing optimum in many chemical systems because of ridges in the response surface. Evolutionary operation (EVOP) strategies (*6*) will succeed in the presence of ridges, but the number of experiments required is large: if k full factorial designs for n factors at m levels are needed to reach the optimum, a minimum of $k(m^n)$ experiments is required.

The sequential simplex (*7*) method of Spendley *et al.* (*8*) is a pattern search technique that requires only $n + 1$ experiments for the initial design and only one additional experiment per move. Thus, if k is the number of simplexes needed to reach the optimum, a minimum of $k + n + 1$ experiments is required. A modification by Nelder and Mead (*9*) of the original simplex algorithm allows expansions along directions that are favorable and contractions along directions that are unfavorable (*10*). The logical and mathematical details of the simplex algorithms have been discussed elsewhere (*5, 9, 11*).

The feasibility of reaction yield optimization by the simplex method is demonstrated here for the synthesis of the known compound π-$C_5H_5Mo(CO)_2CSN(CH_3)_2$ (*12*). The reaction was carried out in two steps as follows:

$$C_5H_5Na + Mo(CO)_6 \xrightarrow[t_1,\ 65°C]{THF} \pi\text{-}C_5H_5Mo(CO)_3Na + 3CO \quad (1)$$

$$\pi\text{-}C_5H_5Mo(CO)_3Na + ClCSN(CH_3)_2 \xrightarrow[22\ hr,\ T_2]{THF} \pi\text{-}C_5H_5Mo(CO)_2CSN(CH_3)_2 + CO + NaCl \quad (2)$$

where THF is tetrahydrofuran. We have investigated the effects of two potentially significant factors on reaction yield: the reaction time t_1 of Eq. 1 and the reaction temperature T_2 of Eq. 2. All other factors were held constant (*13*). The yield at each set of reaction conditions was determined by infrared spectroscopy.

The progress of the simplex toward improved reaction yield is illustrated in Fig. 1. The initial simplex (yields of 16, 74, and 82 percent) is located in a region previously investigated (*12*). Univariate mapping studies were carried out to investigate the individual effects of t_1 and T_2 in the region of the best vertex (yield, 93 percent). These results are shown in the inset in Fig. 1.

It is apparent from Fig. 1 that there is a general trend toward improved yield with increasing t_1. The slight decrease in yield above $t_1 \approx 5$ hours in the mapping study is not statistically significant. A much more dramatic effect is caused by T_2. Up to ~ 55°C, yield is improved by increasing T_2; however, above this temperature as the boiling point (b.p.) of the solvent is approached, yield decreases sharply.

This preliminary study has shown the feasibility of using a simple optimization algorithm to improve the yields of chemical syntheses. The understanding of the behavior of the reactions provided by these

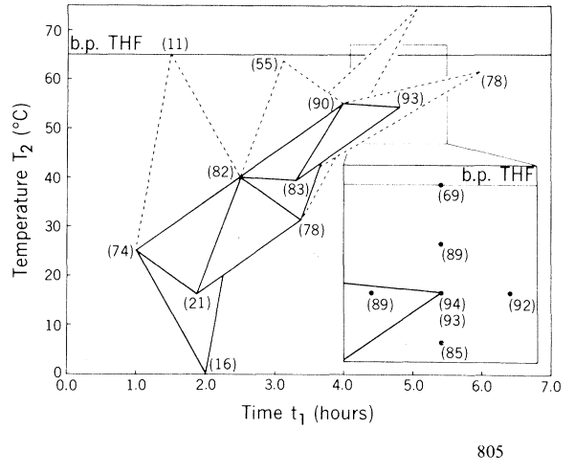

Fig. 1. Simplex improvement of yield as a function of time and temperature. Solid lines indicate simplex movement. Dashed lines show the locations of vertices generated in failed reflections and expansions. Parenthetical numbers beside the vertices are percentage yields.