MONTANA STATE UNIVERSITY

The determination of chlorsulfuron by the inhibition of acetolactate synthase
by Gary William Kagel

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University
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Abstract:
The enzyme acetolactate synthase (ALS, EC 4.1.3.18) is the site of action of the herbicide chlorsulfuron (2-chloro-N-[(4-methoxy-6-methyl-l,3,5,-triazin-2yl)aminocarbonyl]benzenesulfonamide). This enzyme was extracted from plant material and immobilized in a cartridge packed with a silica based activated affinity media. The activity of the immobilized enzyme was inhibited by nanomolar concentrations of chlorsulfuron.

A 60% recovery of radio-labeled chlorsulfuron from treated soil was demonstrated. Analytical standards of chlorsulfuron recovered from soil inhibited immobilized ALS to a greater degree at higher herbicide concentrations.

Results indicate this technique is capable of chlorsulfuron detection down to about 1 ppb (1.12 g/ha).

This technique, therefore, represents a method with the potential to be faster and more economical than the present method which uses high performance liquid chromatography.

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A thesis submitted in partial fulfillment
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APPROVAL

of a thesis submitted by

Gary William Kagel

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Signature _Gary Kagel_

Date _July 31, 1987_

This thesis is dedicated to Mr. and Mrs. Raymond Kagel without whose boundless help, love, and understanding this work could never have been completed.

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ABSTRACT

The enzyme acetolactate synthase (ALS, EC 4.1.3.18) is the site of action of the herbicide chlorsulfuron (2-chloro-N-[(4-methoxy-6-methyl-1,3,5,-triazin-2yl)aminocarbonyl]benzenesulfonamide). This enzyme was extracted from plant material and immobilized in a cartridge packed with a silica based activated affinity media. The activity of the immobilized enzyme was inhibited by nanomolar concentrations of chlorsulfuron.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Chlorsulfuron [2-chloro-*N*-{(4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl}benzenesulfonamide], (Figure 1) is a selective herbicide used for broadleaf weed control in wheat (*Triticum aestivum* L.), oats (*Avena sativa* L.), and barley (*Hordeum vulgare* L.).(Levitt et al., 1981). Chlorsulfuron, and other sulfonylurea herbicides, injure sensitive weed species at extremely low rates of preemergence and postemergence application (16 g/ha) (Ray, 1982). Wheat is tolerant to chlorsulfuron as it rapidly metabolizes the herbicide into nonphytotoxic compounds (Sweetser et al., 1982). Chlorsulfuron represents a major advancement in weed control technology as it is highly active at rates less than 0.5 g/ha, yet has shown little injury to cereal crops and very low mammalian toxicity (Levitt, 1978).

The primary site of chlorsulfuron activity is the enzyme acetolactate synthase (ALS) (EC 4.1.3.18). ALS is the first common enzyme in the biosynthetic pathway for the amino acids valine, leucine, and isoleucine (Dailey and

Cronan, 1984). Chaleff (1984) demonstrated that other
sulfonyl ureas are also potent inhibitors of ALS activity.
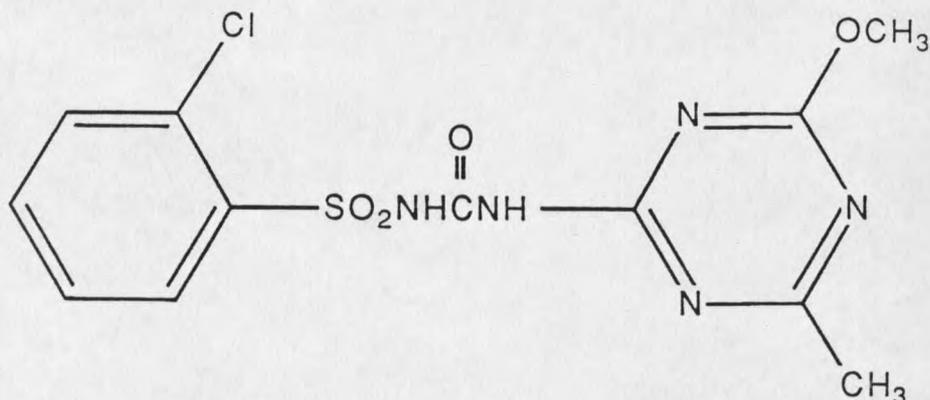
Figure 1. The structure of chlorsulfuron

Synthesis of Chlorsulfuron

Chlorsulfuron is synthesized by adding an equivalent of
2-chlorobenzenesulfonyl isocyanate (Ulrich and Sayigh, 1966)
to a suspension of 2-amino-4-methoxy-6-acetonitrile. After
stirring for 2 to 16 h at room temperature, the mixture is
filtered. The precipitate is washed with ethyl ether to
yield chlorsulfuron which melts at 174-178 °C. Results of
nuclear magnetic resonance, infrared absorption spectra, and
elemental analyses of the product were consistent with the
proposed structure (Huffman and Schaefer, 1963). The
compound is moderately soluble in methylene chloride, less
soluble in acetone and acetonitrile, and slightly soluble in

hydrocarbon solvents. Its solubility in water is 125 ppm at 25° C. Chlorsulfuron is stable to sunlight when dry. Hydrolysis occurs in distilled water with an average half-life of 4-8 weeks at pH 5.7-7.0 and 20° C. Hydrolysis is accelerated to 1-2 days in solutions below pH 5. Polar organic solvents also promote hydrolysis (Hartley, 1983).

Persistence in Soil

Chlorsulfuron residues are degraded biologically by soil microbes (Joshi et al., 1958), and chemically by acid hydrolysis. In some soils, however, they may remain at active levels for several years. Microbial degradation in soil occurs more rapidly when moisture, temperature, pH, nitrogen and carbon content are optimal for microbial growth. Acid hydrolysis, the major mechanism for breakdown (Palm et al., 1980), proceeds more rapidly at high soil temperature, high soil moisture and low soil pH. Since degradation rates are controlled by these soil variables, persistence varies with time and site of application (Walker and Brown, 1983). There is a need for a suitable analysis for chlorsulfuron in field soils. The technique most commonly used at present involves HPLC analysis (Zahnow, 1982). This method of analysis costs more than $200 per sample.

Mode of Action

Chlorsulfuron selectively stops growth by inhibiting cell division (Rost, 1984). Ray (1984) and Chaleff (1984) related this inhibition to ALS activity. A correlation between ALS inhibition and mitosis arrest was also demonstrated by Rost and Reynolds (1985). They demonstrated a reversal in chlorsulfuron-induced inhibition of cell division by supplying isoleucine and valine to treated plants, and Ray (1984) showed a reversal of chlorsulfuron-induced growth inhibition in pea root culture by isoleucine and valine applications.

LaRossa and Schloss (1984) reported that another sulfonyl urea herbicide, sulfometuron methyl, is bacteriostatic as a result of ALS inhibition. These herbicides are potent, selective inhibitors of ALS with I_{50} values as low as 18 nM for chlorsulfuron and 15 nM for sulfometuron methyl.

Another promising new class of herbicides, the imidazolinones, also inhibit ALS activity (Muhitch et al., 1987). However, there is no apparent structural similarity between the two herbicide classes.

Acetolactate Synthase

The biosynthesis of isoleucine and valine occurs through parallel synthetic pathways (Figure 2). The first step involves the ALS catalyzed synthesis of either -acetohydroxybutyrate or -acetolactate from the condensation

of the active acetaldehyde from -ketobutyrate or pyruvate,
respectively (Umbarger and Brown, 1958).

THREONINE
THREONINE DEAMINASE
↓
α- KETOBUTYRATE   **PYRUVATE**

←— ACETOLACTATE SYNTHASE —→

α-ACETOHYDROXY-   **α-ACETOLACTATE**
BUTYRATE

↓   ↓

↓   ↓

↓   VALINE LEUCINE
ISOLEUCINE
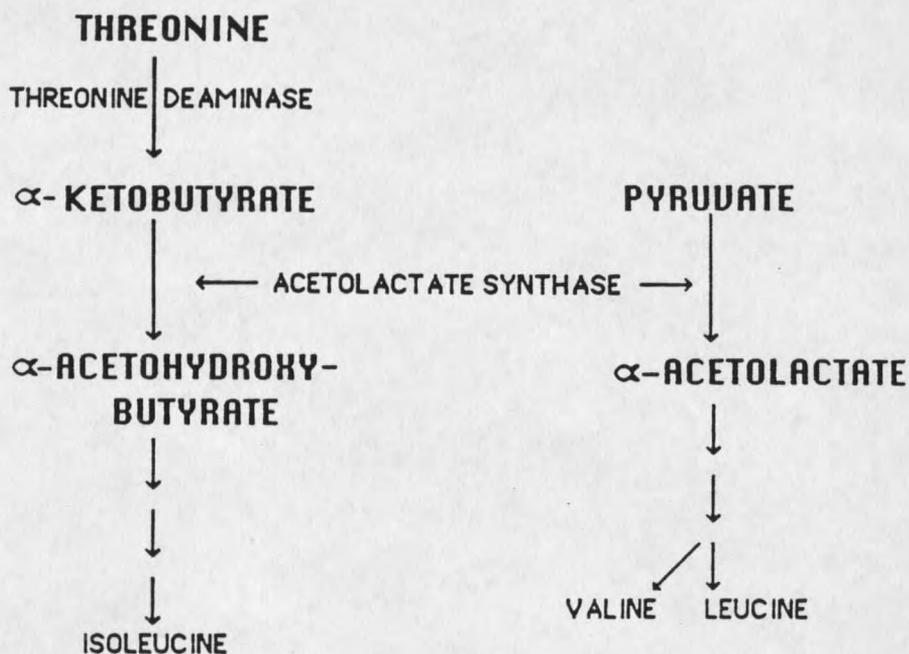
Figure 2.   Acetolactate synthase is the first common enzyme
            in the parallel biosynthetic pathways of valine,
            leucine, and isoleucine.

There are several isozymes of ALS in most species,and
all of those investigated apparently catalyze the formation
of both acetolactate and acetohydroxybutyrate (DeFelice et
al., 1985).   In *E. coli*, six isozymes of ALS have been
found, four of which are encoded by a separate gene (*ilv*B,
*ilv*G, *ilv*H, *ilv*J), and have been described (Schloss et al.,
1985). Only two of these have been purified (Grimminger and

Umbarger, 1979). Later work revealed that the isozyme ALS-I from *E. coli*, consists of two dissimilar polypeptides (Eoyang and Silverman, 1984). The larger subunit (60 kDa) is presumably encoded by *ilv*B and the smaller subunit (9.5 kDa) has been designated as the *ilv*M product. It has also been determined that the stop and start codons of *ilv*G and *ilv*M overlap. Overlapping stop and start codons have also been observed for several other genes whose gene products are produced in equal amounts.

Schloss et al. (1985) developed an improved method for the purification of ALS-II from *Salmonella typhimurium* and showed that native acetolactate synthase has a molecular weight of about 140 kDa and consists of two large and two small subunits weighing about 59.3 and 9.7 kDa, respectively.

The contribution of the different subunits to enzyme function has yet to be systematically examined. The ALS-III isozyme is encoded by the *ilv*I (large) and *ilv*H (small) subunits (DeFelice et al., 1947), and is sensitive to feedback inhibition by L-valine. Some *ilv*H mutations lead to production of a valine-resistant enzyme (Squires et al., 1981), suggesting that the small subunit of ALS-III plays a role in the valine sensitivity of that enzyme. These mutations also effect the specific activity of ALS-III, at least for acetolactate formation. ALS sensitive to end-product inhibition by valine from *E. coli* strains W and K-12

show sensitivity characteristics that are consistent with
the two-site model proposed to account for the regulation of
enzyme activity by specific small molecules (Bauerle et al.,
1964). *Escherichia coli* mutants which are missing one of
the two valine-sensitive ALS enzymes have major membrane
defects (DeFelice et al., 1985). ALS-I catalyzes the
reaction with acetolactate more efficiently than with
acetohydroxybutyrate, although the opposite effect is
measured for ALS-II (DeFelice et al., 1978). This indicates
that the isozymes of ALS function in a complimentary
fashion. Cara and De Felice (1979) separated these isozymes
by low pressure ion exchange chromatography. Grimminger et
al. (1979) purified ALS-I to near homogeneity and reported
that the isozyme consists of two subunits of approximately
60,000 molecular weight each with an isoelectric point of
4.9 and a pH optimum of 7.5. Leavitt and Umbarger (1961)
reported that ALS requires thiamine pyrophosphate (TPP),
Mg^{++}, and flavin adenine dinucleotide (FAD) (Stormer and
Umbarger, 1964). This requirement for FAD is unusual for an
enzyme catalyzing a reaction in which no net oxidation or
reduction occurs.

Chlorsulfuron determination

Chlorsulfuron is persistent in soil, therefore an
accurate soil assay method is needed. Detection is
exceedingly difficult since the herbicide is applied to soil
at rates as low as 4 g/ha. Phytotoxic residues can occur in

soil at concentrations as low as 35 parts per trillion.

Computer software has been developed to model the
decomposition of chlorsulfuron in soil. These programs take
into account the soil variables which affect rates of
degradation (Walker and Brown, 1964). The method is not
widely used because it requires detailed soil histories.

Zahnow (1982; 1985)developed high performance liquid
chromatography (HPLC) methods for the determination of both
chlorsulfuron and sulfometuron methyl in soil using
selective photoconductivity detection (Walters, 1983). He
was able to quantify chlorsulfuron at levels as low as 0.2
ppb. Slates (1983) designed a similar HPLC method for
determining chlorsulfuron residues in grain, straw, and
green plants of cereals. While these methods utilize the
selective conductivity detector, they suffer from high
background levels of interference.

A sensitive enzyme-linked immunosorbent assay (ELISA)
has also been developed for chlorsulfuron with a detection
limit of 0.4 ppb. This technique can quantify nanogram
levels of chlorsulfuron in crude soil extracts (Kelley et
al., 1985). Although this has shown promise, more research
is needed to develop consistent antibodies.

Bioassays are also useful in detecting the presence of
low levels of chlorsulfuron in soil. Nilsson (1981)
detected chlorsulfuron residues in soil treated at a rate of
10 g/ha 11 months before sampling. In our research group we

have demonstrated a useful method of measuring chlorsulfuron levels in soil by plant bioassay using five plant species ranging in their sensitivity to the herbicide (Fay and Fellows, 1987). Although bioassays can be very sensitive, they are time consuming and difficult to quantify.

Enzymes as Analytical Reagents

Specific chemical substances often inhibit enzymes at very low concentrations, and have been used as analytical transducers (Guilbault, 1969; Bowers, 1986). It was demonstrated that *in vitro* inhibition of cholinesterases isolated from different animals could be useful for the highly sensitive and selective assay of pesticides (Guilbault, 1970). Enzymes are normally very substrate-specific due to the three dimensional nature of the binding site. This is advantageous because of the reduced requirement for resolution of compounds from a matrix. In addition, if the enzyme product is easily detected, an enzymatic amplification is possible (Bowers, 1986).

A radiometric enzymic method was developed by Horvath (1982) to measure organophosphorus and carbamate pesticide residues in water. By fluorometric assay of phosphatase, Guilbault et al. (1969) selectively determined bismuth, beryllium, and selected pesticides. Enzyme electrodes have also been developed which directly determine the products formed by the enzyme immobilized on or near the surface of

the appropriate ion selective electrode. This enables the
determination of substrates, cofactors, or inhibitors
(Guilbault and Montalvo, 1970). More recently Haginaka et
al. (1987) developed a postcolumn reactor consisting of
immobilized lactamase for the detection of HPLC-separated
lactamase inhibitors in human serum.

The major problems with enzymatic analyses is the
lability of the enzymes, and the high cost associated with
obtaining large quantities of the enzymes. One solution to
this problem is to immobilize the enzyme to a matrix which
permits reuse and/or increased stability (Guilbault, 1970).
In addition to added stability, immobilization of enzymes is
more convenient and adaptable to automation (Fukui and
Tanaka, 1984). Classically, the term "immobilized enzyme"
has been used to describe an enzyme that has been chemically
or physically attached to a water-insoluble matrix,
polymerized into a water-insoluble gel, or entrapped within
a water-insoluble gel matrix or microcapsule (Zaborsky,
1973).

The sensitivity of ALS to the sulfonyl urea herbicides
at low concentrations may facilitate the use of this enzyme
as an analytical tool for the determination of chlorsulfuron
residues in soil.

Immobilized Enzymes

Immobilization of enzymes is attracting attention for
several reasons. Possibly the most important area is the

biotechnology industry which needs new techniques for the
immobilization of enzymes, antibodies and other biological
materials. These materials, once immobilized, are vastly
more amenable to analytical and preparative needs of
biological research.

An important attribute of immobilized enzymes is their
usefulness in flowing systems, where reactants are
constantly flushed into the immobilized catalyst and
products are constantly flushed off, or where the enzyme
affinity for a particular molecule is utilized for
chromatography. In addition to use as analytical
transducers, immobilized enzymes are presently used in
purification of useful compounds by stereospecific and/or
regiospecific interactions, selective treatment of specified
pollutants to solve environmental problems, and affinity
purification of bio-engineered products from crude ferments
and extracts.

A number of materials have been used to immobilize
enzymes including physical entrapment in crosslinked
polymers, gel matrices, microcapsules, liposomes or hollow-
fibers (Menecke and Polakowski, 1981). Other methods
include chemical attachment by covalent or ionic bonding of
enzyme to water-insoluble, functionalized polymers, and the
more recent use of macroporous silica covered with a
hydrophilic layer before functionalization (Alpert and
Regnier, 1979). Enzymes are also immobilized by

intermolecular crosslinking of enzyme with multifunctional,

low molecular weight reagent (Zaborsky, 1973).

CHAPTER 2

EXTRACTION AND ASSAY OF ACETOLACTATE SYNTHASE

Introduction

Acetolactate synthase is not commercially available therefore it must be obtained by extraction from a suitable plant source. The source of the enzyme must be available in large quantities and contain chlorsulfuron sensitive ALS. Alfalfa sprouts were the plant source chosen because large quantities can be grown in seven days in the dark which eliminates chlorophyll interference. Alfalfa is very sensitive to chlorsulfuron (Burkhart, 1985) and actively growing sprouts are a rich source of ALS because the enzyme is required for cell division.

Materials and Methods

Alfalfa Sprouts. Common alfalfa seed was obtained from the Montana State Seed Testing Laboratory. The seed was surface sterilized in concentrated sulfuric acid for fifteen minutes and rinsed quickly with sterile water to avoid injury from heat accumulation from acid dilution. Sterile seeds (100 g) were placed on one-half of a 24 by 15 cm sheet of germination paper. The germination paper was moistened and folded over to cover the seed. Nine sheets containing

seeds were placed on a rack and covered with a second layer of germination paper. Each rack was wrapped with clean polyethylene wrap and placed in a dark incubator at approximately 20° C. The etiolated sprouts were harvested after seven days and stored in sealed plastic bags at 5° C for no more than one day before enzyme extractions. Fresh sprouts (10 g) were dried at 70° C for 3 days to determine dry weight.

Reagents. Total protein assay dye reagent was purchased from BIO-RAD (Richmond, CA). Biological chemicals, including acetoin (acetyl methyl carbinol), cocarboxylase (thiamine pyrophosphate), dithiothreitol (DTT), flavin adenine dinucleotide (FAD), sodium pyruvate, creatine, and a-naphthol were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Enzyme extraction. The extraction procedure was adapted from the method of Ray (1984). Sprouts were homogenized for 30 sec in a chilled 250 ml Waring blender receptacle with one volume of ice-cold buffer containing: 0.1 M potassium phosphate, 1.0 mM sodium pyruvate, 10 uM flavin adenine dinucleotide (FAD), 15% (v/v) glycerol, and 5 mM dithiothreitol (DTT). The homogenate was filtered through eight layers of cheesecloth. The volume of the filtrate was noted and phenylmethylsulfonyl fluoride (PMSF), dissolved in a minimum of acetone, was added to a final

concentration at 1.0 mM PMSF. The homogenization buffer was initially adjusted to pH 8.5. After homogenization the pH decreased to the desired pH of 7.5 due to the release of acidic compounds. ALS substrate pyruvate, and the cofactor FAD were included to stabilize ALS during extraction. Glycerol was included to prevent enzyme denaturation by forming strong hydrogen bonds to decrease the activity of water. Glycerol also minimized foaming which can cause protein denaturation from surface tension effects (Scopes, 1984). Dithiothreitol was necessary to maintain a reducing environment to prevent oxidation of phenolic compounds which could deactivate ALS, and to maintain the sulfhydryl groups of ALS in a reduced state. The protease inhibitor PMSF was added to prevent ALS deactivation during homogenization. The filtered homogenate was centrifuged at 25,000 g for 30 min at 4° C. The pellet was discarded and the lipid layer was removed from the surface. The supernatant volume was measured and chelex-treated ammonium sulfate was added gradually to the chilled and constantly stirred solution to obtain 20% saturation. Since the addition of $(NH_4)_2SO_4$ acidifies the solution, one molar KOH was added dropwise to maintain constant pH. It was necessary to occasionally soak the pH electrode in a 10% pepsin solution at pH 1-2 for 30 minutes to remove protein adsorbed to the glass electrode. The solution was again centrifuged as described above, and the precipitate discarded. Additional ammonium sulfate was

added to the supernatant to bring the solution to 60%
saturation. Stirring was continued for at least 10 min to
allow complete equilibration between dissolved and
aggregated protein. The mixture was again centrifuged for
one hour. The resulting supernatant was discarded and the
precipitated protein dissolved over ice for 5 to 10 min in 1
to 2 volumes of the precipitate with desalting buffer
containing: 20 mM phosphate (pH 7.5), 10 mM pyruvate, and
0.05 mM $MgCl_2$. The protein solution was centrifuged at 500 g
for 5 min to remove denatured protein and desalted on a 300
mm by 22 mm glass column slurry packed with 15 g (dry wt) of
Sephadex G-25. The packed column was equilibrated with
desalting buffer for 3 h at room temperature prior to use.
Desalted protein was used immediately for assay, or quickly
frozen at -40° C. Frozen protein was thawed over a 50° C
water bath with agitation for further use.

Enzyme assay. ALS activity was measured in assay
buffer containing: 20 mM phosphate (pH 7.5), 20 mM pyruvate,
0.5 mM thiamine pyrophosphate (TPP), 0.5 mM $MgCl_2$, and 10 uM
FAD. One hundred ul of desalted enzyme solution, or
collected chromatographic fraction, was added to 400 ul of
assay buffer. The solution was mixed and incubated at 30° C
in a water bath. The reaction was stopped after 60 min by
adding 25 ul of 12 N sulfuric acid. Acetolactate produced
by the enzyme was hydrolyzed to acetoin by heating in a
water bath at 60° C for 15 min. The reaction steps are

illustrated in Figure 3. Acetoin was quantitatively measured by a modified version of the method developed by Westerfield (1945). Five ml of 0.5% creatine in water, and 0.5 ml of 5% alpha naphthol in 2.5 N NaOH were added in rapid succession with mixing. The alpha naphthol must be mixed immediately prior to use as it rapidly oxidizes in air. The mixture was returned to the 60° C water bath for 15 min to complete color development. Absorbance at 525 nm was measured in a Varian series 634 dual beam spectrophotometer (Varian Instrument Co., Walnut Creek, CA). The reference cell contained either a reagent blank or a blank containing substrate and ALS "killed" with 12 N H_2SO_4. This latter blank eliminated interference of product formed before the assay, or the quenching effect of protein on the color development. A standard curve for the acetoin assay (Figure 4) was developed using known concentrations of acetoin and plotting the absorbance of the assay solution vs. the weight of acetoin.

Protein assay. Protein concentrations were measured by the method of Bradford (1976) with a prepared total protein dye reagent containing 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 nm to the detection wavelength of 595 nm. Bovine serum albumin protein standard was dissolved at specific concentrations in the ALS

desalting buffer described above. Solution containing from 10 to 100 ug protein in a volume up to 100 ul was pipetted into 10 ml test tubes. The dye reagent concentrate was diluted with four volumes of glass distilled water and filtered slowly to prevent foaming, which can result in particulate formation. Five ml of diluted reagent were added to each test tube and the contents mixed. Absorbance at 595 nm was measured after 2 min and before 1 h in 1.5 ml cuvettes against a reagent blank prepared from 100 ul of the same buffer as above and 5 ml of protein reagent. A standard curve was plotted as the weight of protein against the corresponding absorbance.

Pyruvate

$$CH_3 - \overset{\overset{\displaystyle O}{\|}}{C} - COO(-)$$

ALS $\longrightarrow$

Acetolactate

$$CH_3 - \overset{\overset{\displaystyle OH}{|}}{\underset{\underset{\displaystyle COO(-)}{|}}{C}} - \overset{\overset{\displaystyle O}{\|}}{C} - CH_3$$

Acetoin

$$\overset{H_2SO_4}{\underset{Heat}{\longrightarrow}} \quad CH_3 - \overset{\overset{\displaystyle OH}{|}}{CH} - \overset{\overset{\displaystyle O}{\|}}{C} - CH_3$$
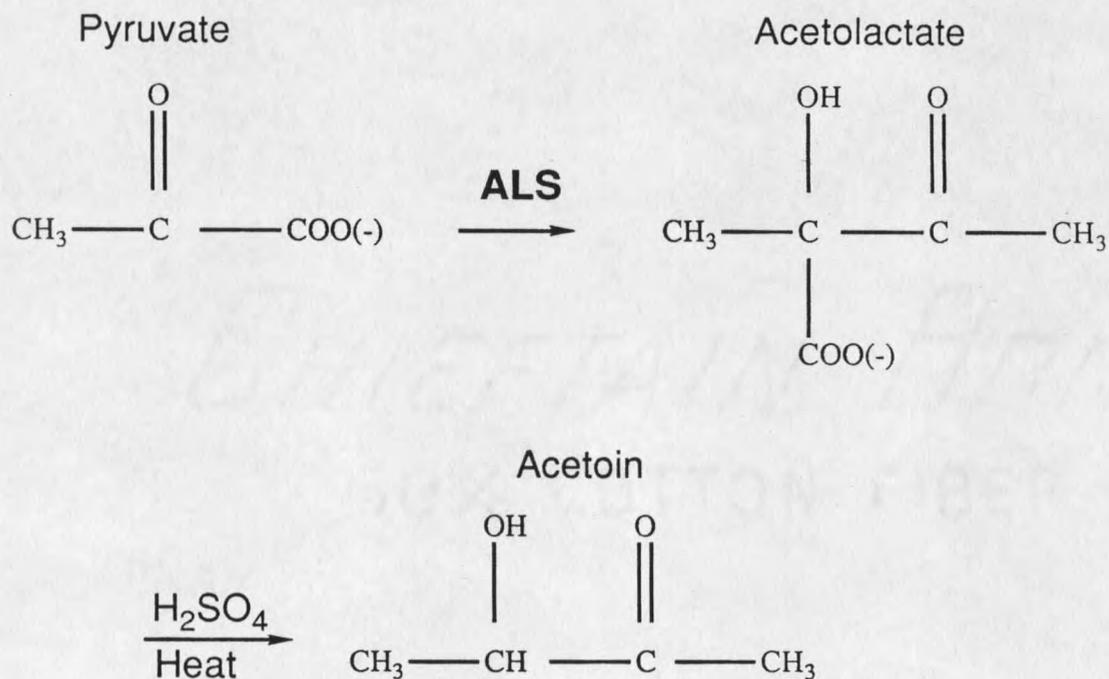
Figure 3. To determine ALS activity, the enzyme is reacted with pyruvate to form acetolactate which is converted to the measured compound acetoin, by acid hydrolysis.