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ABSTRACT

Title of Thesis: A Study of Parameters Indicating Microbial Activity in Mixed Aerobic Cultures

William Smith, Master of Science in Environmental Engineering, 1985

Thesis directed by: Dr. Gordon A. Lewandowski
Associate Professor of Chemical Engineering

Mixed liquor suspended solids (MLSS), oxygen uptake rate (OUR) and general dehydrogenase activity, as expressed by the reduction of the electron-acceptor dye 3-(4',5'-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT), were compared for their ability to measure microbial activity in mixed aerobic cultures. A fourth parameter, specific enzyme activity, was dropped from consideration because of the difficulty of developing a satisfactory experimental technique. Measurement of the parameters was applied to the biodegradation of phenol and o-chlorophenol by activated sludge from a municipal sewage treatment plant, and by two commercially available bacterial preparations.

MLSS, although the most commonly used of the three parameters tested, is often irrelevant to the true microbial activity and is therefore inappropriate for

use as a metabolic parameter. The reduction rate of MTT is potentially useful as a metabolic parameter, but requires a more complex preparation and provides less reproducible results than OUR. OUR has the clearest demonstrable relationship to substrate removal rate and provides the most reproducible results. Substrate removal rates that are normalized with respect to OUR are much more consistent than the original SRR data. OUR can be readily measured using an electrochemical probe, making it the easiest technique to apply.

A STUDY OF PARAMETERS INDICATING MICROBIAL ACTIVITY IN MIXED AEROBIC CULTURES

by
William Smith

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering
1985

APPROVAL SHEET

Title of Thesis: A Study of Parameters Indicating Microbial Activity in Mixed Aerobic Cultures

Name of Candidate: William Smith
Master of Science in Environmental Engineering, 1985

Thesis and Abstract Approved:

9/3/85

Dr. G. A. Lewandowski
Associate Professor
Department of
Chemical Engineering
and Chemistry

Date

9/3/85

Dr. B. C. Baltzis
Assistant Professor
Department of
Chemical Engineering
and Chemistry

Date

9/3/85

Dr. C. A. Venanzi
Assistant Professor
Department of
Chemical Engineering
and Chemistry

Date

VITA

Name: William Smith

Permanent Address:

Degree and date to be conferred: Master of Science in Environmental Engineering 1985

Date of birth:

Place of birth:

Secondary education: Governor Livingston Regional High School, Berkeley Heights, New Jersey 1973

Collegiate institutions attended	Dates	Degree	Date
New Jersey Institute of Technology	9/82-12/85	M.S.	1985
New Jersey Institute of Technology	9/79- 5/83	B.S.	1983
Michigan State University	9/73-12/77	B.S.	1977

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I. INTRODUCTION

The fundamental purpose of biological oxidation is to convert soluble or colloidal organic materials into mineralized products or insoluble biomass. This involves two processes, synthesis and respiration. Synthesis requires organic substrate and some inorganic compounds as material for the production of protoplasm. Synthesis also requires a net input of energy, which is derived from the organic substrate.

Energy for synthesis of cell compounds and maintenance of cellular functions is provided through respiration. In aerobic systems, the end result of respiration is the transformation of organic compounds to carbon dioxide, water and mineral salts with the release of metabolically useful energy. However, the process can also terminate before the substrate is completely oxidized. In activated sludge, the microorganisms primarily responsible for degradation of organic substrates are bacteria and fungi.

The ability of a cell to degrade a substrate is determined by several factors. The first is the ability of the compound to penetrate the cell wall. This ability is controlled by the size of the molecule

and its polarity. Small molecules penetrate more readily than large ones, which must be hydrolyzed by extracellular enzymes before being taken in. Hydrophobic molecules are taken in more readily than hydrophilic ones, although the cell may have transport systems for specific simple acids [1].

As with all major cellular reactions, those involved in biodegradation are mediated by enzymes. The enzymes which catalyze oxidation and reduction of chemical bonds are classified as oxido-reductases. The major group of these enzymes are the dehydrogenases, which catalyze the oxidation of specific bonds by the transfer of hydrogen atoms from their substrate to cyclic intermediate acceptors such as nicotinamide adenine dinucleotide (NAD) [2]. Enzymes are specific to particular compounds or structures. If there is no dehydrogenase for a particular compound, that compound may not be oxidized at any appreciable rate.

The catalysts (i.e., enzymes) utilized in biodegradation processes may be inactivated by toxic agents present in solution. Such agents may be other chemicals, or the substrate itself at higher concentrations. A study cited by Gaudy [3] demonstrates that increasing initial concentrations of

phenol to 100 ppm in an activated sludge batch process results in an increase in growth and respiration rates. At greater concentrations both rates are depressed. The same pattern is observed for other compounds and parameters [3,4].

Since biodegradation is a low-temperature catalytic (i.e., enzymatic) oxidation process, kinetic rate constants should be expressed in terms of the amount of active catalyst present. This quantity may be obtained by direct measurement of enzyme activities or by measuring some other parameter of metabolic activity used as a general indicator of catalyst activity.

The parameter most commonly used as an estimate of available catalyst is the weight of the portion of the dried mixed liquor solids that will volatilize at 600°C. This measure is known as the mixed liquor volatile suspended solids (MLVSS). The assumption behind the use of this parameter is that the amount of volatile organic material present in the solids is proportional to the microbial activity. Contributions to MLVSS from dead or dormant microorganisms, or from other suspended organic matter, are not taked into account. MLVSS is widely used as a critical parameter

in the operation of wastewater treatment plants. It is a key variable in the empirical relationships developed for the control of the biological treatment process [5]. In practice, it is necessary to have constant operating conditions to successfully apply this parameter. Changes in MLVSS occur very slowly compared to the rapidity with which changes in hydraulic conditions or influent compositions occur at treatment plants or in bench-scale experiments. Changes in these operating conditions cause immediate changes in degradation rates [6].

Other measures of active biomass have been proposed. Measuring the concentration of a class of biochemical compound, such as organic nitrogen, protein, lipid or carbohydrate, has the same drawbacks described above for the use of MLVSS. The methods do not distinguish between material from dead and dormant cells or between living and non-living matter, and the parameters change more slowly than observed changes in degradation rates. DNA is associated with living cells, but does not distinguish those which are active from those which are dormant. Changes in DNA content would still be limited by slow response to changes in operating conditions. Some researchers have made total

cell counts using agar plate culture techniques [7,8,9], but this method gives only an approximation of those organisms which can grow and reproduce on the highly selective media.

Another approach is to perform biochemical analysis of the suspended material resulting from cell disruption. Specific enzyme activities can be determined by measuring the rate of transformation of a specific substrate in an extract prepared from the microorganism culture. The amount of catalyst is defined by the number of micromoles of substrate transformed per minute at 25°C, or at some other standard condition. Another parameter which has been suggested is the concentration of energy-transfer or energy-storage compounds, in particular the high-energy compound adenosine 5'-triphospate (ATP). ATP concentrations have been demonstrated to vary directly with parameters such as total cell number or oxygen uptake rate [10, 11].

A third approach has been to measure the overall activity of intact cells. General dehydrogenase activity has been determined by incubating suspensions of intact cells with electron-acceptor dyes in their oxidized state and measuring the rate at which the dyes

are reduced [10]. This parameter has potential for use in studies of biodegradation kinetics because dehydrogenase enzymes are the principal class of enzymes involved in biodegradation of organic compounds.

Several other parameters have been explored. The activity of the culture has been measured by observing the uptake rate of a readily metabolized substrate, such as glucose, and the rate at which the oxidation product (carbon dioxide) is generated [12]. Oxygen uptake rate (OUR) has also been explored as a parameter of microbial activity [13] and has been demonstrated to be successful in predicting the behavior of aerobic biodegradation systems [14].

After a brief review of the available literature, it was decided to pursue specific enzyme activity, oxygen uptake rate, general dehydrogenase activity, and, for the purpose of comparison, MLSS, as parameters of catalytic activity in microbial suspensions. These parameters were selected as being suitable for making a number of rapid determinations of microbial activity without an inordinate increase in capital investment.

In this study, the substrates used as sole source of carbon for the microorganisms of the mixed cultures

were phenol and o-chlorophenol. These compounds have several advantages for use in this study. They are water soluble at high concentrations, relatively non-volatile and known to be readily biodegradable from previous work in this same laboratory. Phenolic compounds are also of environmental interest, mostly because of their presence in waste streams from processes which use coal as an energy source or chemical feedstock.

Three mixed cultures were chosen as biodegradation media. Activated sludge mixed liquor was obtained from the municipal wastewater treatment plant in Livingston, NJ. This source was chosen because the plant treats primarily domestic sewage, and has not been exposed to significant concentrations of industrial organic compounds. The other two cultures were grown from commercially available preparations. Hydrobac (Polybac Corporation) is supplied in a dry form, in which the bacterial suspension has been dried onto bran flakes. LLMO (General Environmental Sciences) is supplied as an aqueous suspension, saturated with hydrogen sulfide to suppress biological activity during storage. Both manufacturers claim that their preparations contain microorganisms

selected especially for their ability to degrade toxic or refractory compounds.

II. OBJECTIVE

The objective of this study is to select a readily measurable parameter of activated sludge metabolism that would indicate the general level of catalyst activity in a mixed microbial population, and to demonstrate experimentally that the parameter selected is reliable for use in the kinetic expressions for the biodegradation of phenol and o-chlorophenol.

III. BACKGROUND AND LITERATURE REVIEW

A. Literature Search Methodology

An extensive literature search was conducted to locate reports of previous work applying measurements of metabolic parameters to biodegradation rates of organic substrates in aerobic cultures.

The bulk of the search was carried out as a customized literature search on DIALOG, an on-line computerized information retrieval service. In searching, the computer identifies key words, authors and categories of information peculiar to a specific database.

The databases searched were Biosis Previews (which consists of Biological Abstracts and Bioresearch Index), and Water Resources Abstracts (a compendium of abstracts compiled by over 50 U.S. water research centers and institutes).

Key words were selected from the terms: activated sludge, microbial degradation, growth, respiration or activity, reduction of tetrazolium dyes, oxygen uptake rates and induction of dehydrogenase enzymes. Abstracts from 1968 to 1984 were reviewed. Out of these, 24 were selected as having possible application

to the present study. Citations found in the articles were also considered.

Several sources, not available in area libraries, were located through a computer search of holdings lists submitted by libraries across the U.S. The computerized search was supplemented by manual searches of the Chemical Abstracts Index and the holdings lists of NJIT and Rutgers in Newark, N.J.; the University of Delaware, Newark, DE; and Lehigh University, Allentown, PA.

B. Mixed Liquor Volatile Suspended Solids and Microbial Viability

Strictly used, the term microbial viability would indicate the ability of the organisms in the activated sludge to reproduce. When applied to the study of biodegradation, however, the term implies the ability of the culture to utilize substrate. In this study, the term is used to describe the ability of cells to reproduce. The cells involved in biodegradation will be described as "active".

The common approach to estimating the potential for substrate utilization is to measure the active biomass concentration in the sludge. The conventional measure has been the organic portion of the sludge dry

weight, referred to as mixed liquor volatile suspended solids (MLVSS). This parameter is seldom accurate because dead or inactive microorganisms, as well as organic compounds of non-biological origin, are measured along with the metabolically active cells.

The fraction of active organisms in the solids can be estimated by counts of viable organisms grown on agar plates. There are several drawbacks to this method. Viable organisms are selected by media that do not reflect the actual environment of the activated sludge process [10]. The technique of agar plate counting does not lend itself to routine or timely analysis [1]. Cells which are incapable of reproduction may still retain metabolic activity.

Walker and Davies [9] surveyed the published estimates of viable cell concentrations and found them to range from 2% to 60% of the MLVSS, with the actual rates of biodegradation being more uniform. In their own experimental work they attempted to correlate changes in oxygen uptake rate (OUR) with both MLVSS and viable cell counts, and found little correlation between changes in OUR and changes in MLVSS. They did find a better correlation between OUR and viable cell counts.

Weddle and Jenkins [7] attempted to correlate a number of metabolic parameters, including OUR and the reduction of the electron-acceptor dye 2,3,5-triphenyl-tetrazolium chloride (TTC), against changes in MLVSS and viable cell counts. They concluded that changes in biodegradative activity were due to changes in the number of active cells per gram of MLVSS, rather than being caused by changes in the activities of individual cells. When they compared total MLVSS to plate counts, they determined that the fraction of viable cells in MLVSS increased rapidly between growth rates of 0.3/day and 3.0/day and were constant for growth rates outside of this range. (Growth rate is expressed as the fraction of the MLVSS reproducing itself per day.) Since most biodegradation units operate at less than 0.3/day, Weddle and Jenkins concluded that MLVSS was an adequate expression of cellular activity, with the caution that viable cells constituted less than 20% of the MLVSS. Weddle and Jenkins based their evaluation of MLVSS on observations of continuous laboratory reactor systems operating at steady-state and thus did not take changes in operating conditions into account.

Tebbutt and Paraskevopoulos [8] also correlated a number of parameters, including OUR and reduction rate

of TTC, against MLVSS and viable cell counts. As did the researchers described above, they found that the parameters correlated better with viable cell counts than with MLVSS. Tebbutt and Paraskevopoulos concluded that MLVSS was the best parameter to use as a measure of sludge activity as it produced results no worse than the other parameters they tested and was the easiest to apply. The samples that they used were drawn from a continuous pilot plant, run under controlled conditions of feed composition and sludge wasting. This would have a favorable effect on the reproducibility of their MLVSS determinations. They also preferred MLVSS because it was less affected than the other parameters by changes in system loading or the presence of toxicants. This slow response limits the general applicability of MLVSS as an activity parameter.

The total mixed liquor suspended solids (MLSS) has also been recommended as a control parameter for continuous activated sludge systems. Both Maeda, et al [15], and Nogita, et al [16] claim that by controlling MLSS concentrations in the aeration tanks in wastewater treatment plants, they achieve efficient control of the biodegradation process. The systems described have relatively constant flow rates and COD loadings and the

test periods described are only several weeks long. It is possible that the composition of the MLSS might not change significantly under those conditions. Otherwise, it is surprising that MLSS would be an adequate parameter of sludge activity as it is composed of both MLVSS, which is a questionable parameter in itself, and an inorganic component, which would further reduce the accuracy of using MLSS.

C. Specific Assay of Enzyme Activity

Bergmeyer [17] deals at length with methods of analyzing the activities of specific enzymes, with particular emphasis on the study of animal tissues. One difficulty in preparing extracts for analysis is that most free enzymes are readily denatured by changes in pH or temperature [6]. Another is the need to produce particle-free samples for spectrophotometric analysis. The equipment used to extract cellular enzymes and separate cell solids from the supernatant are designed to keep the samples cold throughout the process. The centrifuges used are operated at high speeds, usually reported as multiples of gravitational acceleration (G = 32.2 ft/sec). This relates to revolutions per minute (rpm) by

$$a \times G = d \times (rpm) / 3600$$

where: a = multiplier for G

d = effective radius of revolution (ft).

These high-speed centrifuges are effective in removing very small particles that would otherwise interfere with spectrophotometric measurements.

Kotze [18] adapted these methods specifically for application to mixed cultures such as activated sludge. One of the major difficulties in preparing the necessary cell-free homogenates for analysis was the presence of humic substances in the sludge culture. These substances can inactivate free enzymes by binding with them in a nonproductive mode. Kotze's procedure for preparing the homogenate required centrifuging the sludge at 35,000 G at 2°C to separate the sludge solids. The solids were washed and suspended in buffer at pH 7.6, then homogenized by sonication at a minimum of 50 watts for 25 minutes at 8°C. The homogenate was then centrifuged for 30 minutes as described above. The resulting supernatant was kept at 1°C to 2°C until assayed. The assays recommended by Kotze were based on those described in Bergmeyer [17]. Kotze did not present data to demonstrate the effectiveness of his method.

Kotze's method was used by Florentz and Hartemann

[19] to detect changes in activity for eight enzymes as a biological wastewater treatment plant cycled between aerobic and anaerobic operation. Seven enzymes active in glycolysis and the tricarboxylic acid (TCA) cycle were measured, along with 3-hydroxybutyrate dehydrogenase. Measurable activities were found for all eight enzymes. No differences in enzyme activities were found between the two operating states.

Sayler, et al [20], measured bacterial population densities and enzyme activities in an attempt to assess the biodegradative potential of an activated sludge toward toluene, phenol, aniline, and 2,4-dichlorophenol. After assaying for a series of enzymes known to oxidize aromatic compounds, they found catechol 1,2-dioxygenase to be the only enzyme activity consistently present. Cells were washed and suspended in a phosphate buffer of pH 7.0. Sonication was at 50 watts for 5 minutes and cell debris were removed by centrifugation at 25,000 G for 30 minutes. The temperatures of the operations were not mentioned. Analysis showed higher oxygen uptake rates for the washed cells from sludges acclimated to the substrates, but little measurable dioxygenase activity in the sludge or the extract. Only cultures maintained on phenol showed any

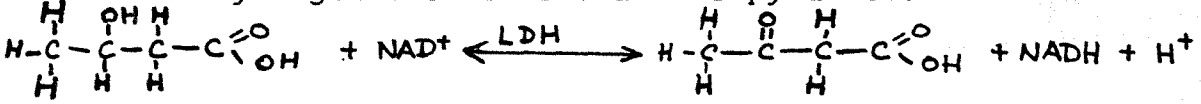
change in enzyme activity attributable to acclimation. The values obtained were near the sensitivity of the assay used. Sayler concluded that his assay was too narrow in scope and that only determinations of broad spectrum enzyme activity could provide unambiguous information.

Kawai, et al [21], successfully analyzed the activity of polyethylene glycol dehydrogenase in mixed cultures by a multistep process. Their assay was preceded by a complex process for purification of the enzyme consisting of precipitation, solubilization and three chromatographic steps. This procedure is too complex for studies where there are a large number of samples to be analyzed.

In brief, the successful published methods for isolating enzymes from activated sludge or other mixed cultures and determining their activities were either too time consuming to be applied to a large number of samples or required specialized equipment unavailable for the present study. Therefore they were not considered further for use in this study. Simpler methods were attempted, based on commercially available enzyme assay kits, as described in section V-A.

D. Use of Electron-Acceptor Dyes

Electron-acceptor dyes have been used to visualize NAD and NADP dependant reactions since Thunberg published his technique of methylene blue reduction in 1917 [17]. As the dehydrogenases are the major class of enzymes involved in substrate utilization, a technique that measures the reduction rate of their common co-enzyme has application to this study. A typical oxidation-reduction sequence involving NAD is the dehydrogenation of lactate to pyruvate:



Other electron acceptors are also used by cells in substrate oxidation. The total cellular pool of these electron acceptors is very small, and their oxidation and reduction very rapid. For aerobic organisms, the ultimate electron acceptor is oxygen. For this reason, measurements of oxygen uptake rate are considered to be an excellent indicator of cellular activity [13]. Diverting the electrons to another acceptor, such as a dye, also provides a measure of cellular activity.

Most of the research reported has involved the use of tetrazolium salts as the electron-acceptor dyes [17]. In particular, the reduction of 2,3,5-triphenyl-tetrazolium chloride (TTC) to its insoluble red

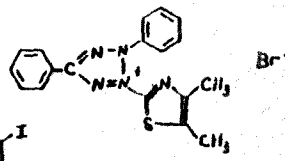
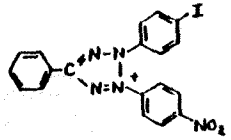
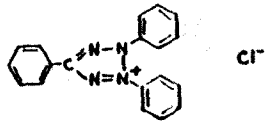
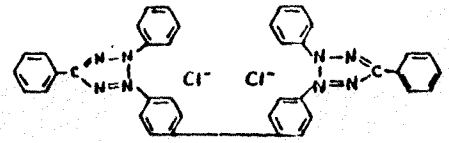
formazan has been applied by a number of researchers to problems of microbial activity. Some commonly used tetrazolium salts are shown in Table 1.

Different tetrazolium salts can be reduced by different metabolites. TTC is reduced by electron carriers with more positive potentials than those which reduce MTT or INT. It is not reduced by nicotinamide adenine dinucleotide phosphate (NADP) or flavin adenine dinucleotide phosphate (FADP) and competes directly with oxygen for available electrons [17]. MTT is reduced by metabolites with less positive electron-transfer potentials, which includes most of the cyclic intermediates [22]. INT can be directly reduced by flavin dehydrogenases, without the need for an intermediate electron carrier [17]. These differences suggest that these dyes would produce different patterns of response in toxicity assays.

The general procedure for determining dehydrogenase activity is to incubate an activated sludge sample with a small amount of dye for a fixed period of time. The formazan is dissolved with an organic solvent, the solids separated and the absorbance of the liquid read at a wavelength characteristic to the formazan. Dehydrogenase activity may be reported as

TABLE 1

COMMONLY USED TETRAZOLIUM SALTS

Abbreviation	Name	Structural Formula	Redox Potential (mV)
MTT	3-(4′,5′)-dimethylthiazol-2-yl) -2,4-diphenyltetrazolium bromide		+110
INT	2-(p-iodophenyl)-3-(p-nitrophenyl) -5-phenyltetrazolium chloride		+90
TTC	2,3,5-triphenyltetrazolium chloride		+460 +240
NT	2,2′-p-diphenylene-3,3′,5,5′- tetraphenylditetrazolium chloride		+170

the quantity of formazan produced, but is more commonly reported as absorbance or extinction.

Each researcher reported developing his variation of this basic method. These variations differ with respect to the blank used, the method of stopping the enzyme reaction, the length of the incubation period, the method of extracting the formazan, the exclusion of oxygen and the substrate included in the reaction mixture.

Lenhard [23] incubated an activated sludge sample with 0.01% TTC and 0.1% glucose in a buffer of triaminohydroxymethane hydrochloride (TRIS-HCl;pH 8.4). Glucose was included to allow expression of the enzyme activity unlimited by substrate availability. Incubation was in the dark at 37°C for 15 minutes, at which time absolute ethanol was added to stop the reaction and dissolve the formazan. Solids were filtered from the solution and the filtrate absorbance was read against a distilled water blank at 483 nm. Oxygen was not excluded from the incubation mixture. Lenhard selected glucose as substrate after trying citrate, succinate and glutamate, none of which allowed expression of the dehydrogenase activity. Lenhard concluded that these substrates did not penetrate the

cell wall. It is more likely that these substrates showed no effect because their co-enzymes would not reduce TTC. Succinate dehydrogenase, for example, reduces FAD, which will not reduce TTC [10], and citrate is not reduced by any NAD or FAD dependant dehydrogenase. It is also possible that the dehydrogenase activities specific to these substrates were too low to be observed. Lenhard did not analyze the mixed liquor to see if substrate was actually consumed.

Lenhard [24] used his method as a measure of the toxic effects of chemicals on activated sludge. The sludge consisted of soil organisms cultured on a medium of glucose, peptone, phosphate, and magnesium and iron sulfates, in a batch reactor. The same medium was used as substrate for the toxicity tests. Lenhard plotted the fraction of dehydrogenase expression (compared to that of an untreated control) against the concentration of several toxicants (silver, mercury and chromium ions, formaldehyde and phenol, and four industrial effluents). In each case, there was a threshold concentration below which no depression of activity was observed. Above this threshold, there appeared to be a linear relationship between the fraction of activity inhibited and the logarithm of the toxicant concentra-

tion. The magnitude of the threshold concentration varied with the age of the culture. Sludges which were exposed to the toxins shortly after being cultured on the substrate medium were affected at a lower concentration than were the sludges with a longer culture period. This observation is consistent with observations of toxicant action on other sludge parameters [6].

In both studies, Lenhard did not consider the effects of dissolved oxygen on the rate of TTC reduction. Higher metabolic rates in the sludge would cause the oxygen content of the samples to be lower, resulting in higher rates of formazan production. Lenhard also observed a lag time of about an hour between the time that the substrate was added to the culture and the time that TTC reduction began, and assumed that the time was needed for enzyme induction to occur. It seems possible that this time was actually needed to deplete dissolved oxygen concentrations to a level that would allow formazan production to begin.

Bucksteeg [25] proposed the use of TTC reduction as a measurement of sludge activity independantly of Lenhard. His method added 0.05% TTC to the sludge

sample, then incubated the sludge sample for 1 hour at 20°C in the dark. Ethanol was again the extraction solvent. The blank was 0.05% TTC in water and was processed with the samples. Oxygen was not excluded and pH was not controlled. The only substrate present in the incubation medium was that carried along with the sludge sample. Over 100,000 observations were made on samples from a laboratory-scale continuous-flow activated sludge plant fed a synthetic wastewater of unspecified composition. TTC reduction was correlated with total sludge solids and rate of BOD removal by the relation:

$$f = y \times a$$

where: f is a constant roughly proportional to influent BOD;

y is the absorbance of the formazan solution; and

a is the rate of BOD removal per mass of total sludge solids.

This would indicate that for a given BOD loading rate, the respiration rate, as measured by the absorbance of the formazan solution, decreases as the rate of BOD removal increases. This relation is contradicted by the observations of Ford [26] and others [6,7] and by

the metabolic interrelation of respiration and substrate utilization. Bucksteeg's results may have resulted from his test being substrate limited, as the largest initial loading used was equivalent to 438 mg BOD/L. As contrast, Lenhard's glucose solution provided a minimum of 1067 mg BOD/L. It is likely that the higher rates of BOD removal resulted in a lower steady-state BOD concentration in the reactor. With a smaller amount of substrate carried over with the sludge sample, TTC reduction would reach its limit sooner. Both BOD removal rate and TTC reduction rate were observed to increase as the BOD loading rate was increased. This increase of microbial activity with substrate concentration is a commonly observed phenomenon. Bucksteeg also determined that the rate of TTC reduction responded immediately to changes in organic loading. His hour of incubation may have allowed the necessary depletion of oxygen in the sample that Lenhard waited for in his reactor.

Ford, Yang and Eckenfelder [26] used Lenhard's method to relate formazan production to sludge age, organic loading, MLVSS and oxygen uptake rate (OUR) in a municipal contact stabilization tank. They also developed correlations to adjust the rate of formazan

production for the effects of pH and temperature. They did not exclude oxygen from their samples. Their findings were that formazan production rate decreases as the sludge ages and increases with MLVSS, organic loading and OUR. These results are in accord with established observations of activated sludge performance [6]. They also observed that the rates of formazan production began to decrease after one-half hour of incubation. They did not account for this decrease. They did mention a lag time between introduction of BOD to the reactor and initiation of TTC reduction. Again this lag was likely the time required to decrease the DO concentration in the contact stabilization tank to a level at which TTC reduction could begin.

Coackley and O'Neill [27] tracked general dehydrogenase activity along the aeration tanks in a municipal wastewater treatment works. Their procedure used 0.03% TTC and 0.06% glucose in the reaction medium. Incubation was at 20°C for 15 minutes. The formazan was extracted with ethanol. Their findings confirmed Bucksteeg's observation that TTC reduction rates increased with organic loading. Elevated dehydrogenase activity was found to continue after the disappearance

of supernatant BOD and COD. They attributed this activity to the metabolism of substrate which had been adsorbed onto the floc and possibly to the metabolism of compounds stored during the earlier respiration. However, observations from other researchers and from this study (section VI-F) show OUR dropping rapidly as supernatant BOD or COD approaches zero. Since Coakley and O'Neill did not examine OUR, no direct comparison can be made. It appears doubtful that metabolism of adsorbed COD could account for the elevated dehydrogenase activities observed. The sustained activity levels they observed in their TTC reduction test can be accounted for by the availability of glucose in the incubation medium. Coackley and O'Neill also noted that TTC reduction increased immediately after the load to the aeration tanks increased and reached its maximum within one-half hour. The incubation time used was shorter than Bucksteeg's and the temperature lower than Lenhard's, yet no delay in TTC reduction was observed. No dissolved oxygen levels were reported, but it is possible that they were low enough to allow immediate reduction of TTC.

Clayfield [28] measured the rate of TTC reduction as one tool in a study relating OUR to denitrifying

behavior in activated sludge. Several important modifications were made to Lenhard's method. Sludge solids were washed twice and resuspended in bicarbonate buffer before analysis. Nitrogen gas was bubbled through the samples to remove oxygen. Extraction was performed with methanol and absorbances were read against a methanol blank. Clayfield compared OUR and TTC reduction rates for sludges in the endogenous state and sludges actively utilizing substrate. Both parameters were used independantly to demonstrate that total sludge activity could be divided into two components. One component was associated with the rate of BOD removal and the other was associated with the endogenous OUR, which he found to be proportional to the MLVSS of the sludge. Clayfield also noted that, for a continuous reactor at steady-state, the specific endogenous respiration rate increased linearly with the BOD loading to which the sludge organisms had been acclimated. This suggests that some change occurred in the composition of the MLVSS, probably an increase in the total number of organisms.

Tebbutt and Paraskevopoulos [8] correlated specific OUR and specific TTC reduction rate on the basis of MLVSS and viable cell count. Their method was based on

Lenhard's, with sodium and cobalt chloride added to the mixture to reduce oxygen. Unit viability was determined by plating samples of activated sludge onto agar with sterilized sewage as substrate and was expressed as the number of cells per gram of MLVSS. Samples were taken from an activated sludge continuous pilot plant operated on municipal sewage. No correlation was found between OUR and TTC reduction rate when both parameters were expressed as activity per gram MLVSS. This observation would imply that there is no relationship between OUR and TTC reduction, which contradicts the findings of all other reports, including the present study. The two parameters showed a strong relationship when they were expressed as activity per viable cell. Tebbutt and Paraskevopoulos suggest that a different proportion of OUR is attributable to dehydrogenase activity in viable cells than in cells incapable of reproduction.

Jones and Prasad [29] compared the effects of various experimental conditions on TTC reduction. Activated sludge from a municipal wastewater treatment plant was incubated with 0.05% TTC and no additional substrate under various conditions of aeration, pH and light. Increasing oxygen concentration in the sample

depressed formazan production and increased the rate of photolytic bleaching of the extract. Increasing pH stimulated both photochemical reduction of the TTC and photochemical oxidation of the formazan. They observed a close correlation between OUR and formazan production for a number of microorganism cultures from different sources.

Klapwijk, Drent and Steenvoorden [30] presented a critical study of the TTC methods published to that date, with particular attention to the blank used, methods of stopping the enzyme reaction, extraction of the formazan, exclusion of oxygen and the organic substrate used in the incubation medium. Since the type of blank used by Lenhard and Ford does not account for chemical reduction of TTC during incubations (as TTC is excluded), Klapwijk created a blank by preparing it in the same way as the samples but stopping the enzyme reaction with concentrated sulfuric acid before incubation. This modification is questionable as the lower solution pH would inhibit chemical formazan production in the blank. Other workers stopped the enzyme reaction by adding organic solvent to extract the formazan. Klapwijk found that formazan production continued, even in the presence of butanol or ethanol.

The reaction definitely ceased when sulfuric acid was added. He also observed that the acid improved formazan extraction, leaving the solids colorless. Heating the samples also improved formazan extraction. To determine the effect of oxygen concentration on TTC reduction, Klapwijk, Drent and Steenvoorden subjected an aerated mixed culture to a shock load of sodium acetate. Over a duration of 4 hours, they periodically recorded the dissolved oxygen (DO) concentration in the reactor for comparison with formazan production rates in two sludge samples. One sample was treated with sodium sulfite and cobalt chloride to reduce oxygen and the other sample was left untreated. As the run progressed the DO level in the reactor decreased, then resumed its original level. In the untreated sample, formazan production increased, then decreased to its original level. These changes occurred at the same time as the changes in DO, with formazan production increasing as DO decreased. In the treated samples, formazan production remained unchanged throughout the run. The rate of formazan production for the treated samples was equal to the maximum rate for the untreated samples, suggesting that the changes in the rate of reduction of TTC were not caused directly by changes in

sludge activity, but were actually the result of effects of competition of TTC with DO. No other report was found where this comparison was attempted. The results of this demonstration may have been affected by the substrate used. Klapwijk compared formazan production resulting from use of lactate, acetate, glucose and a synthetic sewage as substrate in the incubation mixture. Acetate enters the intermediary metabolism bound to a coenzyme, as acetyl CoA. If the available coenzyme is saturated, an excess of acetate would not affect the rate of TTC reduction. Acetate is a substrate for only one dehydrogenase, which reduces acetate to acetaldehyde. This reaction would compete with formazan production as both processes require electrons from NADH. Both factors could account for the lack of results found by Lenhard for acetate [23] and for the constant formazan production found in Klapwijk's analysis of DO effects.

Klapwijk used his method to compare the effects of four toxicants on OUR and TTC reduction in activated sludge. His results indicated poor correlation between OUR and dehydrogenase activity when zinc, cyanide, phenol or 2,4-dichlorophenoxyacetic acid (2,4-D) are added to the activated sludge. He did find a good

correlation between OUR and dehydrogenase activity in unpoisoned sludge. These results were supported by Jorgensen in a study of methylene blue reduction [32].

Ryssov-Nielson [31] presented another modification of the general dehydrogenase assay. Sludge solids were washed and resuspended in oxygen-free phosphate buffer at pH 7.5. The incubation mixture contained 0.005% dye and 0.045% lysozyme. Incubation was in the dark at 37°C for 1 hour in the absence of air. No substrate was used in the incubation mixture. The blank did not include the dye. Both TTC and MTT were tested as electron-acceptor dyes. For both dyes, addition of lysozyme improved the reproducibility of the results for multiple analyses, but did not alter the mean absorbances. Both dyes showed good correlation with OUR and similar patterns of inhibition when the sludge was exposed to mercuric chloride. They showed very different patterns of inhibition when exposed to potassium cyanide. Potassium cyanide first poisons the cytochrome oxidase system, where TTC replaces oxygen as the ultimate electron acceptor. MTT is reduced at a lower redox potential, bypassing the cytochrome system [17] and thus allows a sort of artificial respiration to take place. Ryssov-Nielsen also used TTC reduction

to demonstrate the stimulation and subsequent inhibition of respiration in activated sludge by 2,4-D, lactate and pyruvate. Use of lactate resulted in the highest rate of formazan production. The use of lactate should result in the expression of lactate dehydrogenase activity [31] and of NAD dependant dehydrogenases further down the metabolic path. He estimated a whole-cell Michaelis-Menten half-velocity constant (Km) for lactate dehydrogenase of between 120 and 220 ppm, attributing the high value to active transport of the substrate into the cell. A comparison of the results of the TTC reduction method to those of the standard Warburg respirometer method showed that the two produced parallel results in inhibition tests. This finding contradicted Klapwijk's observation that for the same inhibition tests, TTC reduction rate and OUR were apparently unrelated.

The authors of the aforementioned studies present a range of opinions as to the applicability of tetrazolium dye reduction rates as a measure of biological activity. While all agree that for a given healthy sludge, there is a strong correlation between dye reduction rate and other parameters such as OUR, there is much disagreement as to whether these correlations

hold true for sludges exposed to toxins or sudden changes in process conditions.

At a first look, this dissension may seem odd as the test appears to be quite simple and direct. However, after reviewing the papers discussed above, a picture emerges of a quite complex test with many factors affecting the results.

In the earliest methods, researchers recognized the importance of controlling incubation temperature and duration, pH, the nature of the blank, and the extraction method used to optimize their tests for sensitivity and reproducibility. Other important factors were not always controlled. These were DO, the amount and type of substrate used in the reaction mixture, and the differing reactivities of the different dyes. Sludge age also seems to be an influential factor. Knowledge of the effects of all these parameters is needed to interpret test results.

In particular, the substrate and dye used should be considered more carefully than they have been. As mentioned above, different dyes are reduced at different points in intermediary metabolism. The various pathways can be blocked selectively by different toxins. For example, the reduction of TTC

can be decreased by a chemical that blocks the cytochrome oxidase system. The reduction of MTT would be unaffected or increased since that dye can be reduced independantly of cytochrome oxidase activity.

Similarly, the activity of different pathways can be stimulated or depressed by selecting substrates that enter or bypass them. Several researchers have suggested that the activity of a particular enzyme could be analyzed by using its substrate as the sole energy source in the TTC test. This would provide a temptingly simple method of enzyme analysis, except for three factors. The first is that not all simple molecules are readily taken up by cells. Some enter by diffusion, but some enter by active transport, which is an NAD dependant process [1]. The second is that the tetrazolium dyes can be reduced by different coenzymes. Stimulating succinate dehydrogenase activity would not necessarily stimulate TTC reduction proportionately, as the coenzyme involved (FADH) will not reduce TTC. INT, however, would provide a direct estimate of FAD reduction, as it competes with flavins for active sites. The third is that the change in the activity of the first enzyme in the sequence would not be differentiated from changes in the activities of the dehydro-

genases further along the biodegradative sequence.

E. Measurement of Oxygen Uptake Rate

Oxygen uptake rate (OUR) has been widely used as an indicator of biodegradative activity. Because of the position of oxygen as ultimate electron acceptor in aerobic metabolism, OUR should be useful as a quantitative expression of the turnover rate of high energy metabolic intermediates. Patterson [10], Huang [13], and others have demonstrated a linear relationship between OUR and production of adenosine triphosphate (ATP) in mixed cultures when both are expressed per weight of MLVSS. Linear relationships have also been demonstrated between OUR and substrate utilization rates [33]. Other researchers [7,8] have found OUR and ATP concentration to be constant when expressed per viable cell. However, these parameters varied when expressed per weight of MLVSS, confirming that only a fraction of the MLVSS is active.

Weddle and Jenkins [7] compared OUR, TTC reduction, ATP content, viable cell counts, and analyses for MLVSS, carbon, nitrogen, protein, carbohydrate, and lipid for their relations to net growth rate amd substrate consumption. When expressed per gram of MLVSS, OUR, TTC reduction and ATP content increased

with growth rate up to a rate of approximately 3/day. At greater growth rates, these parameters remained constant. When the same parameters were expressed per viable cell, they were found to be constant throughout the range of growth rates. Weddle and Jenkins concluded that increases in biodegradative activity were due to increases in the number of cells in the MLVSS and not in the activity of the individual cells.

Walker and Davies [9] measured OUR by respirometry and performed viable cell counts in an attempt to correlate both with the net growth rate. Both parameters increased with growth rate, reaching their maximums at growth rates near 0.7/day. At these growth rates, the OUR was 1.46×10^{-12} mg DO/hr-cell. As the growth rate decreased below 0.4/day, the OUR increased rapidly to 9.5×10^{-12} mg DO/hr-cell, then fell off to zero. Walker and Davies' analysis led them to conclude that as growth rates decreased below 0.4/day, an increasing proportion of the microbes are active but not viable. However, the total number of live cells continues to increase until a growth rate of approximately 0.1/day, when the cells begin to die off rapidly. Their conjecture agrees well with known behavior of aging sludges [6]. (Sludge age is the

inverse of specific growth rate.)

Tebbutt and Paraskevopoulos [8] correlated general dehydrogenase activity, OUR, viable cell counts and MLVSS to assess their usefulness as measures of biodegradative activity in activated sludge models. They also correlated OUR due to substrate removal against the endogenous OUR. When the parameters were expressed per gram MLVSS, the only correlation found was between the substrate removal rate and endogenous OUR. On a per viable cell basis, all parameters correlated well with each other. These results suggest that there are different active components of the MLVSS with differing responses to the OUR and dehydrogenase analyses.

Matsumaga, et al [34] used an electrochemical probe to estimate total cell numbers. A small number of cells from a culture in the early growth phase were trapped on the membrane of a galvanic oxygen probe. The current of the probe decreased with increasing cell numbers. There was a strict linear relation between cell number and generated current. Current generated per cell varied between species, but was constant for any single species. Cell numbers were confirmed by viable cell counts. The use of an electrochemical probe to count viable cells is of questionable value

for application to mixed cultures because of the number of species that could be present, each with a different oxygen requirement. Also, the young cultures used by Matsumaga, et al, would be expected to consist primarily of reproducing cells. As the studies discussed above show, older cultures consist of both viable and non-viable cells. The oxygen requirements of cells are presumed to change with age.

Kalinske [35] studied factors affecting OUR. He found that the nature of the floc and the amount of turbulence of the mixture were the major determining factors. Oxygen concentrations from 0.5 ppm to 35 ppm (produced under pressure) had no measurable effect on OUR. This finding was supported by other researchers [6,36].

Huang and Cheng [14] measured the effect of substrate and mean cell retention time (MCRT) on OUR in steady-state continuous reactors using the Monod-type relationship:

$$OUR = \frac{U^{\circ}_{m}\ S\ X}{K^{\circ}_{S} + S}$$

where: U m = maximum specific OUR (per gm MLVSS)

K s = half-rate constant (expressed as substrate concentration)

S = steady-state substrate concentration

X = biomass as gm MLVSS

Equating DO consumed to COD expressed, they were able to predict COD concentrations in their reactor effluent.

F. Summary

It is difficult to see how MLVSS could be used as a reliable measure of biological activity. It is well known that this parameter changes slowly in response to system changes. Large excursions in flowrate or chemical composition of a feed stream can occur before changes in the amount of MLVSS are noticeable [6]. It is also apparent that the composition of MLVSS is quite variable. The portion of the MLVSS that is metabolically active can range from almost the entire mass to only a few percent. At the lower end of this range a small change in substrate or toxin concentration could drastically change the activity of the sludge, with only a small change occurring in MLVSS.

Measurement of other biochemical compounds presents the same problems as the measurement of MLVSS. Even when testing for DNA, a compound closely associated with cellular organisms, there is no way to determine the relative contributions of growing, sene-

scent, or recently dead cells.

Determining unit viability, defined as the number of cells per sludge mass that are capable of reproducing, presents a more accurate picture of the activity of a culture than MLVSS measurement does. Other biochemical parameters show reproducible relationships when expressed as activity per viable cell. The drawback to this method is that the media used to culture viable organisms are always selective. Some species, which are active in the mixed liquor, will not reproduce in the culture medium and will not be counted. Within a species population, individuals which cannot reproduce still respire and take up substrate. Besides these considerations, the time required to culture and count cell colonies makes the method impractical for routine use.

The majority of the methods published for the analysis of specific activities have been developed for use with animal tissues or suspensions of single-specie bacterial cultures. Researchers applying these methods to activated sludge have had limited success, obtaining low activity values that do not always produce significant results. More reliable procedures require elaborate, multi-step separations which are not

suitable for the large number of samples which are required in this study. For these reasons, it was decided not to pursue published procedures but to try methods based on packaged kits for clinical enzyme assays.

The reduction of electron-acceptor dyes, such as the tetrazolium salt TTC, can be used to visualize the general dehydrogenase activity of suspensions of intact cells. Reports by researchers who have worked with TTC reduction show that changes in its reduction rate follow empirically established changes in sludge activity with COD loading, temperature and mean cell retention time. There is a direct correlation between microbial respiration, as determined by OUR, and general dehydrogenase activity, as determined by TTC reduction, regardless of the substrate used as electron donor. This relationship will be disrupted by toxins, such as cyanide ion, which inhibit electron transfer, but not by phenol and o-chlorophenol. Because the method of incubating the dye with a suspension of intact cells is suitable for a larger number of analyses, this method of measuring microbial activity was investigated further.

Oxygen uptake rate (OUR) appears to be the best of

the methods considered for measuring metabolic activity. It is a parameter common to all aerobes and is directly linked to substrate uptake and degradation. OUR changes rapidly in response to changes in feed concentrations and flow rate and is readily measured using an electrochemical probe. The correlation between OUR and substrate removal is not perfect, as some substrate will be converted to biomass instead of carbon dioxide. The amount converted to biomass is related to the ratio of the cells which are growing to those which are senescent, and thus is related to sludge age. OUR can be useful for comparing the toxicities of different compounds or their ability to act as energy sources. Since oxygen is the final electron acceptor in the metabolism of aerobic organisms, any change in metabolism will be reflected by a change in OUR. OUR was also investigated as a measure of microbial activity.

IV. EXPERIMENTAL APPARATUS AND EQUIPMENT

A. Reactor

The reactor used for culture acclimation and biodegradation runs is shown in Figure 1. The 4.0 liter batch reactor was constructed from a 6-inch diameter clear Lucite tube with 1/4-inch wall and a 1/4-inch thick base of Lucite. A 1/4-inch diameter outlet was provided near the base of the reactor and used to remove large volume samples. The reactor was capped with a 1/4-inch thick Lucite piece grooved to fit the top of the reactor and perforated with a 1/2-inch hole to allow tubing to be inserted into the reactor.

Compressed air was provided to keep the liquid supplied with oxygen. The air first passed through a 4-inch long by 2-inch diameter plastic pipe stuffed with glass wool to remove oil droplets. An oil trap was inserted after the filter. This trap was made from an 8-inch tall plastic graduated cylinder capped with a rubber stopper and sealed with adhesive tape. Air flowed downward into the trap through a plastic tube inserted through the stopper, made a 180° turn and exited at the top of the trap. The air then passed through 1/4-inch Tygon tubing, ending in an aquarium diffuser stone at the bottom of the reactor. The air

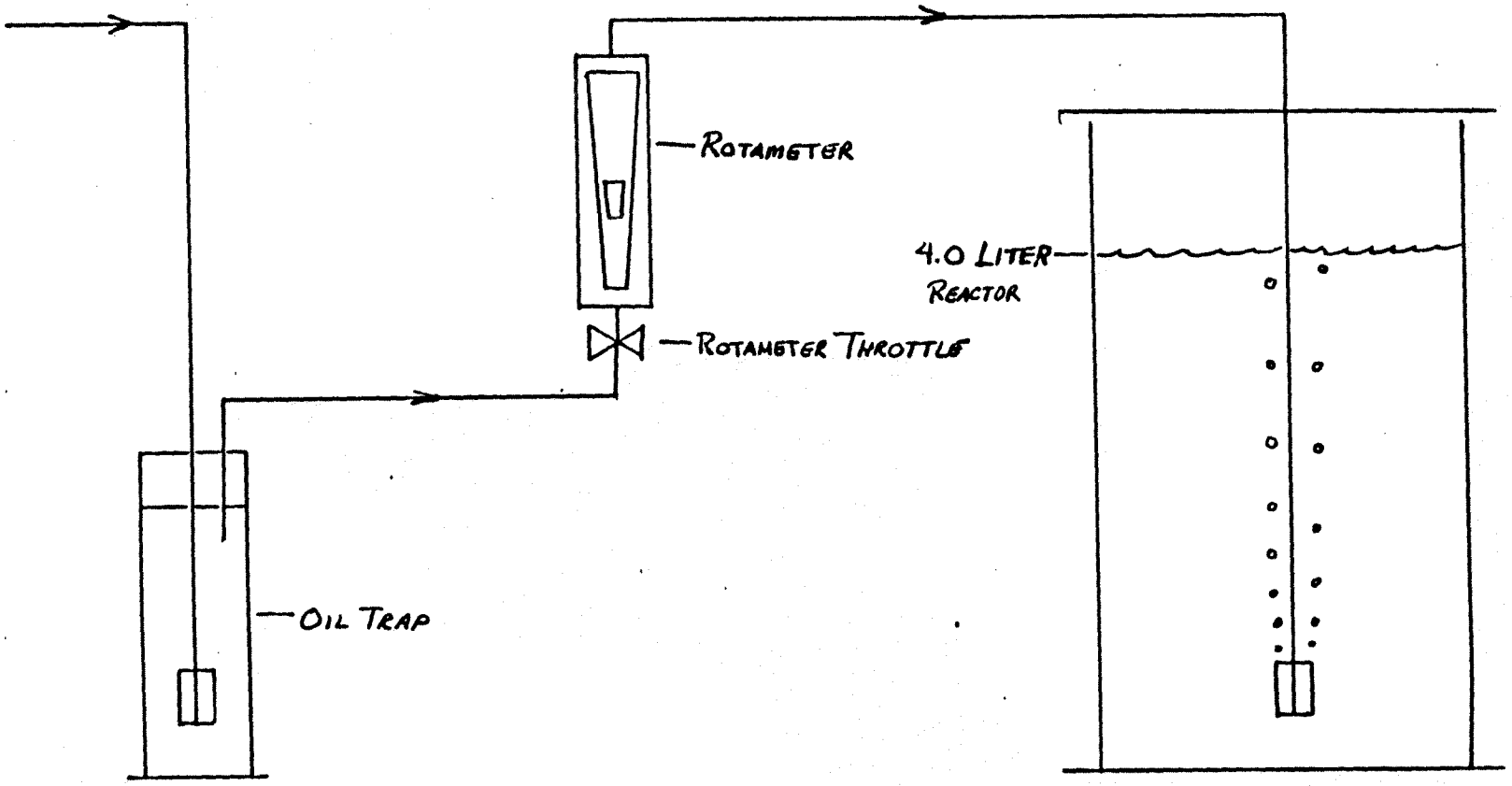


FIGURE 1 - BIODEGRADATION APPARATUS

flowrate was measured with a Gilmont rotameter, and was generally about 1.5 standard cubic feet per hour (750 cc/min).

B. ANALYTICAL EQUIPMENT

1) pH meter: Orion Research model #701A

pH electrode: Orion Research model #91-04

dissolved oxygen electrode: Orion Research model #97-08

ammonia gas electrode: Orion Research model #95-11

2) Gas chromatograph: Tracor model #560

operating temperatures: oven = 120°C

FID/injection port = 250°C

Gas flowrates: N = 45 cc/min @ STP

H = 30 cc/min @ STP

Air = 0.9 SCFH @ STP

GC column: Supelco - 5 ft x 1/8 in S.S.

5% SP-2100 on 100/120 Supelcoport

Integrator: Hewlett-Packard #3390A

3) UV spectrophotometer: Perkin-Elmer model #571

4) Centrifuge: Damon IEC HC-S II

5) Incubator: Blue M Electric Company model #100A

6) COD reactor: Hach model #16500-10

7) Sonic bath: Branson B-220, 50W

8) Pipettors: Labindustries VMP-15H 5-15 µL
Labindustries VMP-1000H 10-1000 µL
Drummond #275 0-100 µL

9) Enzyme Analysis Kits, Sigma Chemical Company:
Lactate Dehydrogenase Colorimetric Method #500
Lactate Dehydrogenase Kinetic Method #226-UV
Glucose-6-Phosphate Dehydrogenase Kinetic Method #345-UV

10) Oven: Lab-Line Instruments, Inc. model #N-3620-5

V. EXPERIMENTAL PROCEDURES

A. Specific Enzyme Assay

There are a number of references describing methods for the in-vitro analysis of specific enzymes. All of them track the formation or disappearance of a substrate or cofactor specific to the enzyme of interest. After reviewing the procedures described in Bergmeyer [17] and in the literature, it was decided to investigate clinical chemistry kits available from Sigma Chemical Company as tools for use in this study. These kits have been developed for routine laboratory use from accepted spectrophotometric methods. They contain all necessary reagents and have been standardized to give reproducible results.

Three tests were selected from those available:

(1) The quantitative ultraviolet determination of glucose-6-phosphate dehydrogenase (G-6-PDH) measures the reduction of nicotinamide adenine dinucleotide phosphate (NADP+) to NADPH in the reaction:

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH} + \text{H}^+$$

where: G-6-P = glucose-6-phosphate and

6-PG = 6-phosphogluconate

Production of NADPH is followed by measuring the increase of its absorbance band at 340 nm. This method

will be referred to as the G-6-PDH method [37].

(2) The quantitative determination of lactate dehydrogenase (LDH) follows the reduction of NAD+ to NADH in the reaction:

$$\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

Production of NADH is followed by measuring the increase of its absorbance band at 340 nm. This method will be referred to as the LD-L method [38].

(3) The third procedure was for the quantitative colorimetric determination of LDH activity. This method is better suited for large numbers of samples than the ultraviolet kinetic (LD-L) method described above. The same enzymatic reaction is involved, but instead of being followed by UV absorbance, it is stopped after a time with a hydrazine derivative, which reacts with the available lactate to form a strongly colored hydrazone. LDH activity is determined from the colorimetric absorbance of the hydrazone. This method will be referred to as the LDH method [39].

Sigma provides procedures, temperature correction data and information on the sensitivity and reproducibility of each test

The methods were checked using standardized enzyme preparations provided by Sigma. The results are summa-

rized in Table 2. Both kinetic methods (G-6-PDH and LD-L) gave average results close to those reported by Sigma but the precision was much worse. The colorimetric method (LDH) gave results with comparable precision to Sigma's report, but the average activities were always higher than predicted. In addition, the colorimetric method did not allow for temperature corrections to be performed, and required longer incubation times and more preparatory steps than the other methods. Therefore only the kinetic methods were pursued further.

All three analyses for the activity of specific enzymes require suspensions of free enzymes rather than intact cells. The most common method of extracting cytosol is to subject the cells to an abrupt pressure change. Lysozyme or other enzymes may be used to weaken cell walls, decreasing the amount of force needed to disrupt the cell membrane.

Sonication, both with and without addition of lysozyme, was tried as the method for extracting cytosol. For the first attempt, 2.0 mL of actively respiring Livingston sludge was pipetted into each of 6 vials. The vials were immersed in icewater in a sonication bath for approximately 5 minutes and then

TABLE 2

VERIFICATION OF SIGMA ENZYME ACTIVITY ANALYSIS METHODS

Method:	Lactate Dehydrogenase Quantitative Colorimetric Method (LD-L)	Lactate Dehydrogenase Ultraviolet Kinetic Method (G-6-PDH)	Glucose-6-Phosphate Dehydrogenase Ultraviolet Kinetic Method (LDH)
Number of Samples:	4	8	6
Experimental Mean (U/L):	363	164	1243
Standard Mean (U/L):	336	162	1233
% Difference	7.4	1.2	0.8
Experimental % Variance:	6.5	10.5	6.1
Standard's % Variance:	5.2	2.4	2.4

sonicated for up to 30 minutes with one vial removed from the bath and placed on ice every 5 minutes.

All samples were centrifuged at 2,000 rpm and 1.0 mL of supernatant was drawn off the top and kept on ice until assayed. Both the G-6-PDH and LD-L assays were run on each sample. No significant change in absorbance was observed over twice the normal assay duration.

In the second attempt, two sludge samples with different solids concentrations were sonicated with glass powder as a grinding agent. A sample of acclimated Livingston sludge was taken shortly after completing a biodegradation run on phenol. A 2.0 mL sample of the culture was filtered, washed with phosphate buffer (pH 7.0) and the solids suspended in 2.0 mL of buffer. Another 50 mL of the culture were centrifuged and the solids washed with buffer and suspended in 5.0 mL of buffer, producing a sample of 10 times the original solids concentration. The filtrates and washes from each sample were combined and stored on ice. Each sample was put in a 10 mL vial and enough glass powder was added to cover the liquid. The samples were sonicated in an ice bath for 15 minutes, then filtered. The filtrates were centrifuged for

10 minutes at 2000 rpm. The filtrates from the sonicated samples, the combined filtrates from the original culture, a phosphate buffer blank, and appropriate enzyme standards were each assayed by the G-6-PDH and LD-L methods. The enzyme standards showed activities within the ranges specified by Sigma. No other sample showed any enzymatic activity. No conclusion can be drawn as to whether the extraction process destroyed the available enzyme or whether enough enzyme was ever present to be detected by the assays.

The effect of lysozyme was tested on a culture of Bi-Chem 1006/7 (produced by Sybron Corporation). An acclimated culture was spiked to 100 ppm with phenol and was allowed 6 hours to consume the substrate. Three 10 mL samples were filtered, the filtrates were combined and saved on ice. The solids were washed with phosphate buffer and resuspended in 2.0 mL of buffer (pH 7.0), to which 1.0 mL of freshly prepared 1500 ug/L lysozyme solution was added. Each sample was mixed by inverting the vial several times. Then each sample was allowed to stand for 10, 20, or 30 minutes at room temperature. At the end of each period, glass powder was added to one sample, which was then shaken vigorously for several minutes and filtered through 0.2 μm

filter paper. The filtrate was saved on ice. The filtrate from each sample, the combined filtrates from the original culture, a freshly prepared 500 µg/L lysozyme solution, and an enzyme standard were analyzed for LDH activity by the LD-L method. The standard showed an activity slightly above the value reported by Sigma. The other samples showed no measurable activity. Again, no conclusion can be drawn as to whether the enzyme was destroyed by handling or whether enough was ever present to assay.

The lysis of the Bi-Chem culture by lysozyme was observed by following changes in the turbidity of the samples at 645 nm [40]. One 10 mL sample of acclimated Bi-Chem culture was centrifuged at 1500 rpm for 2 minutes to remove the easily sedimented particles. A 2.0 mL portion of the remaining suspension was pipetted into each of the 4 cuvettes used in the spectrophotometer. Enough phosphate buffer and lysozyme was added to produce 4 samples of equal volume and final lysozyme concentrations of 500, 150, 50 and 0 ug/mL of lysozyme. The samples were mixed by inverting them several times and changes in absorbance at 645 nm were observed. The sample without lysozyme was used as a control for the settling of particles and its absorbance was subtracted

from the values obtained for other samples. A plot of the results (Figure 2) showed a definite decrease in the suspension turbidity after only a few minutes, indicating that cells were being destroyed in the samples with 150 and 50 ug/L of lysozyme. The lack of a change in the sample with 500 ug/L lysozyme could have resulted from the lysis being complete before the first readings were made.

To determine whether lysozyme was suppressing lactate dehydrogenase activity, duplicate samples were prepared of the enzyme standard, standard with lysozyme, standard with microorganisms, standard with lysozyme and microorganisms, and microorganisms with lysozyme. Sample volumes were adjusted so that the concentration of each component would be the same for each sample. Each sample was handled as in the first lysozyme experiment and was assayed for lactate dehydrogenase activity (Table 3).

The results in Table 3 show no significant depression or elevation of lactate dehydrogenase activity from that observed in the control for samples containing lysozyme. Some loss of activity was observed in the control, probably from handling. No activity was observed in the samples which contained no

FIGURE 2

LYSIS OF BICHEM 1006/7 CULTURE

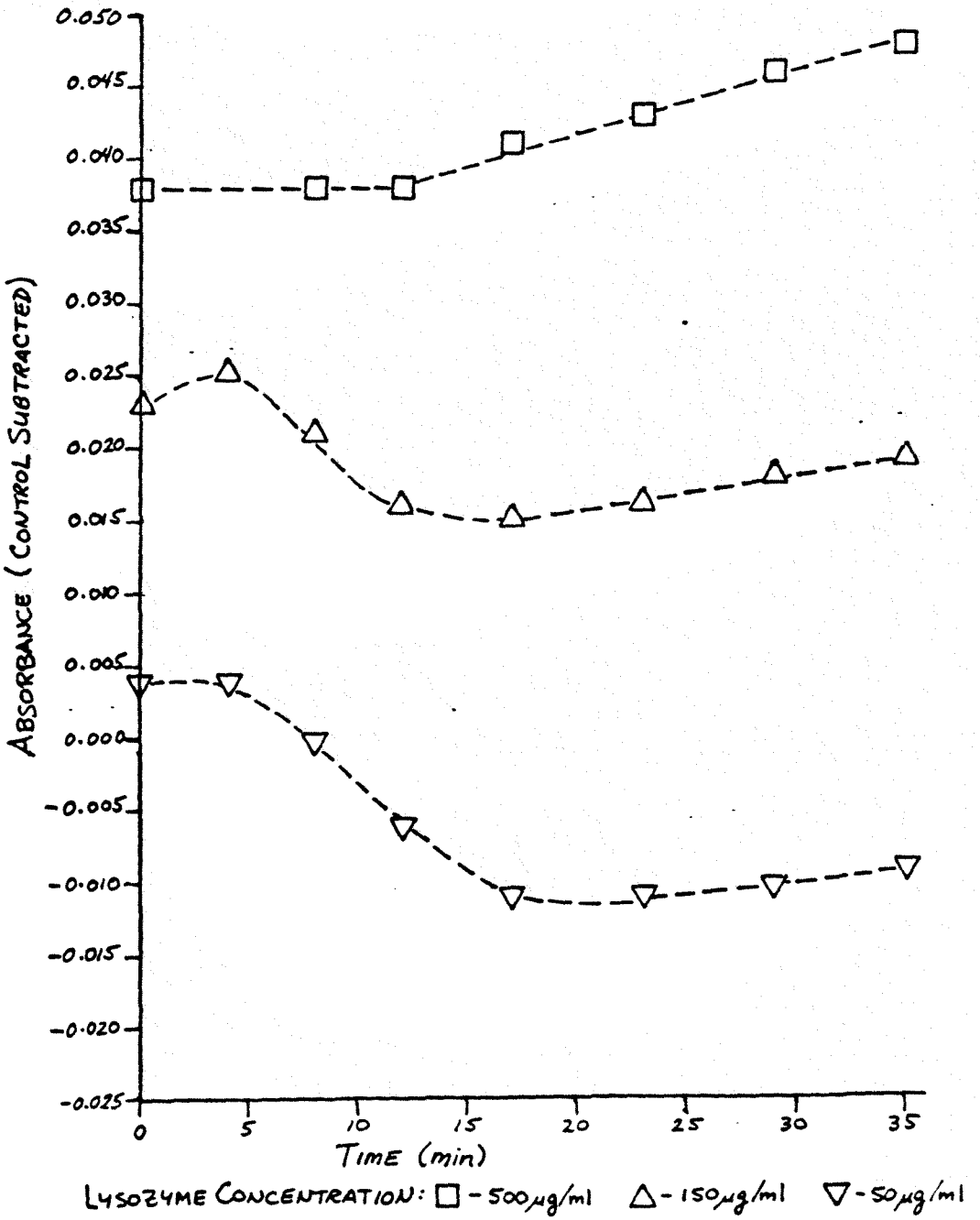


TABLE 3

EFFECT OF LYSOZYME AND SOLIDS
ON LACTATE DEHYDROGENASE ACTIVITY

Sample	Lactate Dehydrogenase Activity Nominal (U/L)	Measured (U/L)	% Change
standard	162	133	-19.7
standard +lysozyme	162	145	-11.1
lysozyme +solids	0	-6.6	
standard +solids	162	104	-43.6
standard +lysozyme +solids	162	143	-12.5

standard.

Since all efforts were unsuccessful, no further investigation of specific enzyme activity was conducted.

There are several possible reasons why no enzyme activity was observed in any test performed on activated sludge or Bi-Chem cultures.

First is the possibility that the enzymes were inactivated by the methods used to extract them. Samples were kept on ice whenever practical, but during filtration and centrifugation, they were not. Samples were also brought to room temperature before being assayed. The enzymes may have been degraded during these warming episodes.

It is possible that naturally occuring substances in the sludge acted as enzyme inhibitors. Humic substances are known to depress enzyme activity [3,18]. Bi-Chem was used for some analyses because it was expected to have less of these humic substances than the Livingston sludge. In addition, sludge solids were washed before lysis was attempted. It is possible that enough inhibitory substance adhered to the cells to inactivate the extracted enzyme.

A major deficiency in all of the experiments

performed, except the final one, was that no enzyme standard was processed along with the samples of microbial culture. Subjecting a known amount of enzyme to the procedures used could have provided some insight as to whether the enzyme was being denatured as it was extracted, or was not extracted in large enough amounts to be detected.

There is the likelihood that the bacterial cells were not ruptured during sonication. Kotze [18] and Sayler [20] both used a piston-type sonicator which transmits high-frequency oscillations directly to the sample. In this study, the samples were immersed in icewater in a bath-type sonicator. Although this instrument operated at the same nominal wattage used by Kotze and Sayler, attenuation of the power by the water and sample container could have prevented the extraction of the enzymes, even in the presence of lysozyme. In future studies where sonication of cells is attempted, the success of sonication in rupturing the cells should be checked directly. This can be done by plating out a portion of the sonicated suspension and attempting to culture the cells. If the cells grow, then they were obviously not ruptured.

A second possibility is that the assays selected

were improper for this application. The kits used were developed by Sigma for particular clinical tests. The lactate dehydrogenase kits (LDH and LD-L) were optimized for the detection of elevated enzyme levels found in blood and urine after acute tissue destruction. The G-6-PDH assay was designed to measure enzyme activity in whole blood.

The LDH and LD-L methods were selected because of the known role of that enzyme in microbial active transport systems [1]. LDH also provides an entry to the TCA cycle in intermediary metabolism. G-6-PDH was selected for study because of its key position in bacterial metabolic pathways [2]. There is no evidence that phenolic compounds are actively transported into microbial cells. The pathways of phenol metabolism described by Sayler suggest that neither enzyme is involved in the process. Their activities might then be suppressed when phenol is the only substrate provided. No data specific to these enzymes was found that could support or refute this conjecture.

It is also possible that the amount of these enzymes present in bacterial suspensions is too small to be detected by the Sigma methods. Neither Bergmeyer or Kotze mention expected enzyme activities for

bacterial preparations, nor the ranges of activity detectable by their methods. Reports from other researchers do not include information on either dehydrogenase. In order for such data to be useful, the specific reaction conditions used in the assay would also need to be known.

B. Use of Electron Acceptor Dyes

The method published by Ford, Yang and Eckenfelder [26] provided the framework for the procedure developed for this study. Because of the limited space available for incubating samples, the volumes used were 1/5 of those described in published procedure.

A number of different solvents were tested for their ability to extract the formazan from the active cells. For these tests, TTC was reduced chemically by adding a few crystals of sodium thiosulfate, a drop of 4N sodium hydroxide and 1.0 mL of distilled water to each tube, in place of the microbial culture. The tubes were then incubated as in the published procedure. At the end of the incubation, the solvents were added and the tubes shaken to extract the formazan. Where two layers formed, the more darkly colored layer was pipetted to a separate container. All samples were examined for intensity of color and

completeness of extraction. The results are presented in Table 4.

The extraction of formazan from an activated sludge sample was then tested. Samples were prepared following the published procedure. After incubation, solvent was added to each tube, which was shaken vigorously. Samples were filtered and the colored layers were pipetted to cuvettes for absorbance readings.

Much red color remained in the cell solids after extraction with toluene or hexanol. Denatured ethanol removed all of the red color from the solids. Absorbances of each sample were not significantly different from that of the blank. The ethanol extract was slightly cloudy, and unsatisfactory for optical measurements (Table 5).

The effect of dissolved oxygen on TTC reduction was then explored. For a quick test, 20 mL each of TRIS-HCl buffer and actively respiring Livingston sludge were mixed and degassed at 20 mm Hg for 20 minutes and quickly divided into twelve 2.0 mL samples in capped vials. Three members of each 4-tube rank received 0.2 mL of TTC-glucose reagent and the remaining tubes were prepared as blanks. After an

TABLE 4

EXTRACTION OF CHEMICALLY REDUCED TTC

Solvent	Volume (mL)	Appearance of Solution
cyclohexane	5.0	pink color translucent
toluene	5.0	intense red color clear solution
cyclohexanol	5.0	intense red color clear solution
1-hexanol	5.0	intense red color clear solution
isopropanol	2.8	red color fades rapidly clear solution
ethanol (CDA)	2.8	red color with intensely red globules cloudy mixture
methanol	2.8	red color fades slowly clear solution
acetone	2.8	red color fades rapidly clear solution

TABLE 5

EXTRACTION OF BIOLOGICALLY REDUCED TTC

Solvent	Volume (mL)	Comments
toluene	5.0	slow extraction poor extraction
1-hexanol	5.0	slow extraction poor extraction
ethanol (CDA)	2.8	rapid extraction red color removed from solids

incubation of 30 minutes, 2.8 mL of alcohol was added to each tube. One set received methanol, one isopropanol and one denatured ethanol (1-B). Samples were processed as above. The results are summarized in Table 6. A measurable amount of formazan was extracted by all three solvents.

Three methods of removing dissolved oxygen were tried. Degassing under vacuum is reported above. Purging for 20 minutes by bubbling nitrogen produced only slightly better results. Depriving the activated sludge culture of air for 30 minutes before adding dye was also tried. This produced comparable results to degassing and purging.

The length of time needed to purge or degas oxygen from samples before TTC reduction could proceed would have prohibited processing the number of samples anticipated from the biodegradation runs. Sealing the samples and allowing the dissolved oxygen to be consumed by the cells would also be unsatisfactory as some active species could die off as the oxygen was consumed.

It was decided to explore the use of electron-acceptor dyes that would be reduced without competition from dissolved oxygen in the incubation medium. Two

TABLE 6

EXTRACTION OF BIOLOGICALLY REDUCED TTC
PRODUCED UNDER LOW D.O. CONDITIONS

Solvent	Volume (mL)	Absorbance at 490 nm	Comments
isopropanol	2.8	.502	color fades slowly
ethanol (1-B)	2.8	.331	cloudy solution
methanol	2.8	.203	color fades quickly

dyes with lower redox potentials were selected for comparison with TTC. The dyes selected were 3-(4',5'-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) (Table 1). A triplicate sample and a blank were prepared for each dye and reduced chemically as described for TTC. Isopropanol was used to extract the dyes. The absorbance of each dye was measured at its wavelength of maximum extinction and checked every 30 seconds at 500 nm for fading (Table 7). INT showed the strongest color and did not fade. MTT and TTC also showed strong colors but faded slightly.

The dyes were then compared for their usefulness in the biological assay. A sample of 20 mL of the aerated phenol-acclimated Livingston sludge was mixed with 20 mL of TRIS-HCl buffer and the dissolved oxygen concentration was measured to be 4.7 ppm. The sample was divided into 2.0 mL portions to produce 3 sets of 5 tubes each. Four members of each set received 0.2 mL of a 0.2% solution of either TTC, MTT, or INT. The remaining tube of each set was prepared as a blank. After mixing and incubation for 15 minutes at 37°C, each tube received 2.8 mL of isopropyl alcohol. The

TABLE 7

ABSORBANCES OF CHEMICALLY REDUCED FORMAZANS

Dye	Wavelength of Peak Absorbance (nm)	Absorbance	Absorbance at 500 nm Initial	Absorbance at 500 nm 5 minutes
TTC	490	2.426	1.560	1.550
MTT	540	3.331	2.934	2.928
INT	620	4.230	0.632	0.633

contents were mixed by inversion and filtered. The absorbance of each dye was read at its optimum wavelength (Table 8). The filtered solids were deeply colored by the INT formazan, indicating that it was poorly extracted by isopropyl alcohol. Comparatively little TTC formazan was produced. Therefore, only MTT was studied further.

First the effect of dissolved oxygen on MTT reduction was checked. Two samples were prepared of 40 mL actively respiring Livingston sludge and 40 mL TRIS-HCl buffer. Nitrogen was bubbled through one sample for 5 minutes to purge it of oxygen. Dissolved oxygen concentration was measured as being 0.79 ppm in the purged sample and 4.81 ppm in the control. Three sets of 5 tubes each were prepared from each sample. MTT-glucose reagent was added to 4 tubes from each set and the remaining tubes were prepared as blanks. Incubation and dye extraction were performed as above. Student's t-test was used to compare the mean absorbance of the samples. The decision to reject the hypothesis that the means were equal was marginal. When outlying values were excluded, the hypothesis was not rejected. Dissolved oxygen content did not affect the biological reduction of MTT (Table 9).

TABLE 8

ABSORBANCES OF BIOLOGICALLY REDUCED FORMAZANS

Dye	Wavelength of Peak Absorbance (nm)	Absorbance	Comments
TTC	490	0.015	little color produced
MTT	540	1.366	strong color produced
INT	620	0.170	poor extraction from solids

TABLE 9

EFFECT OF D.O. ON MTT REDUCTION

Sample:	Oxygenated	Purged (N_2)	Oxygenated	Purged (N_2)
D.O. (ppm):	4.81	0.79	4.81	0.79
Number of tests:	12	12	10	10
Mean absorbance at 540 nm:	0.913	0.988	0.939	0.987
Standard error:	0.080	0.091	0.055	0.057
t	2.15		1.74	
t	2.08		2.10	
H | X1=X2	Reject H		Do not reject H	

Comparison of means: two-tailed t-test

H | X1=X2

$t = (X1-X2)/((1/n1 + 1/n2) * (\Sigma(x1-X1)^{**}2 + \Sigma(x2-X2)^{**}2)/(n1+n2-2))^{**}0.5$

If $t > t$, then reject H

where: X1 + X2 are mean absorbances for a set of samples

x1 + x2 are absorbances of individual samples

n1 + n2 are number of samples in each set

The effect of sludge dilution on the formazan production rate was also determined. A quantity of Livingston sludge was killed by autoclaving. This suspension was used to produce a series of dilutions of actively respiring sludge. These mixtures were processed as above and the absorbances due to formazan were plotted against sludge concentration (Fig. 3). A straight line was demonstrated to be a reasonable fit to this plot.

The effect of temperature and light on formazan stability was also checked. The formazan samples resulting from one biodegradation run were divided into two groups. One group was kept in the dark at 0°C for approximately 60 hours. The other group was kept on the laboratory window sill for the same period. The samples kept cold and in the dark showed an average increase in absorbance of 0.5%. The samples left out in the laboratory showed an average decrease in absorbance of 6.4% (Table 10). This demonstrates that light or warm temperatures can cause a breakdown of MTT formazan, although not as great as that demonstrated for TTC formazan by Jones and Prasad [29]. As a result of this comparison, formazan samples were kept refrigerated until their absorbances were read.

FIGURE 3

EFFECT OF SLUDGE DILUTION ON MTT REDUCTION

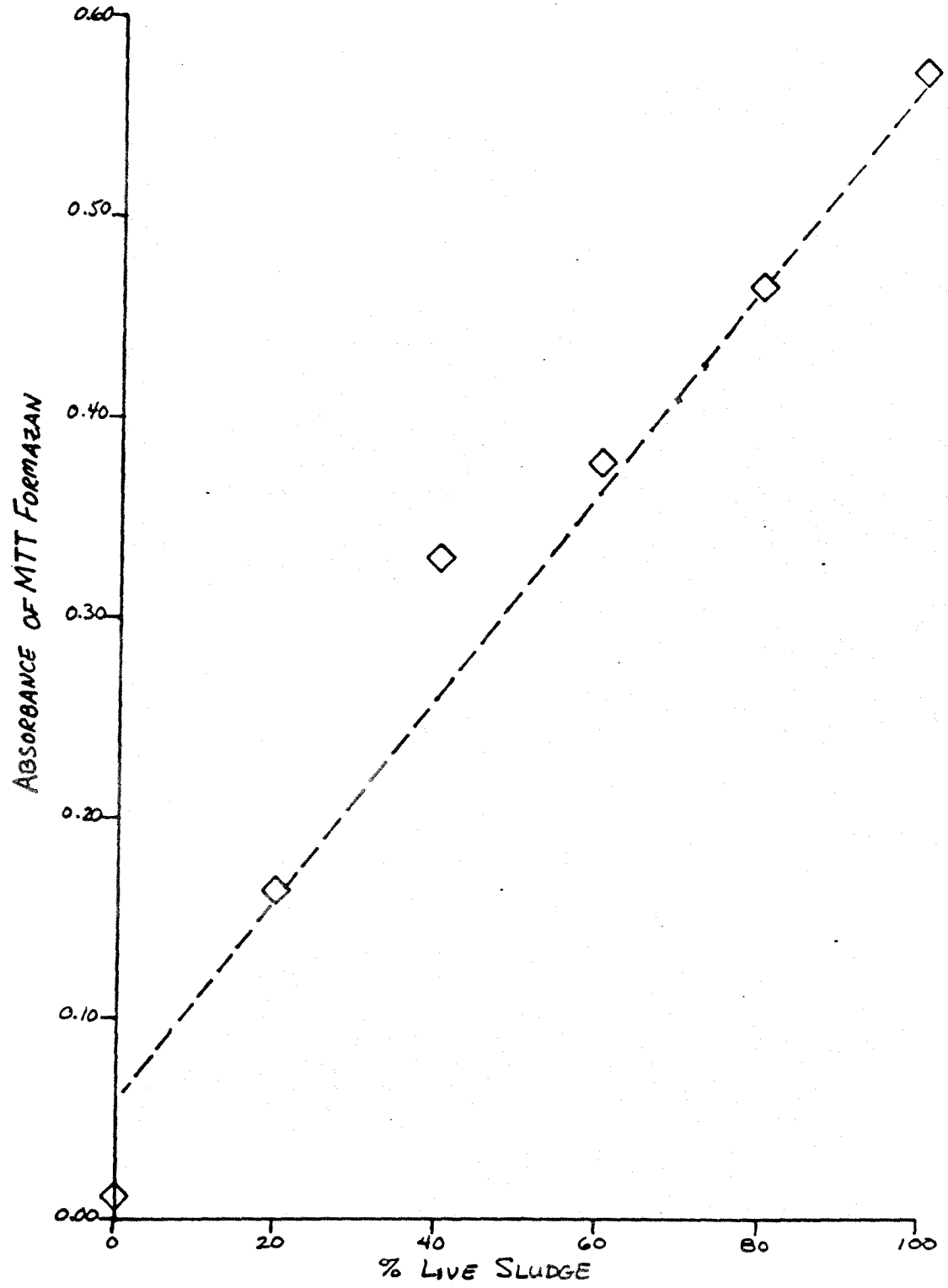


TABLE 10

EFFECT OF LIGHT AND TEMPERATURE ON THE ABSORBANCE OF MTT FORMAZAN

Test Conditions	Mean Absorbance of Set at 540 nm Before Exposure	After 60 hr Exposure		% Change
0 C/Dark	1.287	1.299		0.9
	1.551	1.516		-2.3
	1.331	1.297		-2.6
	1.132	1.201		5.9
			Mean =	0.5
20 C/Light	1.366	1.285		-6.1
	1.323	1.213		-8.7
	1.228	1.152		-6.4
	1.179	1.128		-4.4
			Mean =	-6.4

The rate of reduction of MTT was used as a measure of general dehydrogenase activity in subsequent experiments.

C. Preparation and Maintenance of Bacterial Cultures

The bacterial cultures used as media for the biodegradation studies were maintained in the batch reactors shown in Figure 1. Each culture was kept for approximately one week. During this period, they were acclimated to 100 ppm phenol as sole energy and carbon source, then subjected to one or two biodegradation runs. Additional nutrients were added to the mixture during the acclimation period in the form of ammonium and phosphate salts for a C:N:P ratio of 50:14:3. The presence of these nutrient salts did not affect the biodegradation rates for acclimated sludges, but had a great effect during the acclimation period. Cultures provided with nutrients acclimated readily to phenol, as demonstrated by their ability to consume this substrate. When cultures were not provided with these nutrients, the phenol was not consumed, even after several days. Other researchers state that additional phosphorous and nitrogen are required to synthesize the enzyme systems active in biodegradation [6, 41].

The cultures were kept at room temperature, which

varied from 18°C to 26°C over the study period. Solids wasting only occurred when samples were removed. The initial reactor volume was 4 L for each run. At the most, 100 mL were removed during the acclimation period, but almost a liter would be removed during a run. Volume losses were not made up.

Activated sludge from the Livingston, N.J., wastewater treatment plant was aerated overnight with no added nutrients, to bring it into a state of endogenous respiration. The mixture was then spiked to 100 ppm phenol. Approximately 24 hours later, a sample was analyzed by gas chromatography (GC) for phenol. If no phenol was present, the culture was spiked to 100 ppm phenol again. This procedure was repeated until the culture degraded 100 ppm of phenol to below detectable limits (1 ppm) within a few hours. The culture was then used for biodegradation test runs. Livingston sludge always showed an immediate ability to degrade phenol to below the concentrations detectable by GC.

Hydrobac is a microbial preparation produced by Polybac Corporation. Prepared from microorganisms isolated from toxic waste landfills, it is intended to augment the ability of biological treatment systems to degrade refractory organic compounds. The culture

comes dried onto flaked bran, along with what the manufacturer calls "growth factors". A 25 gm portion of the dessicated preparation was stirred for 2 to 3 hours in 2 L of distilled water. Meanwhile, 2 L of water was aerated in the reactor. At the end of this time, the bran mixture was allowed to settle for about 1/2 hour, after which approximately 1.8 L of the remaining suspension was decanted into the reactor. The volume in the reactor was then made up to 4 L with distilled water.

After being aerated overnight, the Hydrobac cultures were progressively spiked to 25, 50 and 100 ppm phenol as they demonstrated their ability to degrade this compound. Variable lag times occurred before degradation began. These were probably due to the presence of another energy source, possibly one of the "growth factors", taken up by the culture in preference to phenol. DO bottle tests showed a high rate of DO consumption during the lag periods. Literature from the manufacturer does not discuss the effect or purpose of these "growth factors" toward Hydrobac's biodegradative ability.

One phenol biodegradation run was made using LLMO (General Environmental Science) as the culture. This

is a liquid preparation containing hydrogen sulfide to maintain shelf life. A 25 mL portion of the preparation was added to a liter of distilled water and aerated overnight to strip out the hydrogen sulfide. Acclimation began with spiking the culture to 10 ppm phenol, increasing the concentration gradually to 100 ppm as the culture increased its biodegradative ability. The volume of the mixture was also gradually increased until a final volume of 4 L was achieved. LLMO required an acclimation period of 13 days.

D. Biodegradation Runs

In general, 4 to 6 measurements were taken at 20 to 40 minute intervals during each biodegradation run. In order to cut down on the time required for sampling and to decrease the chances for error, a routine sampling cycle was developed and adhered to for each run.

Two successive sampling cycles were performed on phenol acclimated cultures that had been demonstrated (by GC) to be free of substrate. A run began when the phenol acclimated culture was spiked to the desired concentration of substrate: 100 ppm for phenol and 20 ppm for o-chlorophenol. Sampling began immediately after the culture was spiked.

Samples for substrate analysis, measurement of general dehydrogenase activity and oxygen uptake rate (OUR) determination were routinely taken in every sampling cycle. The frequency of sampling for the other parameters was varied according to past experience. The period of the cycle was also varied according to circumstances and past experience. The principle factor determining the length of the sampling period was the OUR. When OUR changed, sampling frequency was increased. When OUR reached a constant rate, samples were taken less frequently.

The sampling cycle was as follows:

1. note time (sample number)
2. mixed liquor suspended solids (if measured)
3. general dehydrogenase activity
4. substrate analysis
5. chemical oxygen demand (if measured)
6. dissolved oxygen concentration (if measured)
7. oxygen uptake rate
8. pH (if measured).

Absorbances of the formazan produced in the general dehydrogenase activity assay were read at the completion of the run. Ammonia determinations were made on selected samples, preserved for up to 2 months

after the run.

(1) Mixed Liquor Suspended Solids

Mixed liquor suspended solids (MLSS) were measured for at least three samples during each biodegradation run. Exactly 10.0 mL of mixed liquor from the reactor was pipetted into a preweighed numbered aluminum weighing dish. All samples were dried overnight in an oven maintained between 103°and 105°C, as described in Standard Methods [42], and weighed on the subsequent day. The difference between this weight and the weight of the empty dish was taken as the total weight of suspended solids in the mixed liquor. MLSS was then calculated in parts per million (ppm). The error in the method was estimated as $\pm$ 100 ppm.

(2) General Dehydrogenase Activity

The general dehydrogenase activity of the microbial cultures was determined by measuring the rate of reduction of MTT to its formazan. Screw cap tubes of approximately 10 mL capacity were prepared before the biodegradation run with 1.0 mL of 0.05 M TRIS buffer (adjusted to pH 8.4 with concentrated hydrochloric acid). Enough tubes were prepared to allow triplicate tests and one blank for each of the maximum number of samples anticipated during the run. The capped tubes

were stored in an incubator at 37°C until used. A portion of the prepared MTT reagent (0.20 gm MTT and 1.500 gm glucose in 100 mL of distilled water) was placed in a vial, which was wrapped in aluminum foil to exclude light, capped, and allowed to come to room temperature. The reagent stock bottle was kept wrapped in aluminum foil and stored at 2°to 8°C to prevent deterioration of the reagent by light or heat. Based on recommendations in the literature [22], fresh reagent was prepared every two weeks.

The assay for general dehydrogenase activity was performed regularly throughout each biodegradation run. Approximately 50 mL of mixed liquor was pipetted from the reactor to a beaker and stirred vigorously with a magnetic stirrer. Four tubes were removed from the incubator and opened. Each received 1.0 mL of mixed liquor from the beaker. The blank received 0.2 mL of distilled water from a dispensing micropipette. The remaining tubes each received 0.2 mL of the MTT-glucose reagent from a dispensing micropipette. Tubes were capped and inverted several times to mix the reagents, labelled with the time incubation began, and incubated at 37°C for 15 minutes. The remaining mixed liquor was returned to the reactor.

At the end of the incubation, each tube received 2.8 mL of isopropyl alcohol to stop the reaction and extract the formazan. Tubes were stored in the dark at 0°C until the absorbance of the formazan could be read.

The absorbance of the dissolved formazan was read on a visible light spectrophotometer at 540 nm and 25°C. Samples were filtered to remove solids, then allowed to come to room temperature in the dark, before pipetting a portion of each solution into cuvettes. The average of the triplicate readings was taken to represent the general dehydrogenase activity of the sample. The standard error of the triplicate readings was almost always less than 10% of the mean value. For more than half of the samples it was less than 5%.

A calibration curve was prepared for each new batch of MTT-glucose reagent. A series of volumes of reagent ranging from 0 to 0.1 mL were diluted in 0.025 M TRIS-HCl buffer to a volume of 2.2 mL. MTT was reduced to its formazan with sodium thiosulfate as described by Reca [22]. The solutions were incubated and the formazan absorbance measured as above. The absorbances were plotted against MTT concentration to demonstrate that Beer's Law was applicable. With each biodegradation run, samples of known MTT concentration

were reduced chemically and checked against this curve as a quality check for the reagent.

(3) Substrate Analysis

After spiking the mixed liquor in the reactor to the desired initial concentration of substrate, 15 mL samples of mixed liquor were taken every 20 to 40 minutes until the substrate concentration decreased to below detectable limits or until the measured OUR reached the value measured before spiking the reactor. The samples were centrifuged for 4 minutes at 2500 rpm and analyzed by GC according to methods previously developed in this laboratory [43]. Areas of peaks were determined with an electronic integrator previously calibrated with known standards at the GC operating conditions. Thymol was added to the samples as an internal standard, resulting in an accuracy of $\pm$2 ppm for the method (based on measurement of standards). All samples were preserved with 1000 ppm copper sulfate as described by Colish [44]. The effectiveness of the sample preservation technique was examined by Colish and found to be satisfactory.

For samples taken during the biodegradation runs, triplicate analyses showed a standard error within $\pm$2 ppm for 142 of 163 samples. Only one sample showed

a standard error greater than 5 ppm. While the method was less reproducible than the desired ±1 ppm, the results were still suitable for analysis.

(4) Chemical Oxygen Demand Determination

Chemical oxygen demand (COD) analysis was performed to determine whether complete or partial mineralization of phenol or o-chlorophenol was occurring during biodegradation. The theoretical COD (mg DO/mg substrate) of the substrates were calculated from balanced equations and were determined to be 2.380 for phenol and 1.680 for o-chlorophenol. These values were used to convert the total COD measured in each sample to equivalent amounts of substrate.

Samples for COD analysis were taken from the mixed liquor in the reactor and filtered immediately. Approximately 10 mL was taken as a sample. Two drops of concentrated sulfuric acid was added to each filtered sample as a preservative, and the samples were stored at 4° to 8°C until analyzed.

The procedure used to determine COD is a modification of the method presented in Standard Methods [42] and was taken from Colish [44]. A digestion solution was prepared by adding 7.5 gm potassium dichromate, 10.0 gm silver sulfate, and 5.0 gm mercuric sulfate to

a 2.5 L bottle of concentrated sulfuric acid. This reagent is extremely corrosive and toxic, and great care must be exercized in its preparation and use. The mixture was heated overnight, with stirring to dissolve the salts. When the salts were completely dissolved, the solution was left standing to cool to room temperature.

A 5.0 mL portion of cooled digestion solution was pipetted into a 16 mm x 100 mm screw-top vial, 2.0 mL of filtered sample added, and the cap screwed on tightly. Two blanks of distilled water were run with each set of up to twelve samples. Duplicate standards of 100 ppm phenol or 20 ppm o-chlorophenol were also run with each set of samples. The vials were placed in a Hach COD reactor, preheated to 150°C, for 2 to 3 hours. The reactor was then shut off and the samples allowed to cool to room temperature. The cooled sample was added to approximately 50 mL of distilled water in an Erlenmeyer flask and the inside of the vial rinsed into the flask. Five drops of ferroin indicator were added to the flask and the solution was titrated to the bright orange endpoint with 0.025 M ferrous ammonium sulfate (FAS). The FAS titrant was prepared by adding 19.6 gm FAS to approximately 1000 mL of distilled

water, adding 20 mL of concentrated sulfuric acid, cooling the solution to room temperature and diluting it to 2000 mL with distilled water. Blanks, standards and samples were titrated in the same manner.

The following equation was used to determine the COD of the sample:

$$(A - B) \times (N) \times (8000/C) = \text{mg COD/L}$$

where: A = volume of FAS solution used to titrate blank

B = volume of FAS solution used to titrate sample

N = normality of FAS solution

C = volume of sample in mL

Colish estimated the error of this method to be $\pm$10%. Titration of standards in this study showed the estimate to be good for phenol standards in the range of 50 to 200 ppm. For o-chlorophenol standards of 10 and 20 ppm, the error was approximately 67%. An attempt was made to increase the precision of the method by using less concentrated oxidant and titrant. This attempt failed since the capacity of the dilute oxidant was frequently exceeded.

(5) Dissolved Oxygen Levels

During the biodegradation runs, dissolved oxygen

(DO) levels in the reactor were monitored to demonstrate that activity in the reactor was not limited by availability of DO. The DO probe was inserted directly into the reactor mixed liquor and the first stable reading was taken to indicate the DO concentration. In every run there was some decrease from the initial DO level. As air flow rate was kept constant, reassertion of the initial DO level was taken as a signal to check for the absence of substrate. DO levels only dropped below the limiting concentration of 0.5 ppm for phenol runs, and then seldom for more than one reading. These readings were preceded by the highest OUR measurements.

The accuracy of the DO probe was checked against the manganous oxide (Winkler) method for DO determinations [42]. Two samples each of distilled water and Livingston sludge remaining from a biodegradation run were checked. All samples were aerated overnight before analysis. The results obtained by the Winkler method were from 3 to 9% greater than those obtained with the DO probe.

(6) Oxygen Uptake Rate

The method for determining oxygen uptake rate (OUR) was the same as described in Standard Methods [42]. A sample of 300 mL of mixed liquor was

transferred into a BOD bottle through the outlet in the bottom of the reactor and a DO probe inserted into the bottle. After allowing 1 to 2 minutes for the reading to stabilize, DO concentration readings were taken at regularly spaced intervals (between 5 and 15 seconds) until twelve measurements were taken or the DO concentration fell below 0.5 ppm. Changes in DO were found to be linear with time above this concentration, similar to observations reported in the literature [35,36]. Least squares regression was performed to fit a straight line to the data and the slope of the line was taken as the OUR. A modification of Students' t-test was used to determine the validity of the regression. All values falling outside the 95% confidence limit in the one-tailed test were rejected. Only OUR values obtained using DO readings of 0.5 ppm or less fell outside the confidence limit.

(7) Hydrogen Ion Concentration

In the initial runs, pH was checked from 3 to 5 times during the run using a pH probe and digital meter. Measurements were made by pipetting approximately 10 mL of mixed liquor into a vial containing a small magnetic stir bar. The vial was placed on a magnetic stir plate and a combination pH probe immersed

in the liquid. After several minutes of stirring, the pH was read. The pH was never observed to vary outside the range of 7.0 to 8.0. As this consistency of pH in the batch reactor was supported by the observations of other workers in the lab [43,44], pH measurements were discontinued after the first few runs.

(8) Ammonia Determination

Ammonia concentrations were determined in selected samples from most runs. The samples measured were taken from those used for substrate analysis. Previous work by Salerno [45] showed that the preservation technique used for substrate samples was adequate for ammonia samples. For each sample, 0.1 mL of sample was diluted in 10.0 mL of distilled water in a vial containing a small magnetic stir bar. While the sample was stirred, the ammonia probe was inserted and three drops of concentrated sodium hydroxide were added. After 2 minutes, a value in millivolts (mV) was read from the meter. All samples were read in duplicate and the readings averaged. After a set of samples was processed, an ammonium chloride sample, prepared to be in the same concentration range, was measured in the same way. All readings were compared against a mV-to-ppm ammonia-N calibration curve which had been prepared

from standards using this same method.

The precision of this method was at best ±5%, due to the precision of the mV readings on the digital meter.

VI. RESULTS

In the following sections, the values measured and patterns observed over 16 biodegradation runs will be presented. Data obtained from the biodegradation runs are presented in tables in Appendix A and in graphs in Appendix B.

A. Mixed Liquor Suspended Solids

Results of the MLSS measurements taken during the runs are summarized in Table 11. Within a given run, variations in measured MLSS usually did not exceed the estimated precision of the method (±100 ppm). MLSS concentrations would not be expected to increase significantly, as no more than 100 ppm of substrate was ever added during a single run. Empirically determined yield coefficients [5] show that typically 60% of BOD will go to biomass, so at most MLSS would have increased by 60 ppm. This increase is within the precision of the MLSS measurement.

In all, 4 cultures of Livingston sludge, 3 of Hydrobac and 1 of LLMO were used. All Livingston sludge cultures, collected over a 2 month period, showed MLSS concentrations of 2100 ± 200 ppm. The Hydrobac cultures had an MLSS concentration of 1100 ppm

TABLE 11

MIXED LIQUOR SUSPENDED SOLIDS
(ppm)

Substrate	Media	Run	Range	Mean
Phenol	Livingston Sludge I	1	2140 - 2280	2176
	Livingston Sludge I	3**	2170 - 2235	2235
	Livingston Sludge III	1	2020 - 2040	2033
	Livingston Sludge III	2	2020 - 2120	2090
	Hydrobac I	1	390 - 590	480
	Hydrobac I	3**	460 - 680	540
	Hydrobac II	1	1080 - 1140	1102
	Hydrobac II	2	1100	1100
	LLMO	1	400 - 500	436
o-Chlorophenol	Livingston Sludge II	1	1620 - 1790	1686
	Livingston Sludge II	3**	1930 - 1980	1956
	Livingston Sludge IV	1	1910 - 1980	1945
	Livingston Sludge IV	2	1980 - 2000	1990
	Livingston Sludge IV	3	1940	1940
	Hydrobac III	1	1050 - 1100	1098
	Hydrobac III	2	*	*

* - MLSS data not taken
** - Data not taken for run 2

when prepared full-strength, while the LLMO culture had an average MLSS of 436 ppm.

B. Ammonia Nitrogen

Ammonia measurements were made on samples from 14 biodegradation runs, representing 8 cultures. Results are summarized in Table 12. Where more than one phenol run was made on a single culture without an intervening recovery period (Livingston sludge III, Hydrobac II), definite decreases in ammonia-nitrogen were observed. The decrease in the amount of ammonia-nitrogen in solution is probably the result of the incorporation of nitrogen into new biomass [41]. Release of ammonia by cellular catabolism could be the cause of increases in ammonia-nitrogen found after recovery, as the cultures were not provided with substrate during recovery periods. Two cultures dosed with o-chlorophenol (Livingston sludge IV, Hydrobac III) showed an increase in ammonia-nitrogen, which may also have been released into solution by cellular catabolism. Ammonium salts were added to the cultures during phenol acclimation, but not during the biodegradation runs. The amount of ammonium salts added to each culture varied in direct proportion (14 mg N:50 mg C) to the amount of phenol needed to acclimate the culture. Results of analyses

TABLE 12

AMMONIA ANALYSIS
(ppm N)

Substrate	Media	Run	Values			Mean
Phenol	Livingston Sludge I	1	46	46	43	45
	Livingston Sludge I	3**	*	*	*	*
	Livingston Sludge III	1	62	62	62	62
	Livingston Sludge III	2	57	53	50	53
	Hydrobac I	1	30	27	21	26
	Hydrobac I	3**	43	37	34	38
	Hydrobac II	1	42	42	40	41
	Hydrobac II	2	39	38	36	38
	LLMO	1	11	7.8	6.4	8.4
o-Chlorophenol	Livingston Sludge II	1	*	*	*	*
	Livingston Sludge II	3**	32	30	30	31
	Livingston Sludge IV	1	71	73	70	71
	Livingston Sludge IV	2	73	76	75	75
	Livingston Sludge IV	3	76	84	87	82
	Hydrobac III	1	43	38	39	40
	Hydrobac III	2	37	45	46	43

* - Ammonia data not taken
** - Data not taken for run 2

for ammonia-nitrogen showed that LLMO had by far the least amount in solution after acclimatiom (11 ppm). All Hydrobac and two Livingston sludge cultures had roughly the same concentrations after acclimation (approximately 40 ppm). Two cultures of Livingston sludge showed much higher ammonia concentrations after acclimation (62 ppm and 71 ppm).

Bacterial cultures require ammonia-nitrogen to maintain growth and for the production of enzymes. Livingston sludge was already a mature culture with a low growth rate and should have put a low demand on solution nitrogen. The Hydrobac inoculum had been provided with nitrogen sources as "growth factors" by the manufacturer. The LLMO culture was started from a much smaller inoculum than the Hydrobac cultures and would therefore have more cells in the growth phase.

C. Substrate Removal Rate

When substrate concentration is plotted against time, a curve similar to that shown in Figure 4 should result. This "S-curve" shows an initial lag time before degradation begins, a portion which is fairly linear and a tail where degradation rate decreases as substrate concentration approaches zero. The plots of phenol degradation can generally be described by this

FIGURE 4 BIODEGRADATION RUN: PHENOL

PHENOL/LLMO - RUN 1

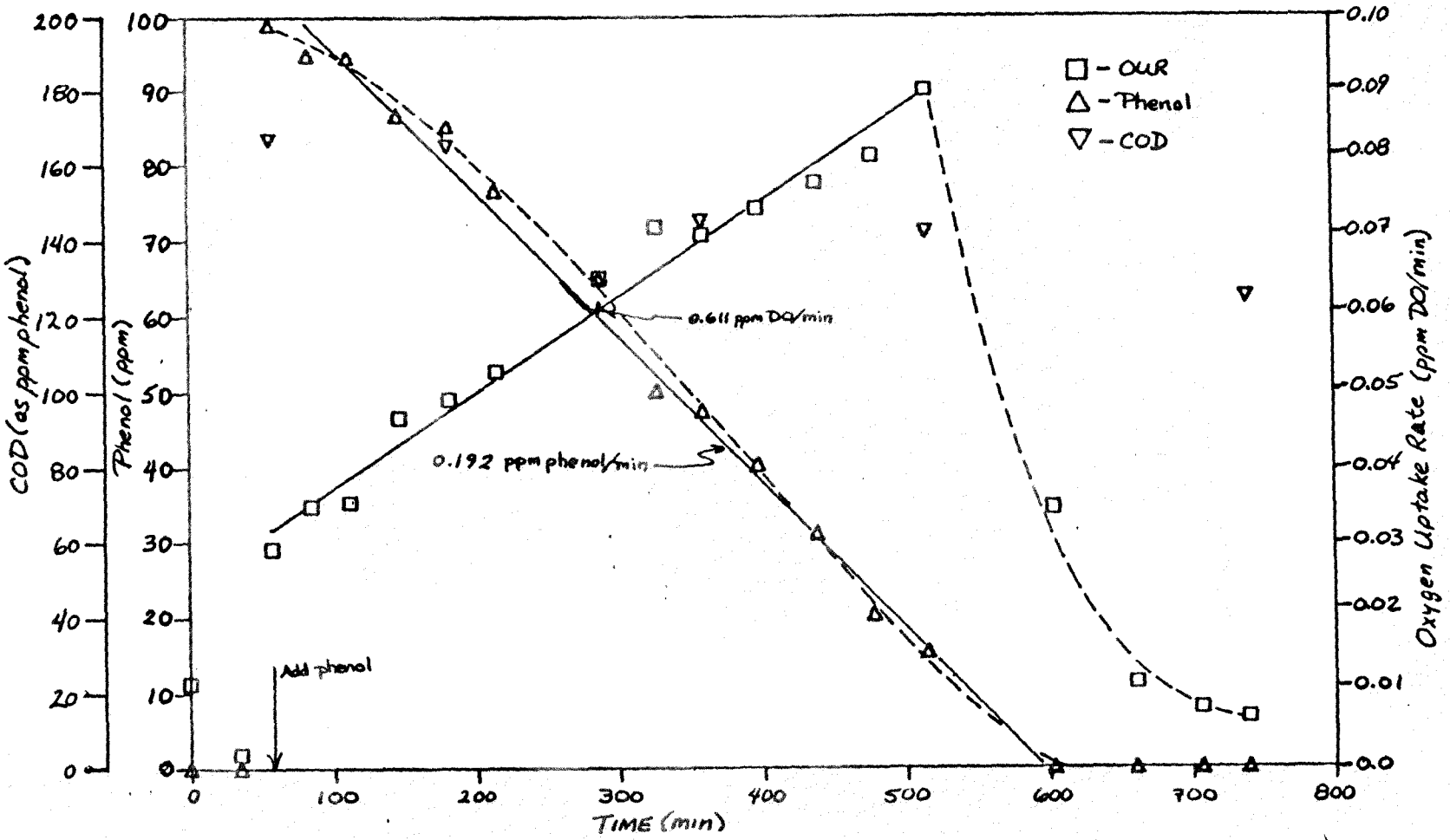
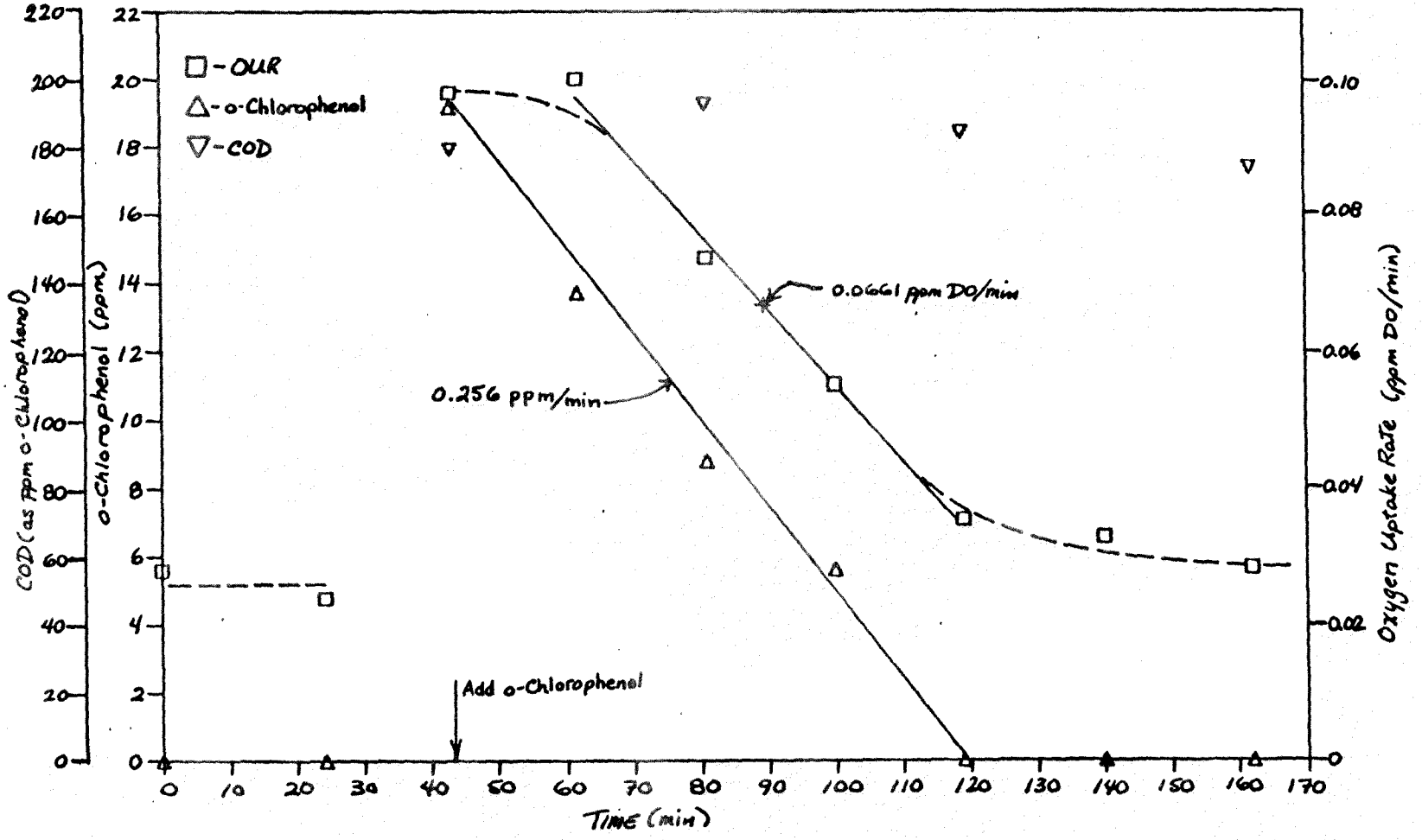


FIGURE 5 BIODEGRADATION RUN: o-CHLOROPHENOL
LIVINGSTON SLUDGE IV - RUN 1



S-curve, although the tail (and sometimes the lag time) are not as well-defined as the linear portion of the curve. Lag times vary in duration, with the lags for Livingston sludge being much shorter than for Hydrobac or LLMO. When a second run was performed on a culture with no recovery period beforehand (Livingston sludge III, Hydrobac II),the lag was not observed.

The S-curve shape is not always evident in plots of o-chlorophenol degradation, although a straight line could usually be fit over part of the curve (Figure 5).

Substrate removal rates were determined by fitting a straight line to the linear portion of the curve and calculating its slope (Figures 4 and 5). When possible, the segment of the curve used was chosen to correspond to the linear part of the OUR or MTT formazan plot for the same run. The equation of the line was determined using the linear regression formula programmed into a TI-66 calculator.

Substrate removal rates are presented in Table 13. It should be noted that substrate removal rates are expressed as mass of substrate per unit time (ppm/min). Because of the higher molecular weight of o-chlorophenol, removal rates for that substrate are presented in terms of the equivalent mass of phenol.

TABLE 13

SUBSTRATE REMOVAL RATES
(ppm/min)

Substrate	Media	Run	Substrate Removal Rate	Correlation (r^2)
Phenol	Livingston Sludge I	1	0.862	0.996
	Livingston Sludge I	3	*	*
	Livingston Sludge III	1	0.377	0.950
	Livingston Sludge III	2	1.020	0.995
	Hydrobac I	1	0.369	0.950
	Hydrobac I	3	0.278	0.924
	Hydrobac II	1	0.512	0.948
	Hydrobac II	2	0.200	0.909
	LLMO	1	0.192	0.992
o-Chlorophenol	Livingston Sludge II	1	*	*
	Livingston Sludge II	3	0.277 (0.203**)	0.995
	Livingston Sludge IV	1	0.256 (0.187**)	0.993
	Livingston Sludge IV	2	0.214 (0.157**)	0.998
	Livingston Sludge IV	3	0.0504 (0.0369**)	0.986
	Hydrobac III	1	0.0260 (0.0190**)	0.998
	Hydrobac III	2	0.0519 (0.0380**)	0.910

* - Insufficient data for analysis
** - Adjusted to equivalent weight of phenol

TABLE 14

SUBSTRATE REMOVAL RATES
(ppm/min)
(McMullen [44])

Substrate	Media	Substrate Removal Rate	Correlation (r^2)
Phenol	Livingston Sludge	1.21	0.970
	Livingston Sludge	1.43	0.988
	Hydrobac	0.477	0.979
	Hydrobac	0.345	0.979
	LLMO	0.119	0.990
	LLMO	0.156	0.999
	LLMO	0.183	0.988
o-Chlorophenol	Livingston Sludge	0.143 (0.105*)	0.976
	Livingston Sludge	0.105 (0.121*)	0.924
	Livingston Sludge	0.230 (0.168*)	0.956
	Hydrobac	0.0991 (0.0725*)	0.927
	Hydrobac	0.0080 (0.006*)	0.724
	Hydrobac	0.0337 (0.0247*)	0.862
	Hydrobac	0.0330 (0.0242*)	0.849
	LLMO	0.0079 (0.0058*)	0.290
	LLMO	0.0064 (0.0047*)	0.546
	LLMO	0.0039 (0.0029*)	0.378

* - Adjusted to equivalent weight of phenol

Rates varied widely, but phenol was consistently removed at higher rates than o-chlorophenol. Livingston sludge degraded phenol more rapidly than either Hydrobac or LLMO.

McMullen [43] obtained substrate removal rates for phenol and o-chlorophenol using Livingston sludge, Hydrobac and LLMO in the same batch reactor used in this study. His results are shown in Table 14. The removal rates obtained in the present study are within the same ranges as those in Table 14, with the exception of the phenol/Livingston sludge runs. There is no obvious explanation for the difference in removal rates between the two studies. The removal rates from the present study are closer to those obtained by Colish (again using Livingston sludge in the same batch reactor used in this study). His values ranged from 0.53 to 1.03 ppm phenol/min [44].

D. Chemical Oxygen Demand (COD)

COD was determined for filtered samples from 13 runs, representing 8 cultures.

There was COD present in solution in all cultures prior to addition of substrate. For Livingston sludge, the COD value increased with the addition of substrate and resumed its initial value at the end of the run,

indicating that substrate accounted for all COD consumed. The inital COD value for Hydrobac was much higher than that for Livingston sludge. COD remaining at the end of a run in the Hydrobac cultures was lower than the values at the beginning, indicating that COD other than substrate was consumed during the run. In Hydrobac cultures, COD decreased during the lag phase, during which phenol concentration remained constant. This probably reflects the presence of the "growth factors" included with the dried culture.

Examination of the plots of the biodegradation runs (Appendix B) shows that for Livingston sludge, total COD was removed at approximately the same rate as phenol. For Hydrobac, the removal of COD was more rapid than for substrate, showing that phenol and additional COD were co-metabolized. For LLMO, COD removal was slower than phenol removal, suggesting that phenol was either incompletely oxidized or incorporated into cell mass.

Results for o-chlorophenol show no appreciable change in COD through any of the biodegradation runs. This could be due to incomplete oxidation or incorporation of o-chlorophenol by the cells. It could also be due to the insensitivity of the COD analysis to changes of concentration in the 20 ppm range.

E. Reduction Rate of MTT

The ability of the culture to reduce MTT was monitored for 11 runs. Of these, 8 produced results that could usefully be compared with other parameters.

For phenol runs, plots of the measured values showed a pattern similar to the corresponding OUR curve (Figure 6) with an immediate increase in absorbance followed by a gradual increase throughout the run. Absorbance at the end of the run ranged from 1.4 to 2.2 times the value at the beginning of the run. The major difference was that MTT formazan concentrations remained elevated at the end of the run, while OUR decreased. This difference is not meaningful with respect to sludge activity, but is an artifact of the tests used. Both parameters are expressions of the active oxidation of a substrate. OUR was controlled by the amount of substrate remaining in the mixed liquor, while MTT reduction was assisted by a large excess of glucose in the test reaction mixture. There was no consistent pattern observed for MTT reduction in the o-chlorophenol runs (Figures B-33 through B-38).

Average formazan absorbances were calculated in the same manner as average OUR. Results are presented

FIGURE 6 OXYGEN UPTAKE RATE AND MTT FORMAZAN ABSORBANCE
PHENOL/LLMO - RUN 1

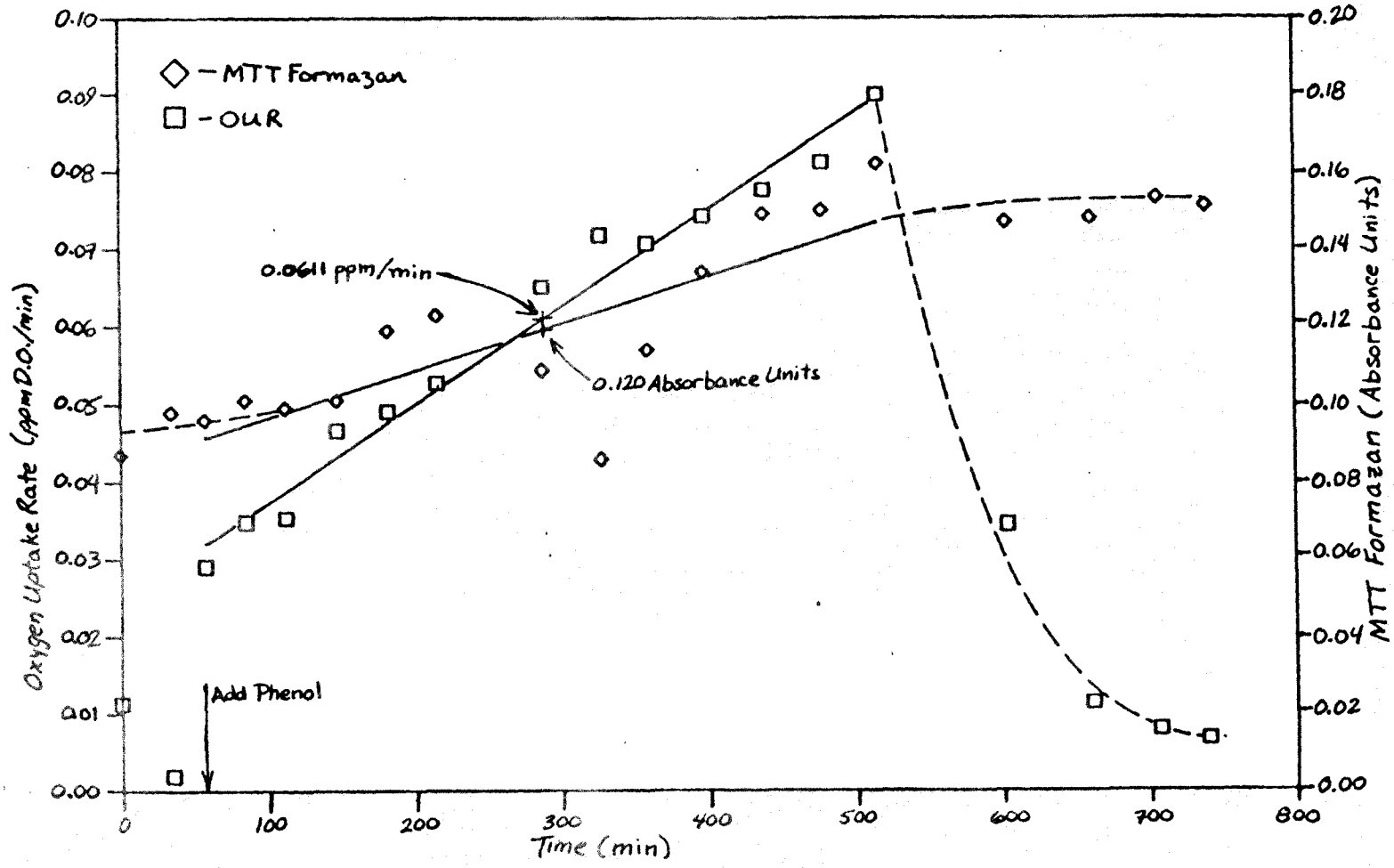


TABLE 15

AVERAGE FORMAZAN ABSORBANCES

Substrate	Media	Run	Average Absorbance	Correlation (r^2)
Phenol	Livingston Sludge I	1	*	*
	Livingston Sludge I	3	*	*
	Livingston Sludge III	1	1.026	0.930
	Livingston Sludge III	2	*	*
	Hydrobac I	1	*	*
	Hydrobac I	3	0.177	0.889
	Hydrobac II	1	*	*
	Hydrobac II	2	0.358	0.766
	LLMO	1	0.120	0.622
o-Chlorophenol	Livingston Sludge II	1	*	*
	Livingston Sludge II	3	*	*
	Livingston Sludge IV	1	0.700	0.861
	Livingston Sludge IV	2	0.512	0.955
	Livingston Sludge IV	3	0.402	0.980
	Hydrobac III	1	0.720	0.769
	Hydrobac III	2	0.740	0.364

* - MTT formazan absorbance data insufficient for analysis

TABLE 16

RATIOS OF FORMAZAN ABSORBANCE TO OXYGEN UPTAKE RATE

Substrate	Media	Run	Ratio
Phenol	Livingston Sludge I	1	*
	Livingston Sludge I	3	*
	Livingston Sludge III	1	3.65
	Livingston Sludge III	2	*
	Hydrobac I	1	*
	Hydrobac I	3	1.33
	Hydrobac II	1	*
	Hydrobac II	2	1.84
	LLMO	1	1.96
o-Chlorophenol	Livingston Sludge II	1	*
	Livingston Sludge II	3	*
	Livingston Sludge IV	1	10.59
	Livingston Sludge IV	2	8.48
	Livingston Sludge IV	3	14.46
	Hydrobac III	1	8.45
	Hydrobac III	2	11.54

* - Data insufficient for analysis

in Table 15. Ratios of formazan absorbance to OUR are presented in Table 16. There are too few values to justify any observation other than that the rate of MTT reduction relative to OUR is much higher for the o-chlorophenol runs than for the phenol runs.

F. Oxygen Uptake Rate (OUR)

Changes in OUR were followed for 12 biodegradation runs and plotted against time. All phenol runs showed the pattern of Figure 4. On adding substrate, OUR increased immediately. This was followed by a more gradual increase in rate until a maximum was reached, after which OUR decreased rapidly. The maximum OUR measured for each run ranged from 2.0 to 3.3 times the OUR at the beginning of the run. It appears that the sudden drop in OUR coincides with a critical phenol concentration between 4 and 10 ppm. Occasionally substrate uptake was so rapid that oxygen was nearly depleted (0.5 ppm or less) before the probe had stabilized. Below 0.5 ppm, OUR is limited by the available DO in the mixed liquor [35,36]. The OUR measured in the BOD bottle, where DO was allowed to deplete, would not reflect the OUR in the reactor, which was aerated. In these cases, the OUR values were not determined.

Biodegradation runs performed on o-chlorophenol

TABLE 17

AVERAGE OXYGEN UPTAKE RATES
(ppm DO/min)

Substrate	Media	Run	OUR	Correlation (r^2)
Phenol	Livingston Sludge I	1	0.394	0.993
	Livingston Sludge I	3	*	*
	Livingston Sludge III	1	0.281	0.971
	Livingston Sludge III	2	*	*
	Hydrobac I	1	0.186	0.999
	Hydrobac I	3	0.133	0.988
	Hydrobac II	1	0.271	0.906
	Hydrobac II	2	0.194	0.933
	LLMO	1	0.0611	0.978
o-Chlorophenol	Livingston Sludge II	1	*	*
	Livingston Sludge II	3	0.108	0.948
	Livingston Sludge IV	1	0.0661	0.993
	Livingston Sludge IV	2	0.0605	0.993
	Livingston Sludge IV	3	0.0380	0.801
	Hydrobac III	1	0.0851	0.983
	Hydrobac III	2	0.0642	0.964

* - OUR data insufficient for analysis

showed a different pattern of OUR of which Figure 5 is typical. There was a significant increase in OUR immediately upon the addition of substrate but OUR decreased from then on. This may have been caused by the initial concentration of substrate being too low to support continued increases in OUR.

Average OUR was calculated from plots of OUR against time. A straight line was fitted to a portion of the curve by linear regression, as was done to calculate substrate removal rate. The OUR value at the midpoint of the straight line segment was taken as the average OUR over that portion of the run (Figures 4 and 5). Average OUR are presented in Table 17. Values obtained for o-chlorophenol are much lower than those obtained for phenol. This could be due to a toxic effect of o-chlorophenol or to o-chlorophenol being less completely oxidized than phenol. However, it is more likely due to the lower initial concentration of this substrate.

G. Ratios of Parameters

The respective ratios of substrate removal rate to MLSS, OUR and MTT absorbance are presented in Tables 18 and 19. Although the limited amount of data prevents the calculation of statistically significant values of

the standard errors, the results shown are instructive. Comparing the standard error to the mean for the set of phenol runs shows little or no improvement in reproducibility using MLSS or MTT, but a significant improvement using OUR. For o-chlorophenol, the lack of data for Hydrobac runs makes it difficult to interpret those results. Concentrating instead on the Livingston sludge data, the use of MLSS and MTT had no effect on reproducibility of SRR results, while again the use of OUR produces some improvement (although not as substantial as that demonstrated for the phenol data).

The inverse of the experimental ratio of SRR to OUR represents the mass of DO consumed per mass of substrate and can thus be expressed as a fraction of the theoretical COD of the substrate (Table 20). The results show that for most cases only about 20% of the available substrate is being oxidized. The higher fractions of theoretical COD expressed in the two Hydrobac runs with o-chlorophenol (Hydrobac III) are anomalous. OUR for these two runs were of the same magnitude as OUR results for the Livingston sludge runs with o-chlorophenol (Livingston sludge II and IV), but the OUR/SRR ratios were down to 10 times as low. This suggests that another substrate present (probably one

TABLE 10

ADJUSTED SUBSTRATE REMOVAL RATES FOR PHENOL

Media	Run	SRR **	SRR/MLSS **	SRR/OUR **	SRR/MTT **
Livingston Sludge I	1	0.862	0.396	2.10	*
Livingston Sludge I	3	*	*	*	*
Livingston Sludge III	1	0.377	0.185	1.34	0.367
Livingston Sludge III	2	1.02	0.488	*	*
std. error/mean		44.5%	43.6%	33.6%	*
Hydrobac I	1	0.369	0.769	1.99	*
Hydrobac I	3	0.278	0.515	2.08	1.57
Hydrobac II	1	0.512	0.464	1.89	*
Hydrobac II	2	0.200	0.181	1.03	0.558
std. error/mean		39.4%	50.0%	27.7%	67.3%
LLMO	1	0.192	0.440	3.15	1.606
overall std. error/mean		64.6%	44.0%	34.6%	63.9%

* - Data insufficient for analysis
** - SRR = Substrate Removal Rate (ppm/min)
MLSS = Mixed Liquor Suspended Solids (1000 ppm)
OUR = Oxygen Uptake Rate (ppm DO/min)
MTT = MTT Formazan Absorbance

TABLE 19

ADJUSTED SUBSTRATE REMOVAL RATES FOR O-CHLOROPHENOL

Media	Run	SRR **	SRR/MLSS **	SRR/OUR **	SRR/MTT **
Livingston Sludge II	1	*	*	*	*
Livingston Sludge II	3	0.203	0.104	1.88	*
Livingston Sludge IV	1	0.187	0.0961	2.83	0.267
Livingston Sludge IV	2	0.157	0.0789	2.60	0.307
Livingston Sludge IV	3	0.0369	0.0190	0.971	0.0918
Hydrobac III	1	0.0190	0.0173	0.223	0.0264
Hydrobac III	2	0.0380	*	0.592	0.0514

* - Data insufficient for analysis

** - SRR = Substrate Removal Rate (ppm/min)
(adjusted to equivalent weight of phenol)
MLSS = Mixed Liquor Suspended Solids (1000 ppm)
OUR = Oxygen Uptake Rate (ppm DO/min)
MTT = MTT Formazan Absorbance

TABLE 20

FRACTION OF THEORETICAL SUBSTRATE COD
EXPRESSED BY OXYGEN UPTAKE RATE

Substrate	Media	Run	OUR/SRR **	%COD **
Phenol	Livingston Sludge I	1	0.457	19.2
	Livingston Sludge I	3	*	*
	Livingston Sludge III	1	0.746	31.3
	Livingston Sludge III	2	*	*
	Hydrobac I	1	0.503	21.1
	Hydrobac I	3	0.481	20.2
	Hydrobac II	1	0.529	22.2
	Hydrobac II	2	0.971	40.8
	LLMO	1	0.317	13.3
o-Chlorophenol	Livingston Sludge II	1	*	*
	Livingston Sludge II	3	0.389	23.1
	Livingston Sludge IV	1	0.258	15.4
	Livingston Sludge IV	2	0.282	16.8
	Livingston Sludge IV	3	0.754	44.9
	Hydrobac III	1	3.28	195
	Hydrobac III	2	1.24	73.8

* - Data insufficient for analysis

of the manufacturer's "growth factors") was metabolized along with the o-chlorophenol. If present at a low concentration (e.g. 10 to 20 ppm), the "growth factor" could compete significantly with the o-chlorophenol as substrate, while not greatly influencing the removal of phenol at its higher concentration of 100 ppm. With phenol as substrate, the fraction of the theoretical COD that was expressed as OUR did not differ greatly between Hydrobac and Livingston sludge.

The ratios of OUR to the removal rate of the experimentally determined solution COD (CODRR) are shown in Table 21. COD measurements are expressed as ppm of substrate. CODRR was estimated from the change in total COD over the same time period used to determine the average OUR. For three runs made on phenol (Livingston sludges I and III, Hydrobac I), OUR/CODRR ratios were only slightly higher than OUR/SRR, suggesting that phenol was the primary carbon source oxidized by the cultures. Results for the remaining phenol runs (Hydrobac II, LLMO) have CODRR less than SRR, suggesting that partially oxidized phenol remained in solution. The results for the o-chlorophenol runs are too inconsistent to demonstrate a relationship between OUR and CODRR. The maximum change in these runs expected to

TABLE 21

FRACTION OF EXPERIMENTAL TOTAL COD
EXPRESSED BY OXYGEN UPTAKE RATE

Substrate	Media	Run	CODRR*	OUR/CODRR	%COD
Phenol	Livingston Sludge I	1	1.07	0.371	15.6
	Livingston Sludge I	3	**	**	**
	Livingston Sludge III	1	0.506	0.555	23.3
	Livingston Sludge III	2	**	**	**
	Hydrobac I	1	0.440	0.422	17.7
	Hydrobac I	3	**	**	**
	Hydrobac II	1	0.222	1.22	51.2
	Hydrobac II	2	0.145	1.33	56.0
	LLMO	1	0.055	1.12	47.0
o-Chlorophenol	Livingston Sludge II	1	**	**	**
	Livingston Sludge II	3	0.300	3.59	213
	Livingston Sludge IV	1	**	**	**
	Livingston Sludge IV	2	0.0395	1.53	91.2
	Livingston Sludge IV	3	0.192	0.145	8.6
	Hydrobac III	1	0.0296	2.88	171
	Hydrobac III	2	0.0165	3.88	231

* - COD expressed as ppm substrate
** - Data insufficient for analysis

be caused by substrate removal was 20 ppm out of roughly 200 ppm total COD. A change of this size could be masked by the best estimated precision of the method used to measure COD ($\pm$10%).

VII. DISCUSSION

For a metabolic parameter to be useful as a measure of sludge activity, it must have a demonstrable relationship to the activity of interest and it must be applicable to a variety of media and substrates. The method used to measure the parameter should be readily applicable to the number of samples required to monitor the sludge activity.

The sludge activity of interest in the present study was the removal of substrate, in particular phenol and o-chlorophenol, from solution in the mixed liquor. The parameters evaluated were:

1) specific assay of enzyme activity
2) mixed liquor suspended solids (MLSS)
3) general dehydrogenase activity
4) oxygen uptake rate (OUR).

A. Specific Enzyme Activity

The methods for the specific assay of enzyme activities attempted in the present study did not produce any results that were useful for the determination of microbial activity. The results obtained have been discussed in section V-A. There are several possible reasons that this approach failed to give useful results. The first is that the methods of

extracting the enzymes from the mixed liquor resulted in either incomplete extraction or partial inactivation of the enzymes. The researchers cited [18,19,20] employed piston-type sonicators and refrigerated ultra-centrifuges in the preparation of the extract. Neither type of equipment was available for this study. The possibility that enzymes were being denatured by the extraction process was not adequately explored in this study.

A second possibility is that the assay methods selected were inappropriate to this application. The enzyme assay kits used were developed to produce optimum results for particular clinical tests routinely performed on human serum [38,39,40] and may not be useful for suspensions prepared from bacterial cultures.

Another possible reason that the attempted assays did not produce useful results is that the amount of the assayed enzymes present in bacteria is too small to be detected by the methods used. No information was found as to the range of activities to be expected for dehydrogenases in bacterial preparations, but the results of Sayler, et al [20], suggest that the activities of individual enzymes in bacterial

suspensions are too low to measure reliably.

The majority of published methods for enzyme analysis are oriented toward the study of animal tissue. Most of the procedures published for microbial systems have been developed for cultures of single species. Adapting these methods to mixed cultures does not appear to produce reliable results.

B. Mixed Liquor Suspended Solids

During the biodegradation runs, mixed liquor suspended solids (MLSS) were observed to change very little. The small changes that were observed were always within the precision of the method used to measure MLSS. Attempts to normalize substrate removal rates (SRR) by dividing each rate by the average MLSS value for that run resulted in little or no improvement in the reproducibility of SRR between runs (Tables 18 and 19).

During the phenol runs, OUR and the rate of MTT reduction did change, increasing as the run progressed. Since both of these parameters can be assumed to be constant when expressed per viable cell [7,8,9], it is evident that the number of active cells increased during each run. Increases of over 3-fold in the number of active cells (estimated from the increase in

OUR) produced no significant increase in total MLSS. Since MLSS did not respond at all to environmental stress (remaining essentially constant), it was totally inadequate as a measure of biological activity.

C. Substrate Removal Rates

Microbial cultures acclimated to phenol showed an immediate ability to degrade o-chlorophenol, although the substrate removal rates (SRR) for o-chlorophenol were always lower than for phenol. The SRR obtained in the present study, in general, are within the same ranges as values obtained in other studies in the same laboratory [43,44,45]. One exception is that SRR obtained in this study for phenol in Livingston sludge were lower than those obtained in another study [43]. As discussed in section VI-B, there is no obvious explanation for this discrepancy as the reactor, the microbial culture, and the experimental methods used in these studies were the same. Taking the results of the studies as a single group demonstrates that a broad range of unnormalized experimental values can be obtained on the same experimental system.

Another difference between this study and prior studies is the effect of successive shocks of o-chlorophenol on a single culture with respect to SRR. In a

prior study [43], successive shocks resulted in an increase of the culture's ability to degrade the substrate. In the present study, successive shocks resulted in a decrease in SRR, eventually reducing removal to negligible rates. This seems to point to the production of toxic metabolites during the biodegradation of o-chlorophenol. OUR also decreased, but not as greatly as SRR. The difference between the two studies is that in the present study o-chlorophenol was added to the culture as soon as substrate concentrations dropped below the concentration detectable by GC. In the prior study, a period of several hours was allowed between the end of one run and the start of the next. This period may have allowed the sludge to recover from the toxic effects of the o-chlorophenol or its metabolites.

D. General Dehydrogenase Activity

In theory, the general dehydrogenase activity within a microbial culture can be measured by the rate of reduction of an electron-acceptor dye, such as the tetrazolium salt MTT. Since, for aerobic organisms, oxygen is the ultimate electron acceptor for the electron transfers initiated by the dehydrogenases, the results obtained by determining general dehydrogenase

activity should be similar to those obtained by measuring OUR. A number of researchers have demonstrated that the rate of MTT reduction does parallel OUR for non-poisoned sludges [8,26,29,30,31]. Several researchers, in particular Klapwijk [30], and Tebbutt and Paraskevopoulos [8], deny that the relationship is consistent enough to be meaningful. In the present study, some biodegradation runs showed a close relationship between MTT reduction and OUR and some showed none. The presence or absence of a relationship was not associated with the substrate or culture medium. Where there was a relationship, the MTT/OUR ratios were found to be surprisingly consistent (Table 16). The consistency is surprising because OUR determinations were made at the temperature and substrate concentrations prevailing in the reactor, while the MTT reagents were always incubated at 37°C with an excess of glucose. For the portion of the run where OUR was not limited by substrate concentration, MTT reduction and OUR should still parallel each other. No explanation can be offered for the o-chlorophenol ratios being so much higher than the phenol ratios.

Attempts to normalize the substrate removal rate by dividing through with the absorbance of the MTT

formazan resulted in a poorer consistency among the SRR/MTT ratios than among the absolute SRR (Tables 18 and 19).

From the literature search it appears that no single method of tetrazolium dye reduction is generally applicable across a variety of cultures and substrates. Each of the authors cited developed a method that would work under his particular conditions. Ryssov-Nielson, Lenhard and Klapwijk demonstrated that changing the substrate used in the test reagent altered the results obtained. Toxic substrates would also produce variable results, depending more on the nature of the toxic effect than on the rate of substrate removal. Substrate carried over with the sludge itself would further confuse interpretation of the results. It is disappointing to note that workers were seldom successful in applying each others' methods. Furthermore, the procedure requires considerable advance preparation, plus more space and equipment, than does OUR measurement. Adding steps to the procedure, such as washing the sludge solids before adding reagents, would increase the amount of time needed for the test and make it less practical for large numbers of samples.

The MTT reduction procedure used in this study was a modification of that presented by Ford, Yang and Eckenfelder [26]. The method does not show the actual rate of reduction as it would occur in the biodegradation reactor. Dye reduction was not carried out at the same temperature as substrate removal and an excess of a readily oxidized co-substrate (glucose) was supplied in the incubation mixture. This procedure would measure the amount of catalyst available in the sludge, rather than the activity expressed in the reactor. A possible modification to the test would be to add a portion of dye to an undiluted aliquot of mixed liquor and allow it to react at room temperature, then extracting the dye with a volume of solvent [25]. This test would better reflect conditions in the reactor.

Thoughtful selection of the reaction conditions could produce tests that provide useful information about the activity of microbial systems. By selecting the dye and the type of substrate, it should be possible to answer questions about the biodegradability of the substrate or the nature of its toxic action. It is necessary to understand the effect of each factor in the method in order to interpret the results obtained.

Reduction of electron-acceptor dyes would be preferred over OUR when it is desired to examine the activity of electron-transfer chains that do not terminate in the reduction of oxygen. It has a definite application in low DO and anaerobic systems.

E. Oxygen Uptake Rate

For all runs performed in the present study, there was an immediate increase in oxygen uptake rate (OUR) when either phenol or o-chlorophenol was added to the reactor.

For the phenol runs there was a gradual increase in OUR as the run progressed, suggesting that the ability of the culture to remove phenol improved during the run. This conclusion is supported by the slightly convex shape over most of the substrate concentration curve for most of the phenol runs, which indicates an accelerating removal rate. OUR drops suddenly toward the end of the run, just before the tail of the S-curve of substrate concentration. Both the drop in OUR and the tail of the substrate concentration curve are indicative of a decrease in microbial activity. This decrease is caused by the substrate concentration being too low to support the rate of activity of which the culture is capable. Accepting that OUR is constant

when expressed per cell [7,8,9,34], leads to the conclusion that the number of active cells increased as the run progressed. If this is so, adding additional phenol at the end of a run should cause the sludge to resume its high OUR. For the removal of phenol by Livingston sludge and by Hydrobac, OUR did resume at a high rate when additional phenol was added (Livingston sludge III, Hydrobac II).

Following the initial exposure to the substrate (and the sudden rise in OUR), the runs made with o-chlorophenol showed a decrease in OUR as the run progressed. Successive shocks of this substrate resulted in a decrease of the average OUR over each run, although not as great as the decrease in SRR. This has been discussed in section VII-C.

The results of the present study show that SRR for phenol is more closely related to OUR than to either MLSS or MTT reduction (Table 18). For the Livingston sludge runs, the SRR/OUR ratios vary less between the runs than do SRR or the SRR/MLSS and SRR/MTT ratios. The same observation holds true for the Hydrobac runs. The SRR/OUR ratios for Livingston sludge and Hydrobac are within the same range.

For the o-chlorophenol runs, Livingston sludge and

Hydrobac definitely show different responses. Livingston sludge appears to remove o-chlorophenol at a higher normalized rate than it does phenol (Table 19). Results from the present and prior studies [43,44] show that the absolute SRR for o-chlorophenol is much lower than the rate for phenol. What the normalized rate (SRR/OUR) actually expresses is the mass of substrate removed per mass of DO removed. It does not comment on the fate of the substrate. If o-chlorophenol is less completely oxidized than phenol, its OUR will be smaller relative to its SRR, increasing the normalized removal rate. The presence of a falling OUR curve for the o-chlorophenol runs as opposed to a rising curve for the phenol runs does not affect the normalized SRR, as only the average OUR is of interest, not the direction of its change.

Hydrobac had a much lower ratio of SRR/OUR for o-chlorophenol than it did for phenol. This is probably due to the oxidation of additional COD available from the "growth factors" included in the dessicated mixture. The effect of this additional COD was much greater for the o-chlorophenol runs than for the phenol runs. This may result from the "growth factors" being more readily degradable than o-chlorophenol, but not

phenol (hence, their presence even after phenol acclimation). Furthermore, if the "growth factors" are present at 20 ppm, they will exert a greater influence over the results of the o-chlorophenol runs, where substrate is present at 20 ppm, than over the phenol runs , where substrate is present at 100 ppm. In future studies, the uncertainties caused by the presence of these "growth factors" can be diminished by allowing COD in the culture to drop to a constant low level before beginning acclimation of the culture to phenol.

There are several reasons why OUR and SRR should be closely related. Oxygen is the terminal electron acceptor for the metabolic pathways that degrade substrate. Therefore, absence of oxygen limits the functions of cellular metabolism, including the degradation of substrate. Stimulating or depressing cellular metabolism (e.g. by a change in substrate concentration) affects both the energy requirement of the cell (substrate uptake) and its requirement for electron acceptors (oxygen uptake) in the same manner. Huang and Cheng demonstrated this relationship when they successfully substituted OUR for reaction velocity (SRR) in a Monod-type equation to predict effluent COD

[14].

However, SRR and OUR should not be expected to be related perfectly. A portion of the substrate will be incorporated into the biomass rather than being oxidized. This incorporation will not be expressed as OUR. This proportion will vary, depending on the sludge age, its health, and the species present in the sludge. Some compounds will be incompletely oxidized, in which case SRR will appear higher in relation to OUR than it actually is. This would depend on the capabilities of the species present, and on the presence of toxicants and co-substrates. It should be possible to apply OUR as a sludge activity parameter even under these conditions if additional tests, such as total COD and comparisons with biodegradation of standard compounds, are performed to aid in the interpretation of results.

The DO probe method used in this study is simple to apply and gives accurate results rapidly. The method could still be improved: a number of data points were lost when DO concentrations in the BOD bottle fell below 0.5 ppm. At DO concentrations above this level, OUR is independent of DO. Below 0.5 ppm, OUR is controlled by the DO available in the mixed

liquor [35,36]. During the biodegradation runs, DO in the reactor very rapidly fell below 0.5 ppm. When DO in the BOD bottle fell below this level, the measured OUR was no longer representative of the OUR within the reactor.

A possible modification of the BOD bottle method for determining OUR would be to dilute a small volume of the sludge into a standard solution of substrate before beginning measurements. Although this procedure would not reflect conditions inside the reactor, it would allow the activity of the sludge to be determined without restrictions of low DO or low substrate concentration. It would therefore provide a better estimate of the total amount of catalyst present in the sludge.

VIII. CONCLUSIONS

1) The measurement of specific enzyme activities is not an appropriate method to determine metabolic activity in mixed microbial cultures because the low activities of individual enzymes cannot be measured by the simple, rapid clinical methods designed primarily for human cell cultures.

2) Mixed liquor suspended solids (MLSS) is not an effective parameter for measuring the metabolic activity of mixed microbial cultures, since it has no direct relationship to the viable cell population, and is very insensitive to environmental stress.

3) The reduction of electron-acceptor dyes, in particular tetrazolium salts, has potential for use as a metabolic parameter, but is more difficult to apply and much less consistent than OUR.

4) Oxygen uptake rate (OUR) is a satisfactory parameter for measuring the metabolic activity of mixed microbial cultures. OUR has a direct relationship to substrate removal rate and can be determined rapidly using an electrochemical probe. Substrate removal rates (SRR) that are normalized with respect to OUR are much more consistent than the original SRR data.

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APPENDIX A

DATA SUMMARIES

OF

BIODEGRADATION RUNS

TABLE A-1 BIODEGRADATION RUN

Substrate: Phenol Media: Livingston Sludge I

Initial Concentration: 100 ppm Run: 1 Date: 3/28/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE @ 540nm	SUBSTRATE	COD	NH3
Min.	ppm	ppm	ppm/min		ppm	ppm	ppm
0	2200	3.221	0.0586		0.000	57.4	
15	2140	3.224	0.0486		0.000	54.6	
30	2140	3.002	0.2537		98.870	170.0	46.0
45	2160	2.286	0.2950		99.421	145.0	
60	2130	2.035	0.3512		89.011	200.0	
75	2130	1.837	0.4268		74.617	103.0	
90	2000	1.456	0.5090		61.106	95.8	46.0
105	2160	0.702			37.676	83.4	
120	2280	0.273			7.315	58.8	
135	2220	2.482	0.1441		0.468	58.0	
150	2200	2.735	0.1312		0.000	63.0	43.0
165	2240	2.850	0.1113		0.000	87.0	
180	2230	2.644	0.0955		0.000	57.4	
195	2230	3.022	0.0920		0.000	57.4	

TABLE A-2 BIODEGRADATION RUN

Substrate: Phenol Media: Livingston Sludge I

Initial Concentration: 100 ppm Run: 3 Date: 3/30/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORBANCE @ 540nm	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min		ppm	ppm	ppm	
0	2180	3.205	0.0719	0.711				
30	2170	3.260	0.0744	0.762				
60	2210	2.523	0.5373	1.287				
90	2220	0.230		1.366				
120	2250	1.775	0.2453	1.551				
150	2270	2.879	0.1311	1.323				
180	2270	2.905	0.1026	1.331				
210	2220	3.088	0.0937	1.228				
240	2290	3.066	0.0763	1.132				
270	2270	2.979	0.0809	1.179				

TABLE A-3 BIODEGRADATION RUN

Substrate: Phenol Media: Livingston Sludge III

Initial Concentration: 100 ppm Run: 1 Date: 5/09/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORBANCE @ 540nm	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min		ppm	ppm	ppm	
0		3.832	0.1805	0.611	0.000			
20			0.0335	0.670	0.000			
40	2040	3.429	0.1863	0.809	99.396	196.0	62.0	
59			0.2378	1.009	96.755			
79		2.036	0.2987	1.111	87.919	199.0		
98	2040		0.4020	1.175	78.002			
117		1.250		1.248	56.170	163.0	62.0	
137		0.278		1.238	34.707			
156		0.252		1.303	10.605	130.0	62.0	
176	2020	2.627	0.1250	1.456	2.189			
196		3.190	0.1061	1.281	1.070	117.0		
250		3.467	0.0842	1.231	0.000			

TABLE A-4 BIODEGRADATION RUN

Substrate: Phenol Media: Livingston Sludge III

Initial Concentration: 100 ppm Run: 2 Date: 5/09/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
.0	2020	3.062	0.3393	1.250	115.540	190.0	57.0	
26		1.478	0.4343	1.255	99.956			
45		1.000		1.364	83.484	168.0		
65	2030	0.352		1.402	67.599			
84		0.277		1.361	44.753	117.0	53.0	
103		0.242		1.378	22.322			
123	2190	1.834	0.1835	1.337	2.900	91.4		
143		2.820	0.1401	1.379	0.000			
164		3.144	0.1198	1.311	1.597	145.0	50.0	
183	2120	3.352	0.0999	1.305	0.773			

TABLE A-5 BIODEGRADATION RUN

Substrate: Phenol Media: Hydrobac I

Initial Concentration: 100 ppm Run: 1 Date: 4/11/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
0	430	3.332	0.2058		1.501	102.0		
15		3.380	0.0487		0.000	113.0		
30	440	3.408	0.0828		105.380	249.0	30.0	
45		3.295	0.0767		105.950	175.0		
60	500	3.241	0.0857		107.890	152.0		
75		3.237	0.0996		104.130	174.0		
90	470	3.113	0.1137		104.120	186.0		
105		3.093	0.1323		96.562	122.0		
120	410	3.080	0.1476		90.652	102.0		
135		3.017	0.1658		87.554	129.0		
150	390	2.956	0.1773		84.853	140.0	27.0	
170		2.899	0.2047		76.376	126.0		
190	450	2.803	0.2215		69.485	128.0	21.0	
210		2.717	0.2463		60.559	110.0		
230	590	2.656	0.2673		48.956	98.7		
250		2.588	0.2847		35.503	92.4		
270	560	2.439	0.3240		25.023	90.3		
290		2.185	0.3812		6.819	74.6		
310	560	3.118	0.0728		1.045	83.0		
330		3.200	0.0598		0.609	75.6		

TABLE A-6 BIODEGRADATION RUN

Substrate: Phenol Media: Hydrobac I

Initial Concentration: 100 ppm Run: 3 Date: 4/13/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
0	460	3.422	0.0752	0.128	0.000	44.6		
30		3.457	0.0204	0.125				
60	480	3.302	0.0762	0.149	115.360	>135.0	43.0	
90		3.225	0.0651	0.142				
120	500	3.157	0.0930	0.168	105.180	>135.0		
150		3.117	0.1195	0.159				
180	500	3.005	0.1389	0.174	93.672	>135.0	37.0	
210		2.967	0.1598	0.202				
240	540	2.912	0.1727	0.198	63.593	>135.0		
270		2.887	0.1845	0.223				
300	620	2.653	0.2400	0.300	38.819	109.0	34.0	
330		2.533	0.2920	0.287				
360	680	3.079	0.0707	0.306				

TABLE A-7 BIODEGRADATION RUN

Substrate: Phenol Media: Hydrobac II

Initial Concentration: 100 ppm Run: 1 Date: 5/23/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
0		3.088	0.2157	0.384	1.852			
20		3.072	0.1142	0.401	0.000			
45	1100	3.245	0.1276	0.361	99.536	279.0	42.0	
75		3.267	0.1448	0.356	101.210			
105		3.207	0.1682	0.380	99.258			
135		3.150	0.1808	0.332	102.310	269.0		
165	1090	3.067	0.2020	0.344	99.087			
195		3.052	0.2010	0.343	102.430		42.0	
225		2.972	0.2287	0.326	96.558	231.0		
255		2.917	0.2418	0.296	87.474			
285	1080	2.743	0.2675	0.321	72.190			
315		2.600	0.2948	0.325	49.488	233.0		
349		2.240	0.3813	0.342	24.541		40.0	
383		2.865	0.1480	0.377	4.774	204.0		
417	1140	3.074	0.1169	0.369	0.000			

TABLE A-8 BIODEGRADATION RUN

Substrate: Phenol Media: Hydrobac II

Initial Concentration: 100 ppm Run: 2 Date: 5/23/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
4		3.300	0.1755	0.401	112.490	269.0	39.0	
38		3.205	0.1723	0.375	118.470	269.0		
73		3.207	0.1698	0.336	108.270	284.0	38.0	
107	1100	3.185	0.1822	0.335	104.380	277.0		
142		3.160	0.1957	0.352	99.814	269.0	36.0	
176		3.681	0.2292	0.407	86.894			

TABLE A-9 BIODEGRADATION RUN

Substrate: Phenol Media: LLMO

Initial Concentration: 100 ppm Run: 1 Date: 4/19/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
0	430	3.385	0.0113	0.087	0.000			
35		3.381	0.0019	0.098	0.000			
57	450	3.219	0.0292	0.096	99.000	167.0	11.0	
85		3.144	0.0349	0.101	94.835			
112	400	3.129	0.0354	0.099	94.470			
147		3.079	0.0466	0.101	86.765			
181	430	3.042	0.0491	0.119	85.223	165.0		
215		3.010	0.0528	0.123	76.573			
287	400	2.951	0.0652	0.109	64.884			
327		2.922	0.0718	0.086	50.193			
359	420	2.885	0.0708	0.114	47.540	145.0	7.8	
397		2.868	0.0742	0.134	40.408			
437	440	2.806	0.0777	0.149	31.238			
477		2.816	0.0813	0.150	20.527			
515	500	2.799	0.0900	0.162	15.534	142.0		
603		3.349	0.0346	0.147	0.000			
660	440	3.373	0.0114	0.148	0.000			
705		3.391	0.0079	0.153	0.000			
739	450	3.389	0.0067	0.151	0.000	125.0	6.4	

TABLE A-10 BIODEGRADATION RUN

Substrate: o-Chlorophenol Media: Livingston Sludge II

Initial Concentration: 20 ppm Run: 1 Date: 4/04/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
0	1710	3.233	0.0746	0.710				
30	1790	3.278	0.0647	0.683				
60	1720	3.162	0.1186	0.755				
90	1690	2.999	0.1276	0.681				
120	1650	3.061	0.0923	0.725				
150	1670	3.265	0.0485	0.675				
180	1640	3.322	0.0444	0.661				
210	1620	3.247	0.0393	0.688				

TABLE A-II BIODEGRADATION RUN

Substrate: o-Chlorophenol Media: Livingston Sludge II

Initial Concentration: 20 ppm Run: 3 Date: 4/06/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
0	1930	3.267	0.0831		0.000			
15		3.342	0.0576		0.000			
30	1950	3.204	0.1385		18.192	89.3	32.0	
45		2.974	0.1351		13.703	81.5		
60	1950	2.914	0.1151		10.590	85.4		
75		3.186	0.0865		6.122	73.2	30.0	
90	1970	3.253	0.0628		1.211	87.5		
105		3.272	0.0514		0.000	97.3		
120	1980	3.384	0.0482		0.000	94.3	30.0	

TABLE A-12 BIODEGRADATION RUN

Substrate: o-Chlorophenol Media: Livingston Sludge IV

Initial Concentration: 20 ppm Run: 1 Date: 5/16/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORBANCE @ 540nm	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min		ppm	ppm	ppm	
0	2180	3.583	0.0280	0.513	0.000			
24		3.549	0.0239	0.520	0.000			
43	1980	3.055	0.0979	0.670	20.100	188.0	71.0	
62		2.981	0.0999	0.675	14.404			
81		2.917	0.0736	0.679	9.251	202.0		
100		2.916	0.0553	0.729	5.908		73.0	
119	1910	3.283	0.0354	0.745	0.000	193.0		
140		3.446	0.0327	0.617	0.000			
162		3.488	0.0283	0.626	0.000	182.0	70.0	

TABLE A-13 BIODEGRADATION RUN

Substrate: o-Chlorophenol Media: Livingston Sludge IV

Initial Concentration: 20 ppm Run: 2 Date: 5/16/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORBANCE @ 540nm	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min		ppm	ppm	ppm	
2		3.324	0.0837	0.632	19.403	188.0	73.0	
21	1980	3.152	0.0745	0.650	18.678	193.0		
40		3.191	0.0585	0.561	14.781	193.0	76.0	
59		3.350	0.0482	0.482	10.181	182.0		
78		3.413	0.0374	0.453	6.128	185.0	75.0	
96	2000	3.482	0.0330	0.412	2.860			

TABLE A-14 BIODEGRADATION RUN

Substrate: o-Chlorophenol Media: Livingston Sludge IV

Initial Concentration: 20 ppm Run: 3 Date: 5/16/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
2		3.405	0.0330	0.398	26.876	208.0	76.0	
21		3.524	0.0281	0.428	26.001	226.0		
40		3.535	0.0260	0.400	23.679	190.0	84.0	
61	1940	3.560	0.0259	0.377	22.464	193.0		
80		3.550	0.0249	0.363	21.858	193.0	87.0	
100		3.562	0.0267	0.369	20.540			

TABLE A-15 BIODEGRADATION RUN

Substrate: o-Chlorophenol Media: Hydrobac III

Initial Concentration: 20 ppm Run: 1 Date: 5/26/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min	@ 540nm	ppm	ppm	ppm	
0		3.040	0.0822	0.604	0.000			
19		3.046	0.0757	0.600	0.000			
39	1160	2.750	0.1230	0.770	20.722	160.0	43.0	
72		2.913	0.0805	0.506	13.729			
97		2.981	0.0784	0.541	15.779			
124		3.017	0.0795	0.527	14.589	139.0		
155		2.959	0.0787	0.588	13.346			
188	1100	2.985	0.0800	0.579	11.479	160.0		
213		3.030	0.0774	0.656	10.377			
250		2.993	0.0747	0.587	9.003	172.0	38.0	
286		2.905	0.0795	0.716	8.072			
333	1110	2.902	0.0873	0.697	6.619			
408		2.890	0.0942	0.795	4.823			
453	1050	2.895	0.0861	0.780	3.752	166.0		
494		2.923	0.0787	0.727	5.794			
532		2.963	0.0781	0.800	2.201		39.0	
563		3.023	0.0763	0.757	1.597			
604	1070	3.005	0.0762	0.763	0.567			

TABLE A-16 BIODEGRADATION RUN

Substrate: o-Chlorophenol Media: Hydrobac III

Initial Concentration: 20 ppm Run: 2 Date: 5/26/84

TIME	MLSS	DISSOLVED OXYGEN	OXYGEN UPTAKE RATE	ABSORB-ANCE @ 540nm	SUBSTRATE	COD	NH3	pH
Min.	ppm	ppm	ppm/min		ppm	ppm	ppm	
3		3.040	0.0699	0.854	26.528	212.0	37.0	
35		3.037	0.0672	0.713	25.975	208.0		
67		3.080	0.0624	0.686	25.153	204.0	45.0	
96		3.108	0.0613	0.709	22.266	214.0		
124		3.102	0.0593	0.734	20.448	210.0	46.0	

APPENDIX B

PLOTS OF
BIODEGRADATION RUNS

FIGURE B-1 PHENOL / LIVINGSTON SLUDGE I - RUN 1
SUBSTRATE, COD, OXYGEN UPTAKE RATE

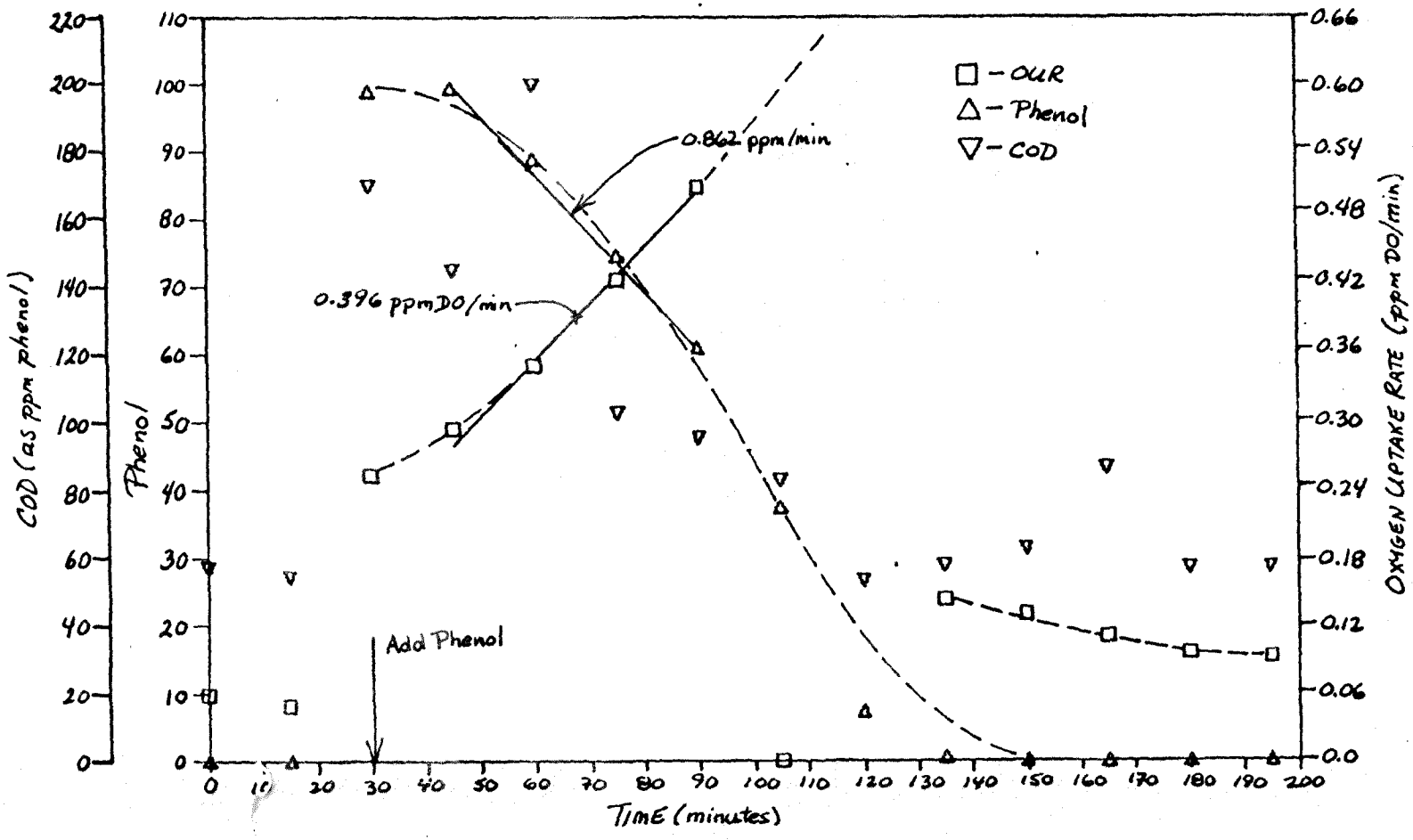


FIGURE B-2 PHENOL / LIVINGSTON SLUDGE III - RUN 1
SUBSTRATE, COD, OXYGEN UPTAKE RATE

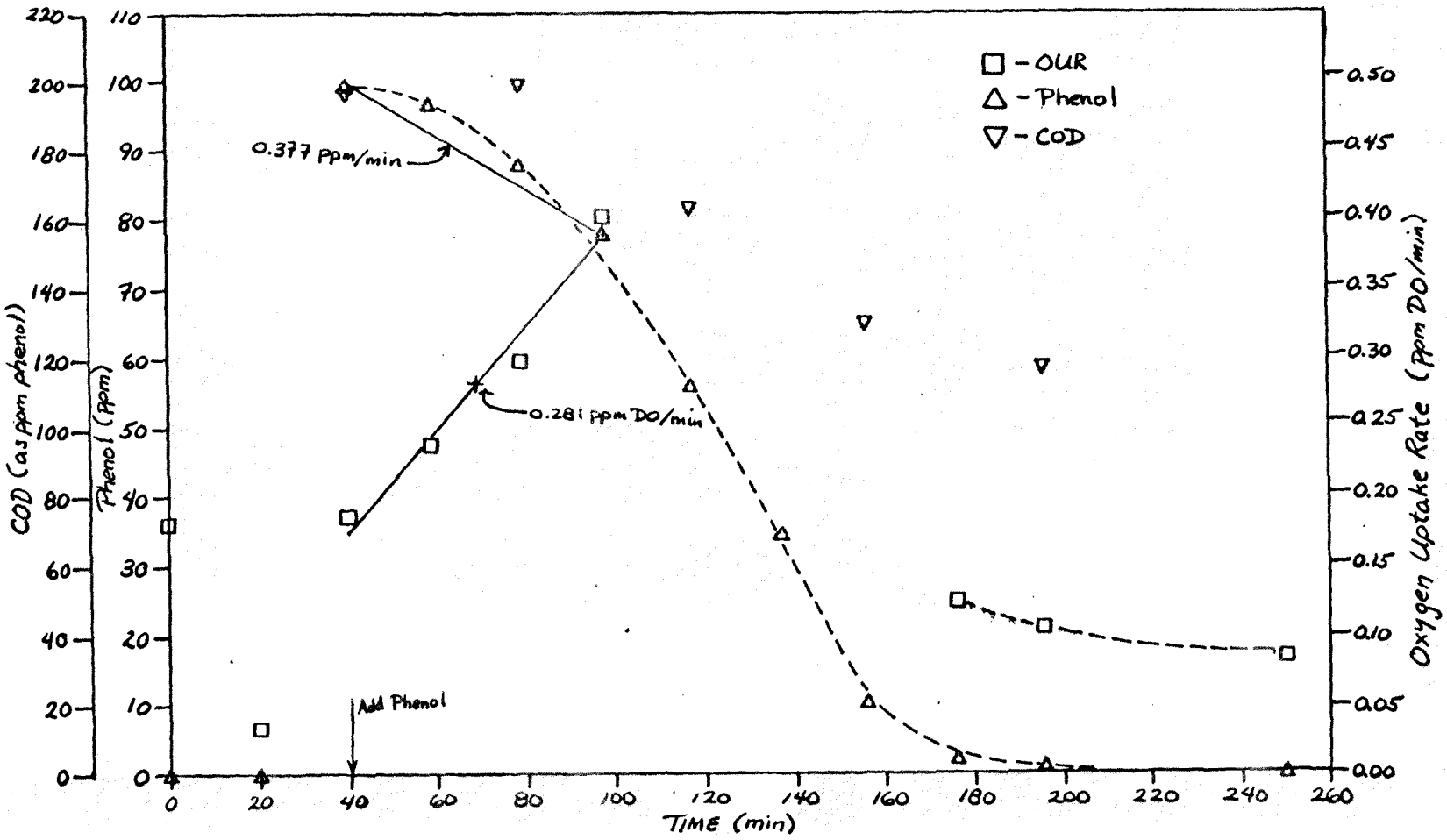


FIGURE B-3 PHENOL/LIVINGSTON SLUDGE III - RUN 2
SUBSTRATE, COD, OXYGEN UPTAKE RATE

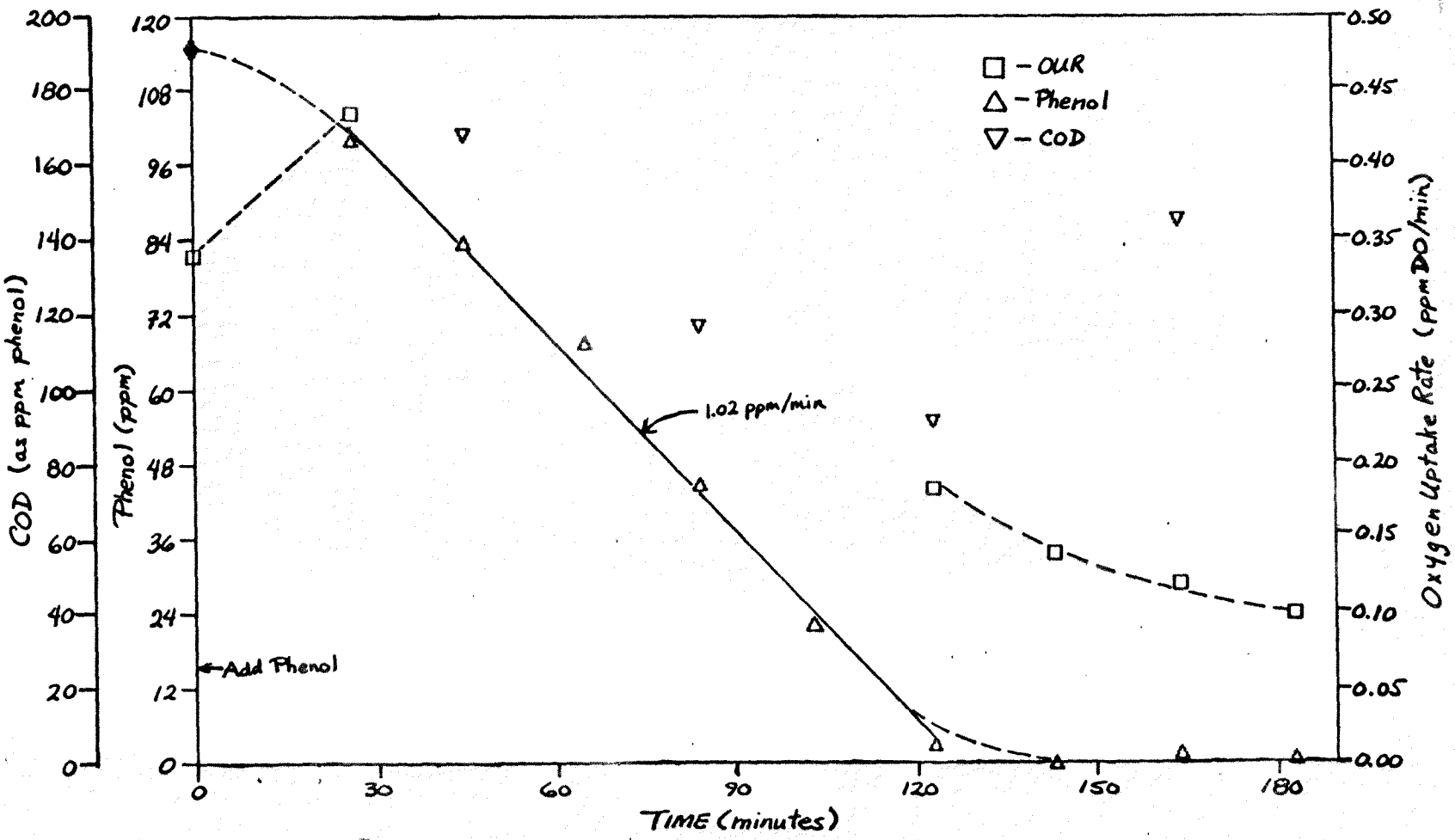


FIGURE B-4 PHENOL / HYDROBAC I - RUN 1
SUBSTRATE, COD, OXYGEN UPTAKE RATE

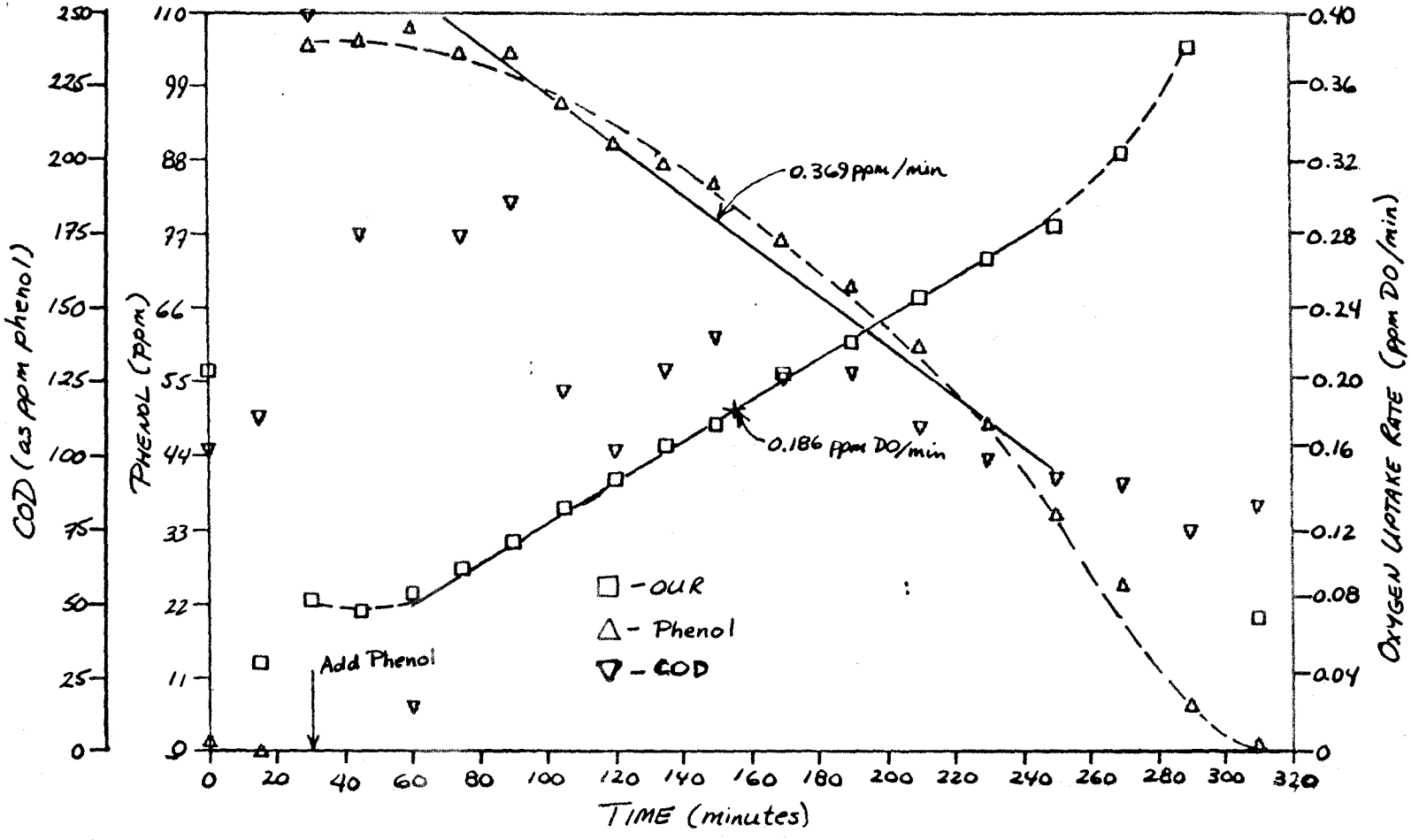


FIGURE B-5 PHENOL / HYDROBAC I - RUN 3
SUBSTRATE, OXYGEN UPTAKE RATE

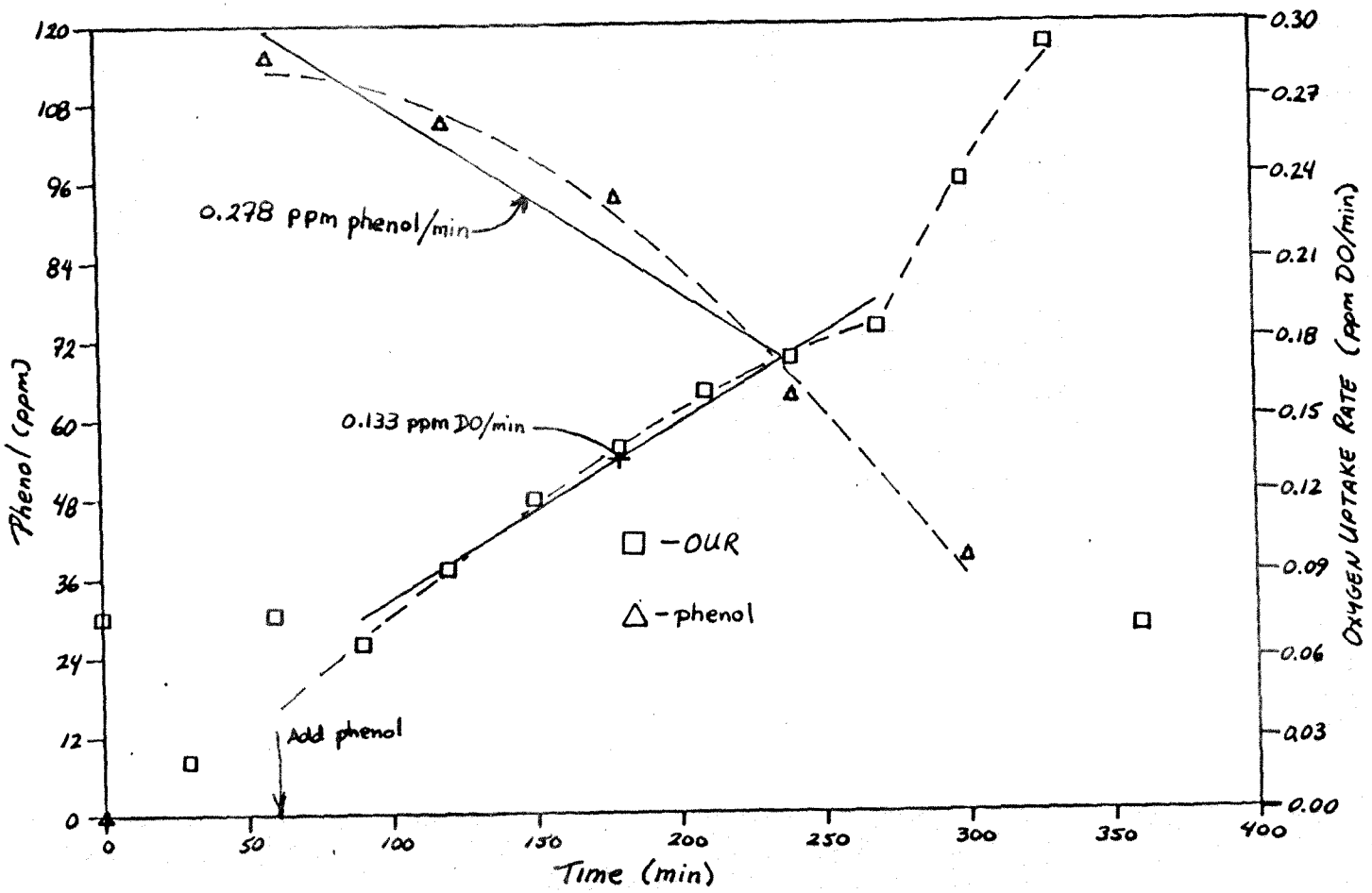


FIGURE B-6 PHENOL / HYDROBAC II – RUN 1
SUBSTRATE, COD, OXYGEN UPTAKE RATE

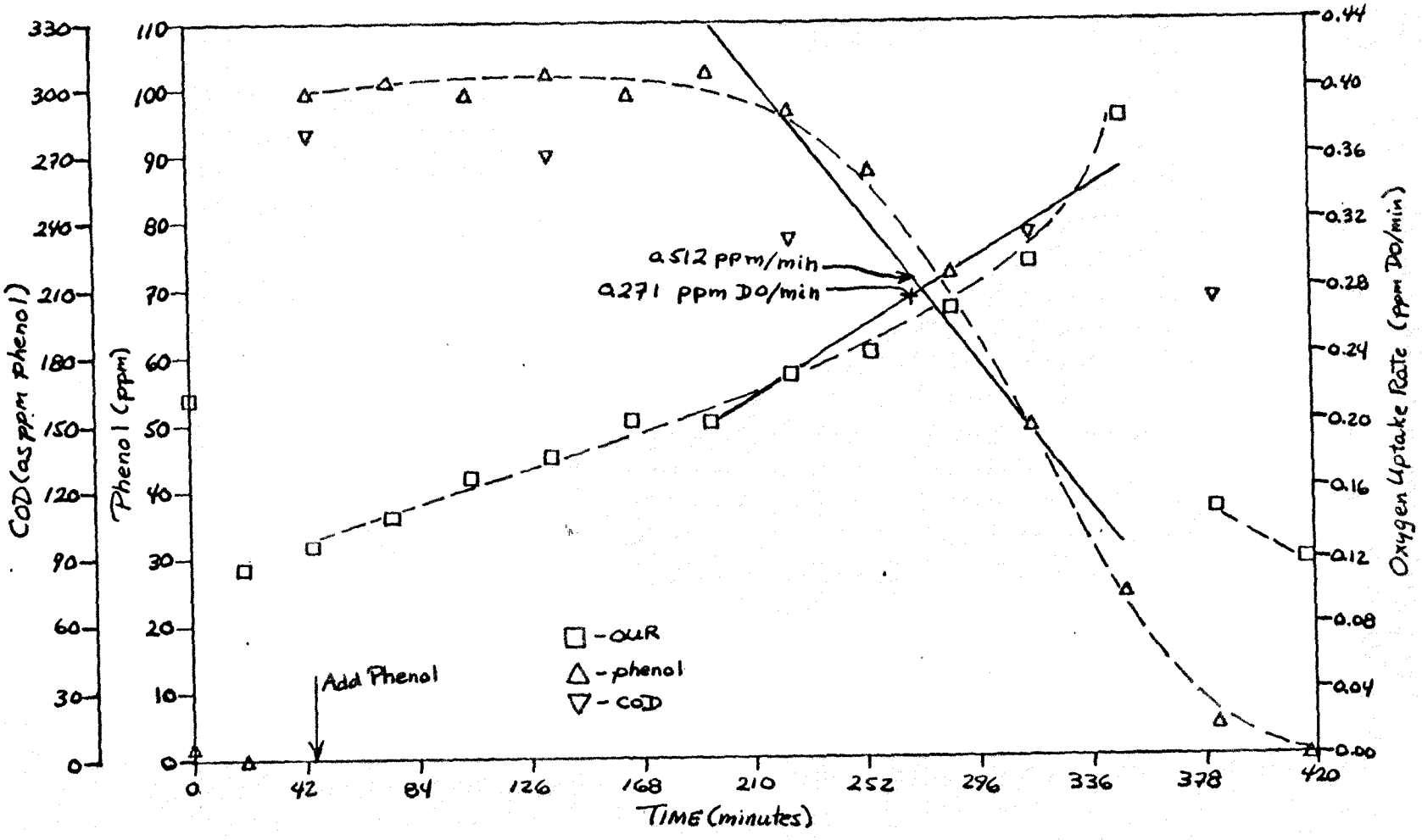


FIGURE B-7 PHENOL/HYDROBAC II - RUN 2
SUBSTRATE, COD, OXYGEN UPTAKE RATE

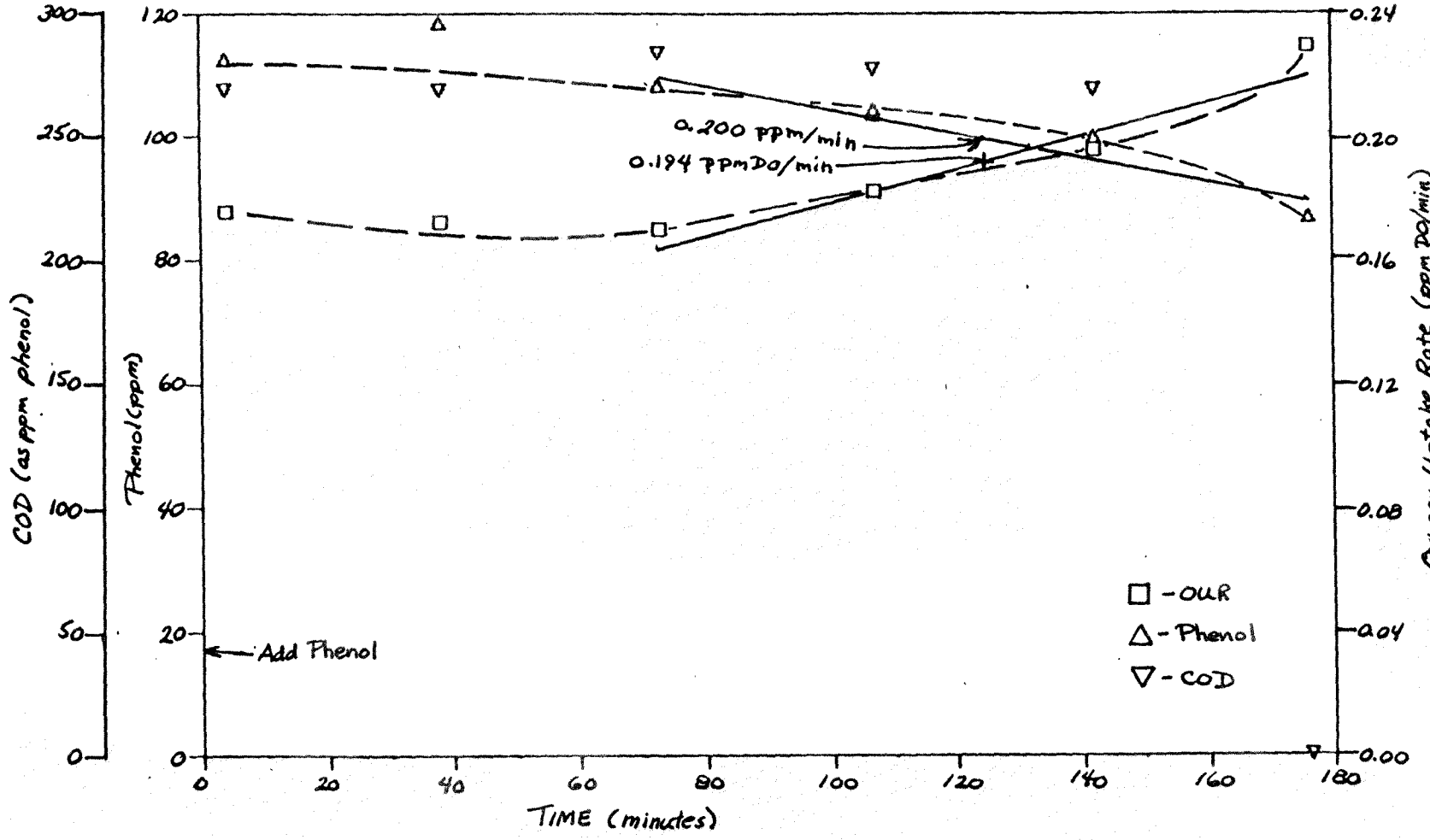


FIGURE B-8

PHENOL / LLMO - RUN 1
SUBSTRATE, COD, OXYGEN UPTAKE RATE

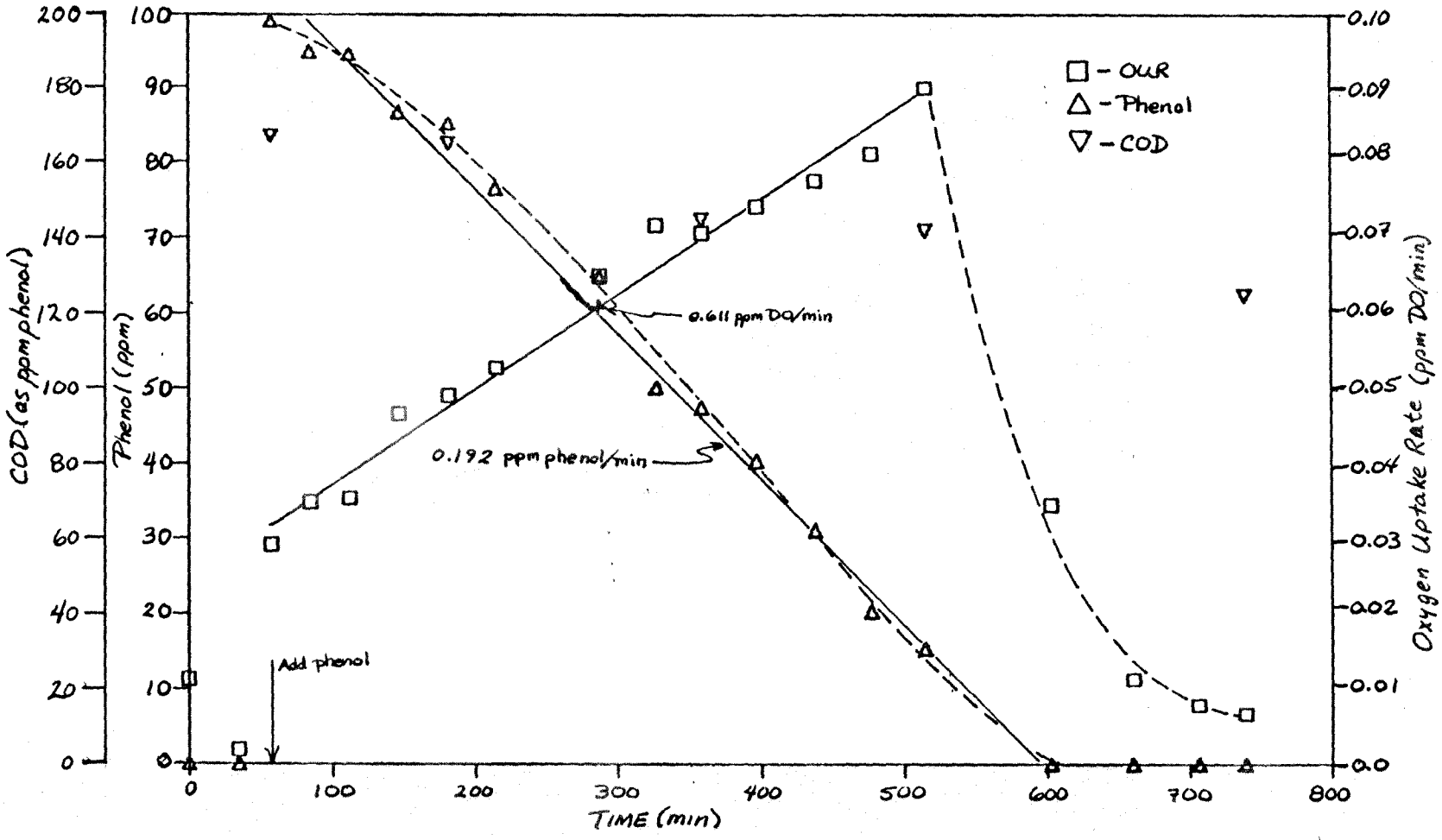


FIGURE B-9 PHENOL/LIVINGSTON SLUDGE III - RUN 1
SUBSTRATE, COD, MTT FORMAZAN

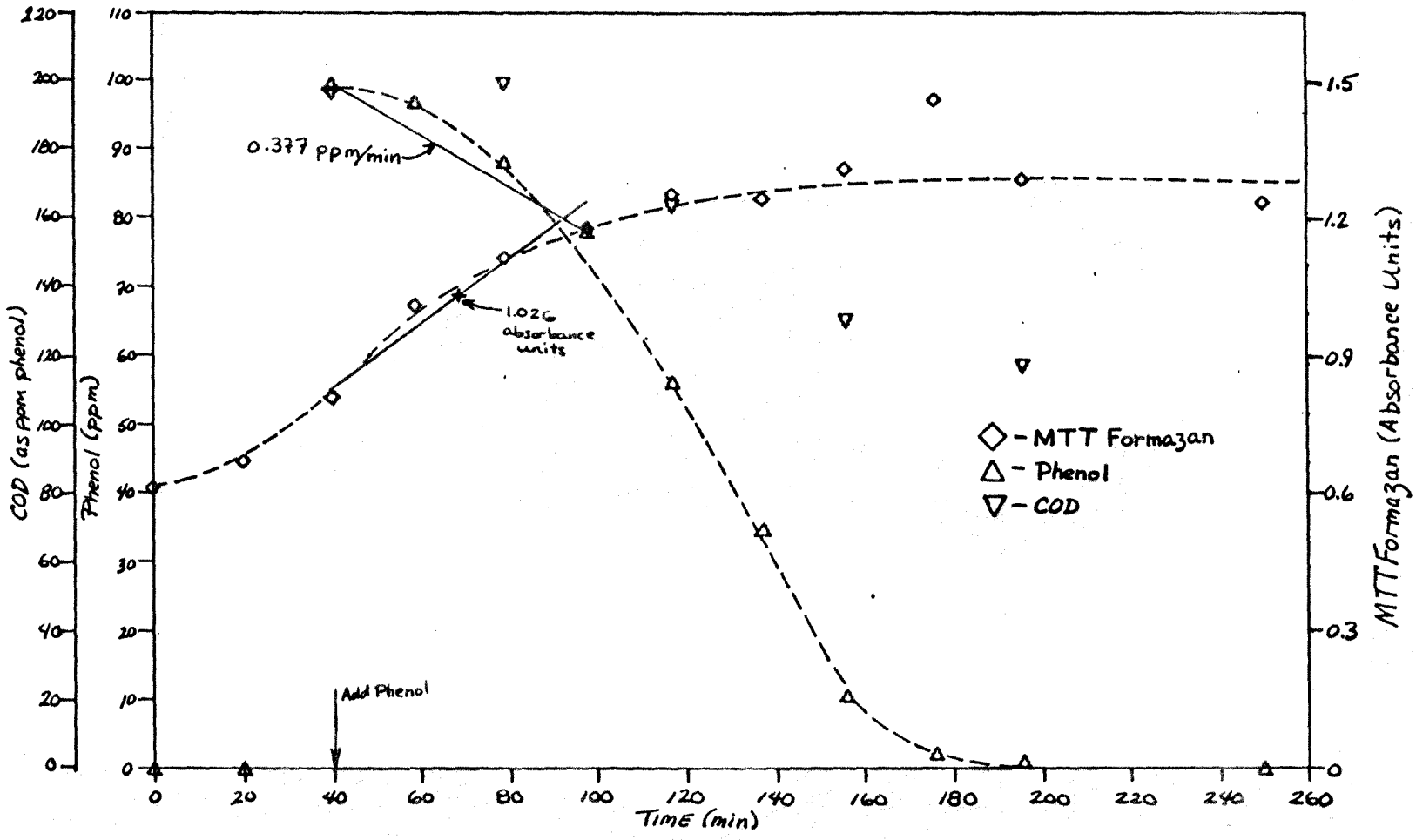


FIGURE B-10 PHENOL/LIVINGSTON SLUDGE III - RUN 2
SUBSTRATE, COD, MTT FORMAZAN

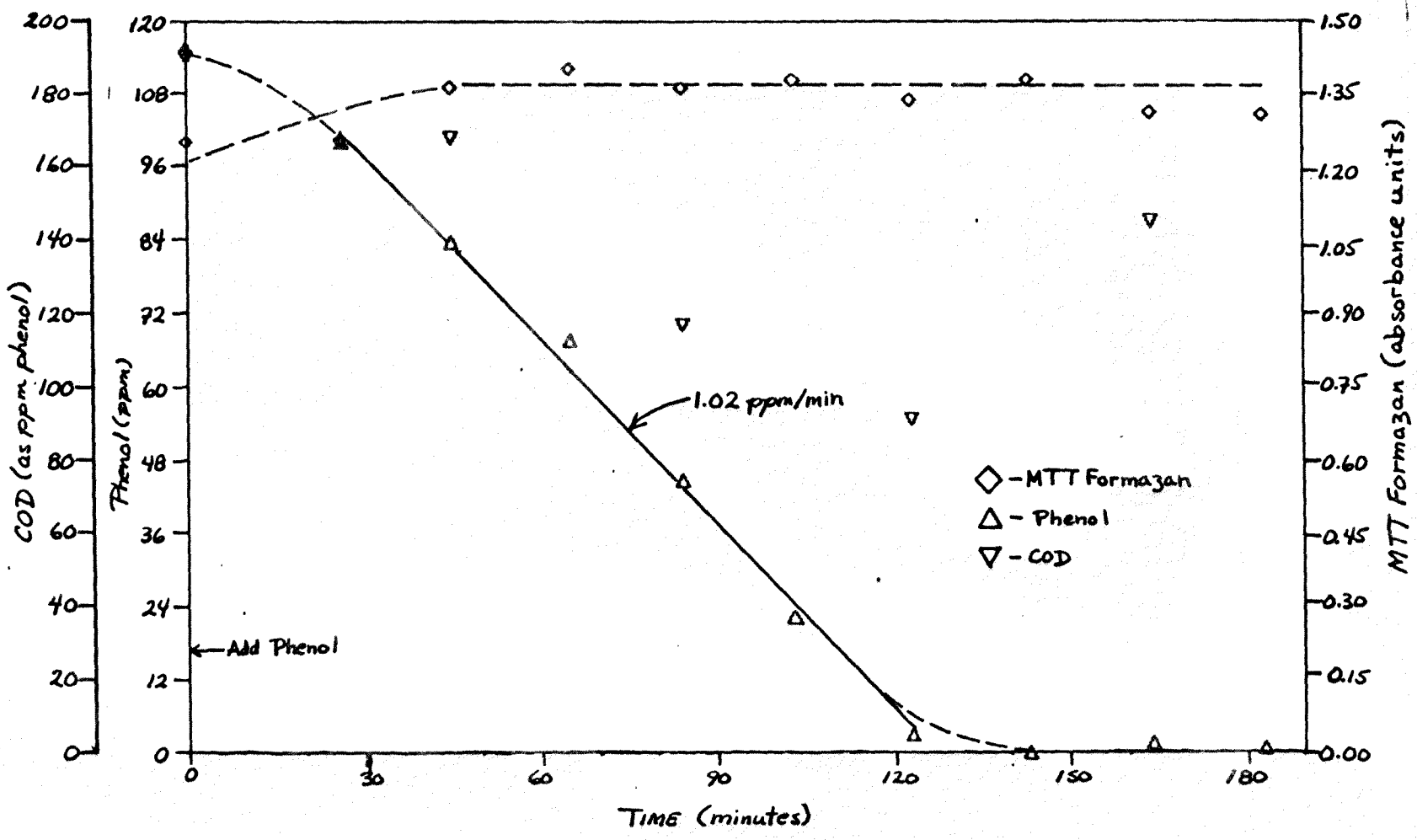


FIGURE B-11 PHENOL/HYDROBAC I - RUN 3
SUBSTRATE, MTT FORMAZAN

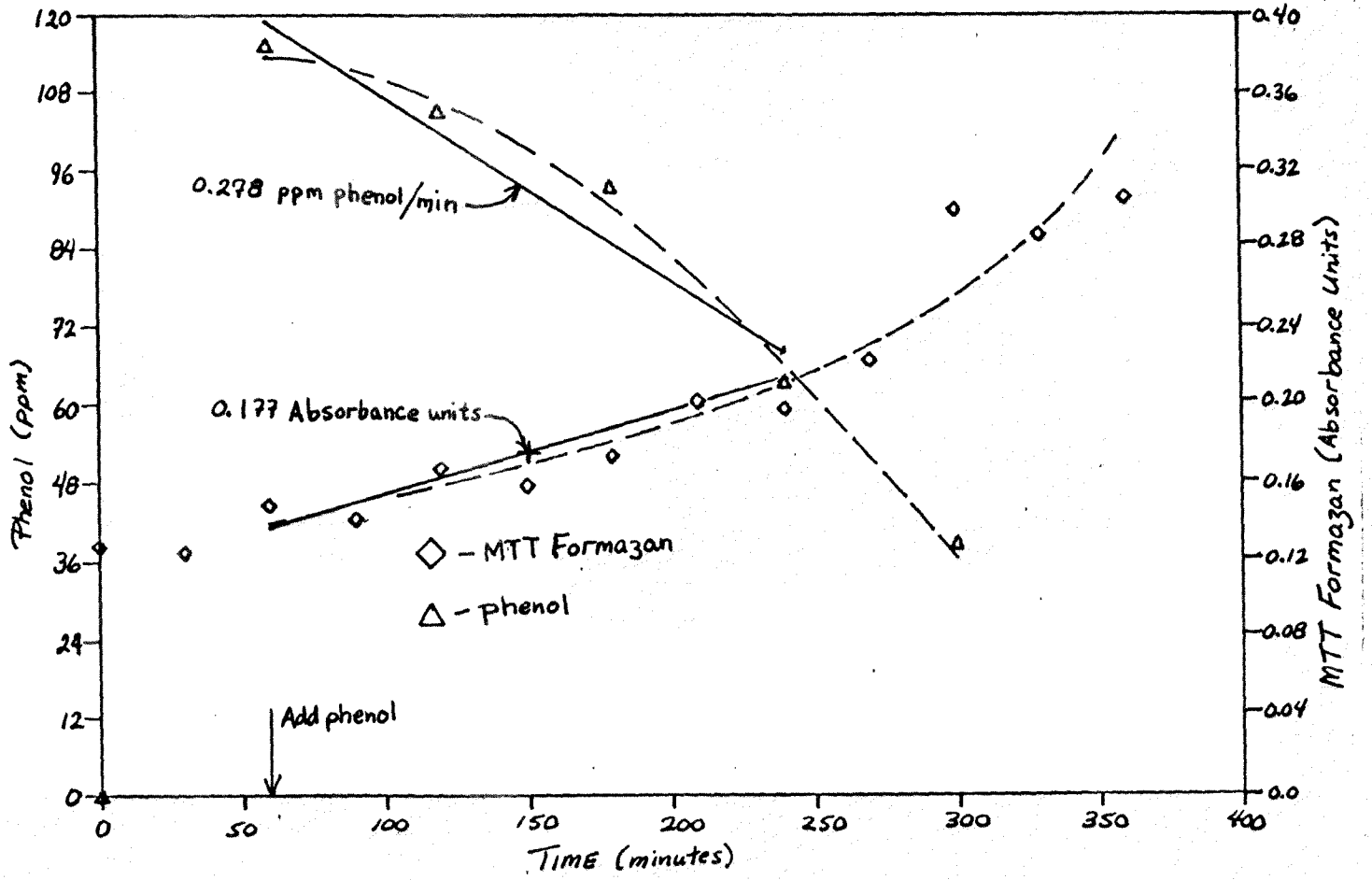


FIGURE B-12

PHENOL / HYDROBAC II - RUN 1
SUBSTRATE, COD, MTT FORMAZAN

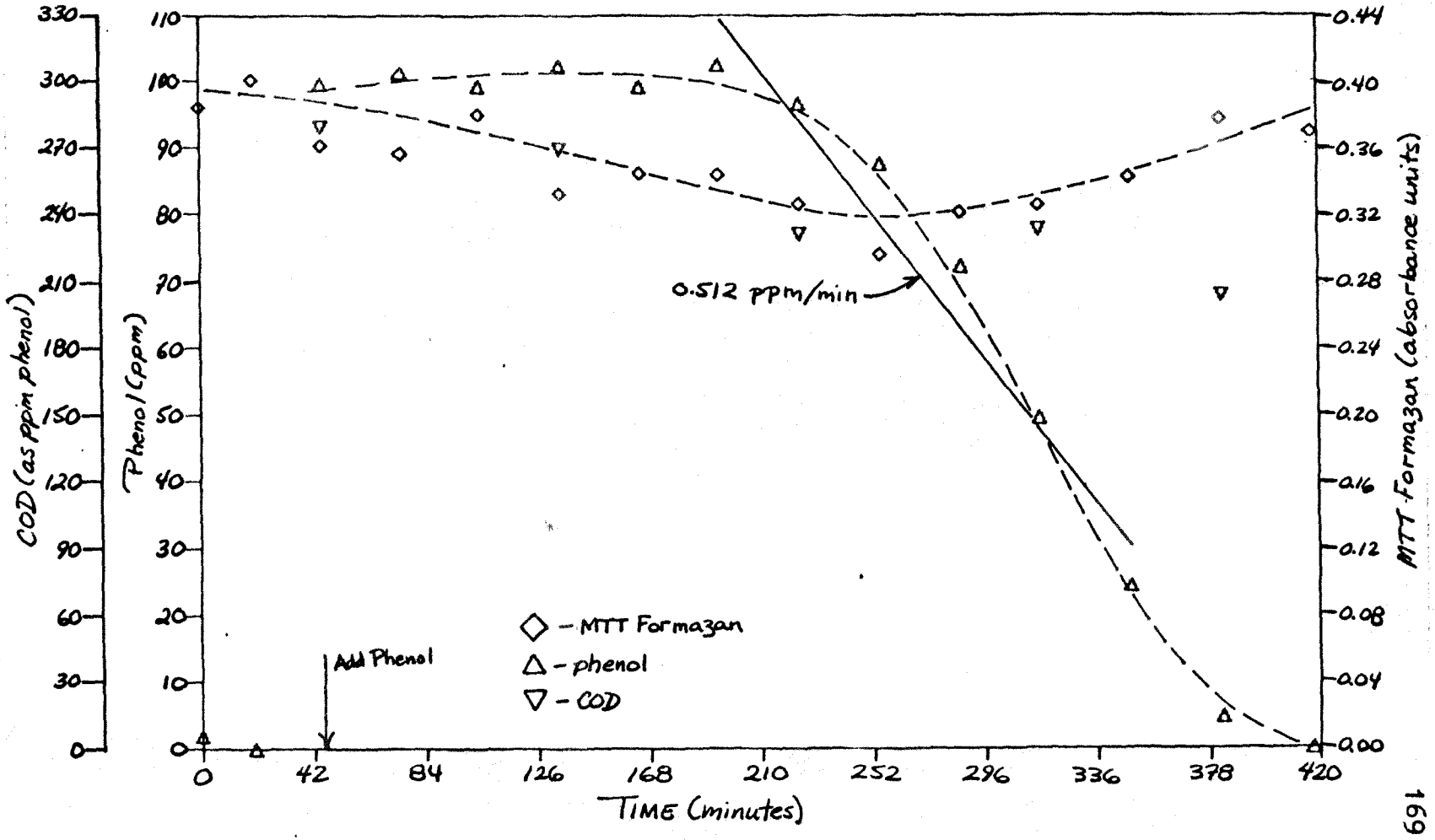


FIGURE B-13

PHENOL / HYDROBAC II - RUN 2
SUBSTRATE, COD, MTT FORMAZAN

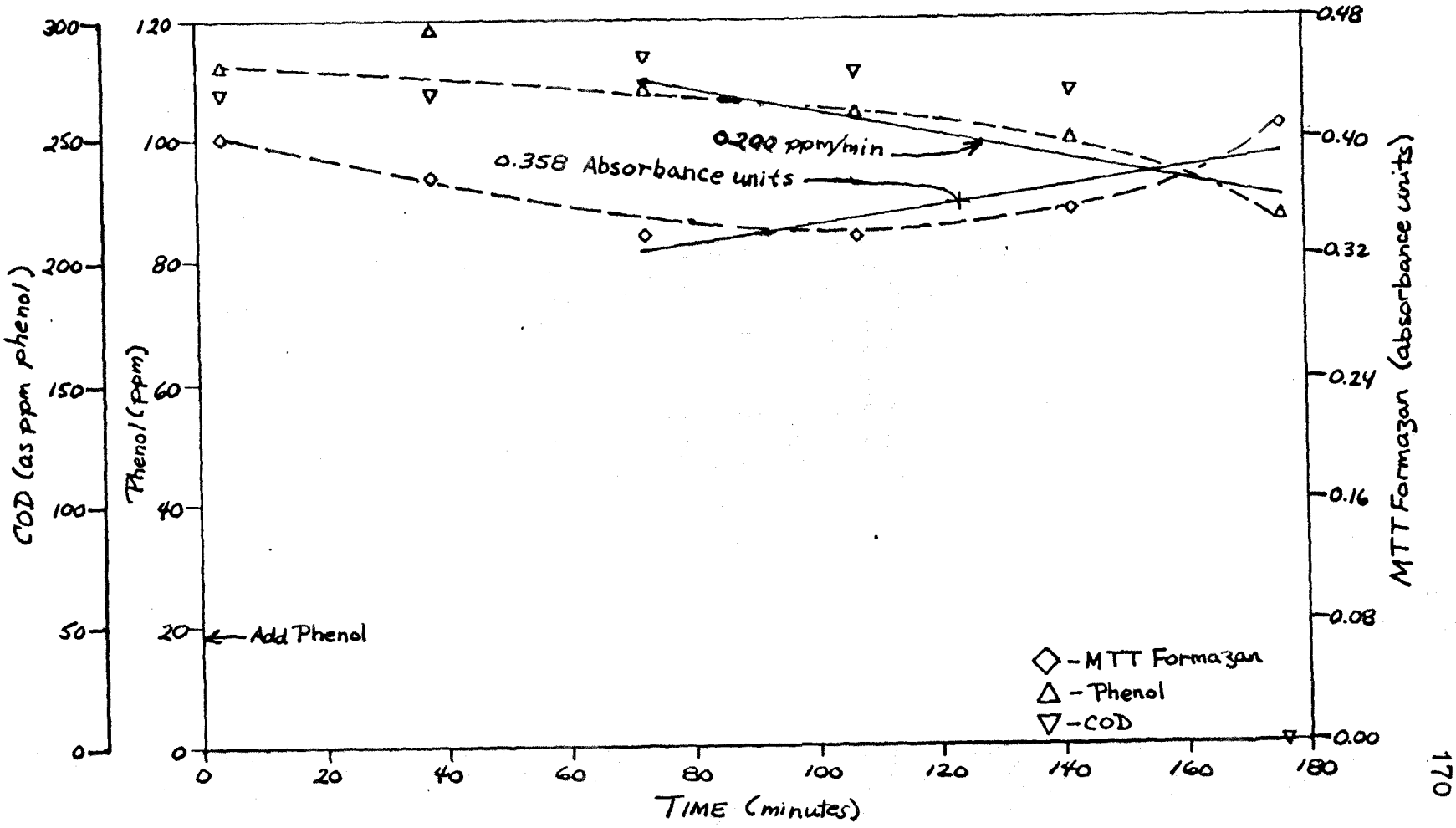


FIGURE B-14

PHENOL/LLMO - RUN 1
SUBSTRATE, COD, MTT FORMAZAN

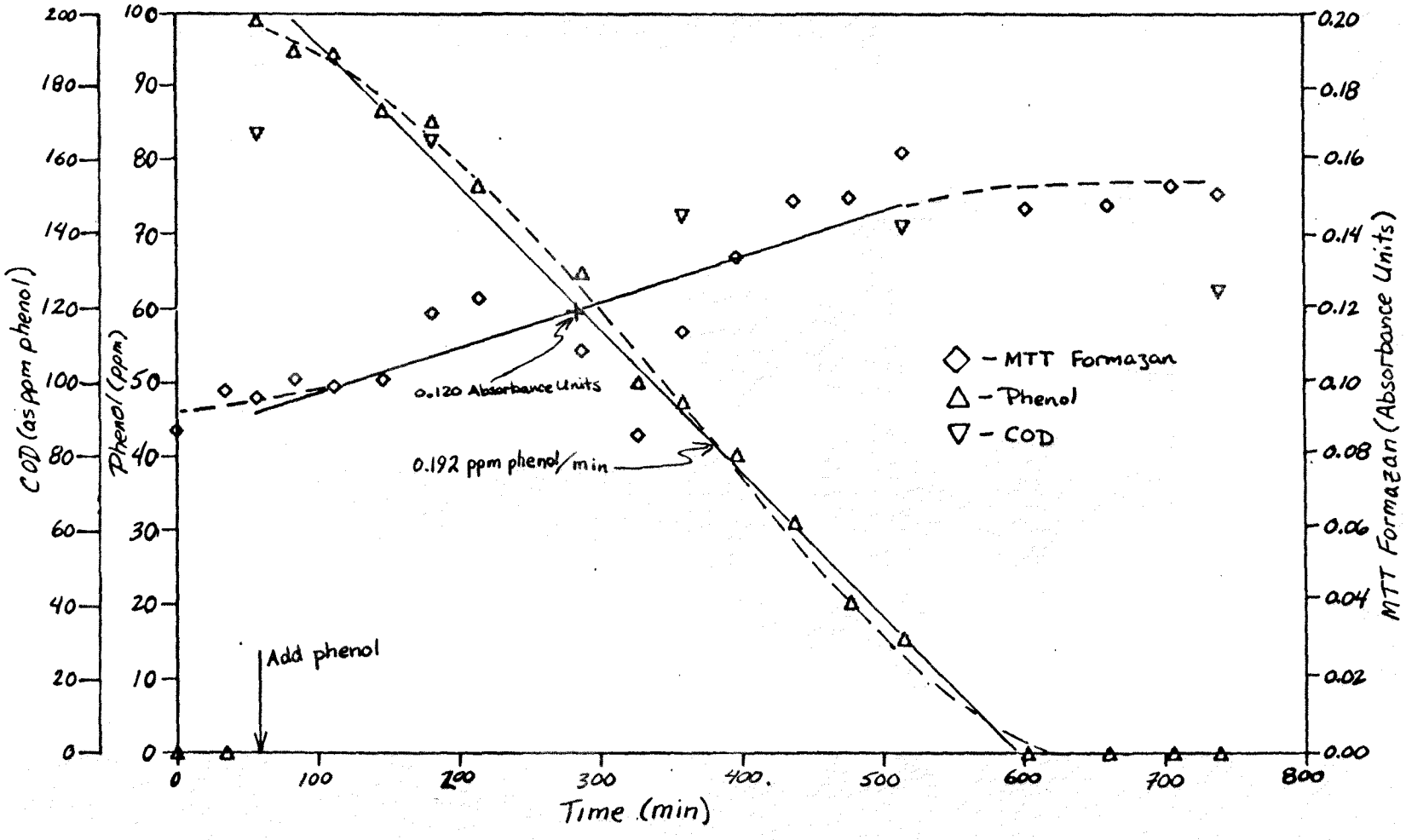


FIGURE B-15 PHENOL/LIVINGSTON SLUDGE I - RUN 3

OXYGEN UPTAKE RATE, MTT FORMAZAN

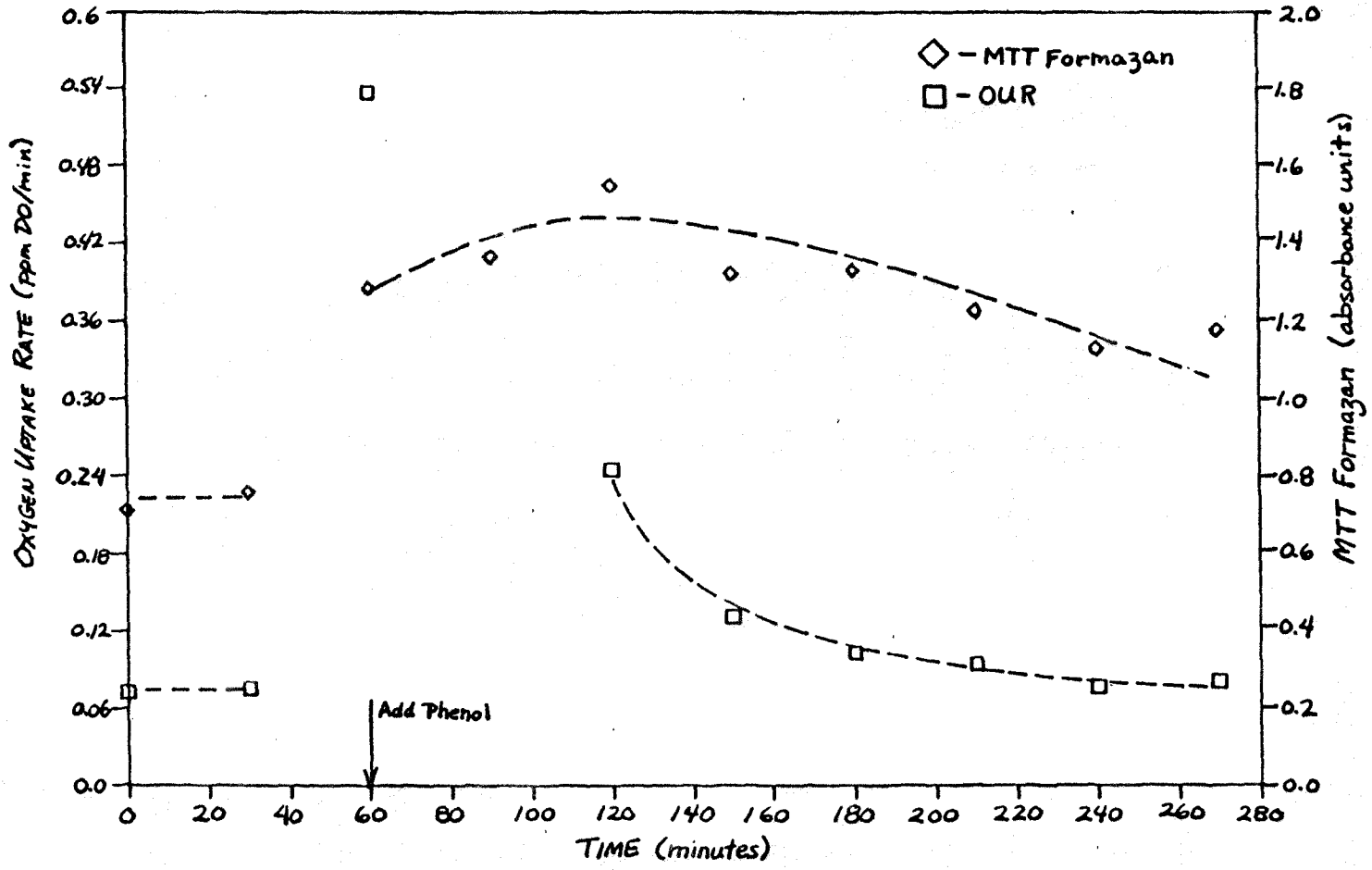


FIGURE B-16 PHENOL / LIVINGSTON SLUDGE III - RUN 1
OXYGEN UPTAKE RATE, MTT FORMAZAN

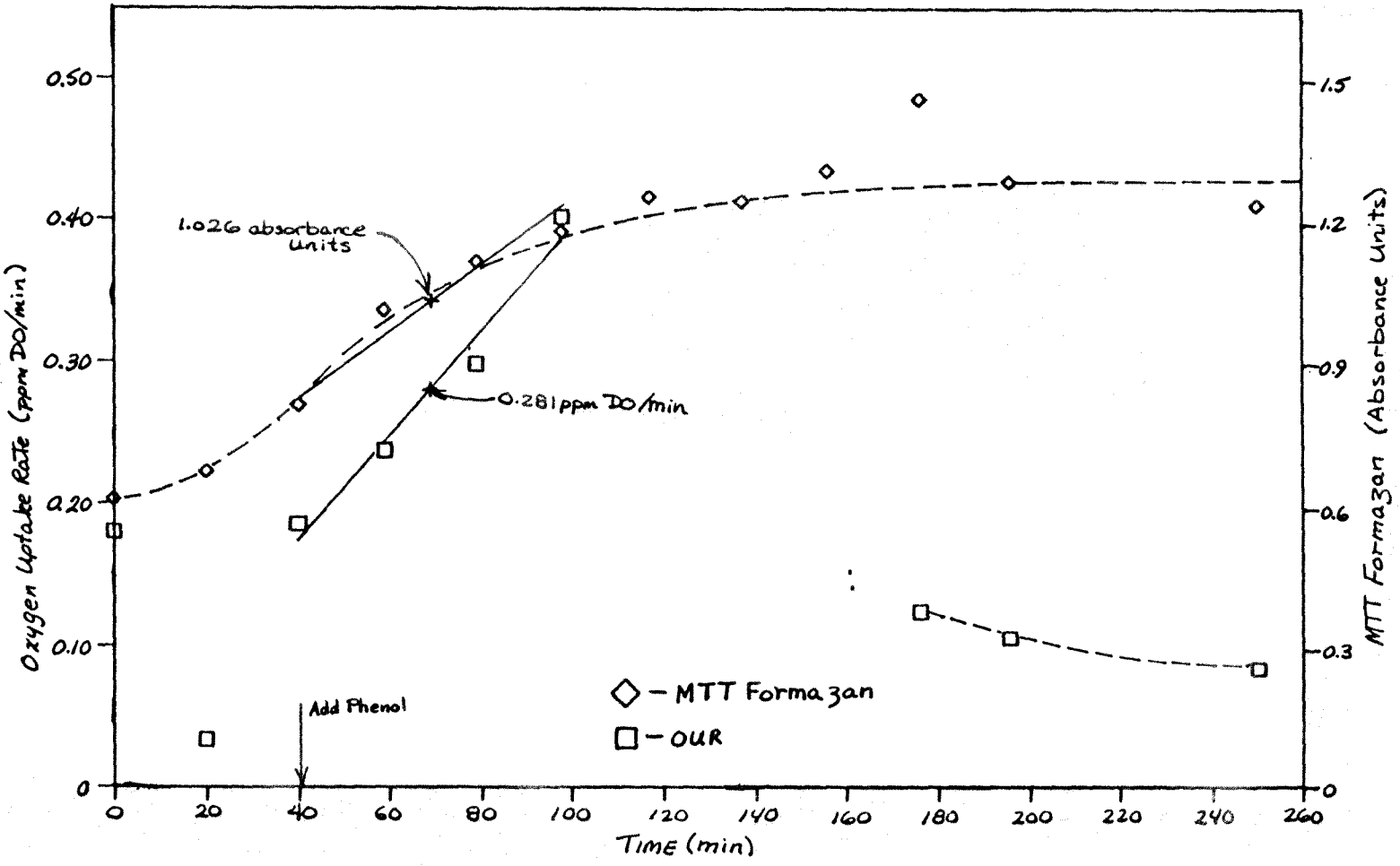


FIGURE B-17 PHENOL/LIVINGSTON SLUDGE III - RUN 2
OXYGEN UPTAKE RUN, MTT FORMAZAN

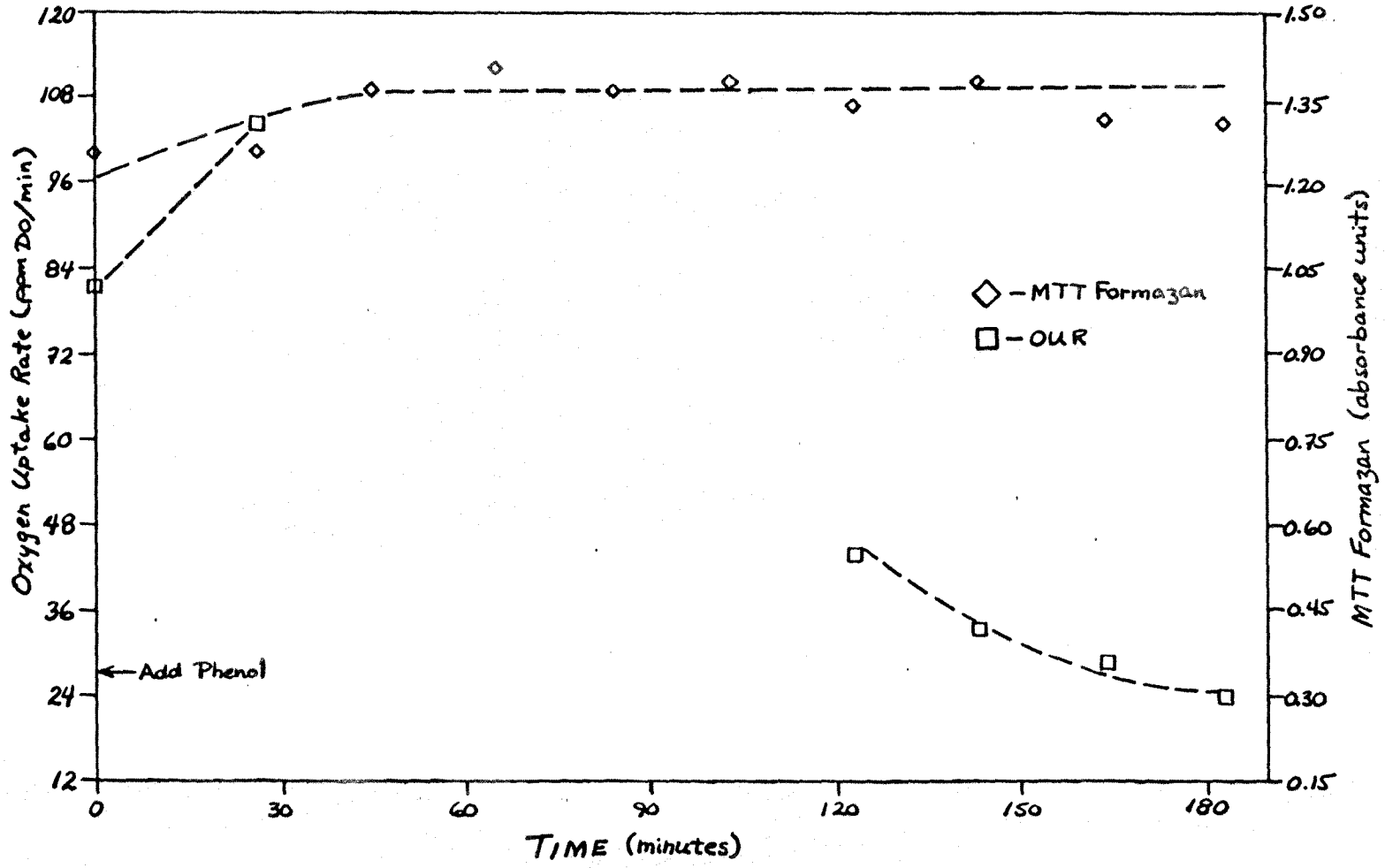


FIGURE B-18 PHENOL / HYDROBAC I - RUN 3
OXYGEN UPTAKE RATE, MTT FORMAZAN

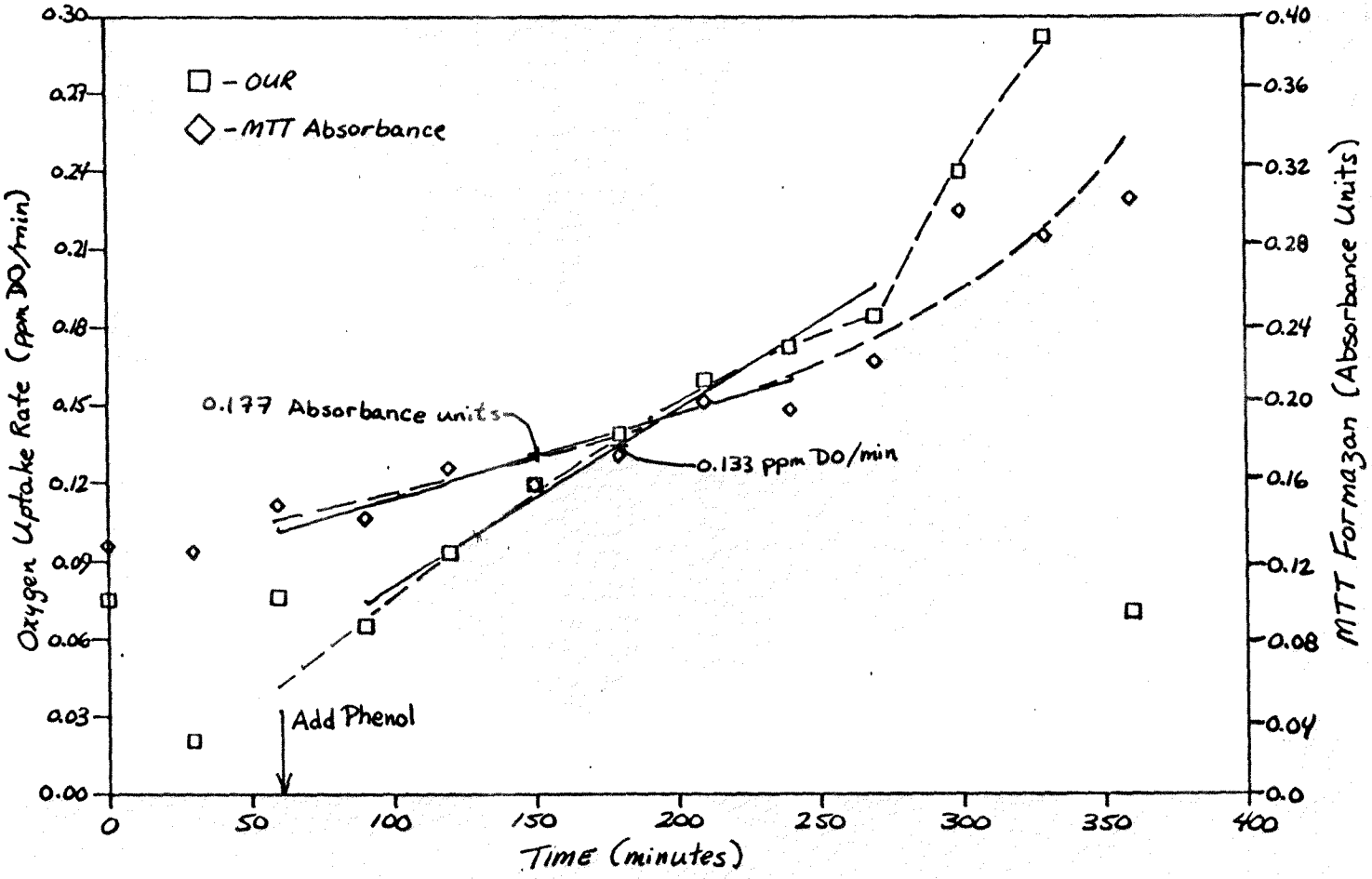


FIGURE B-19 PHENOL / HYDROBAC II - RUN 1
OXYGEN UPTAKE RATE, MTT FORMAZAN

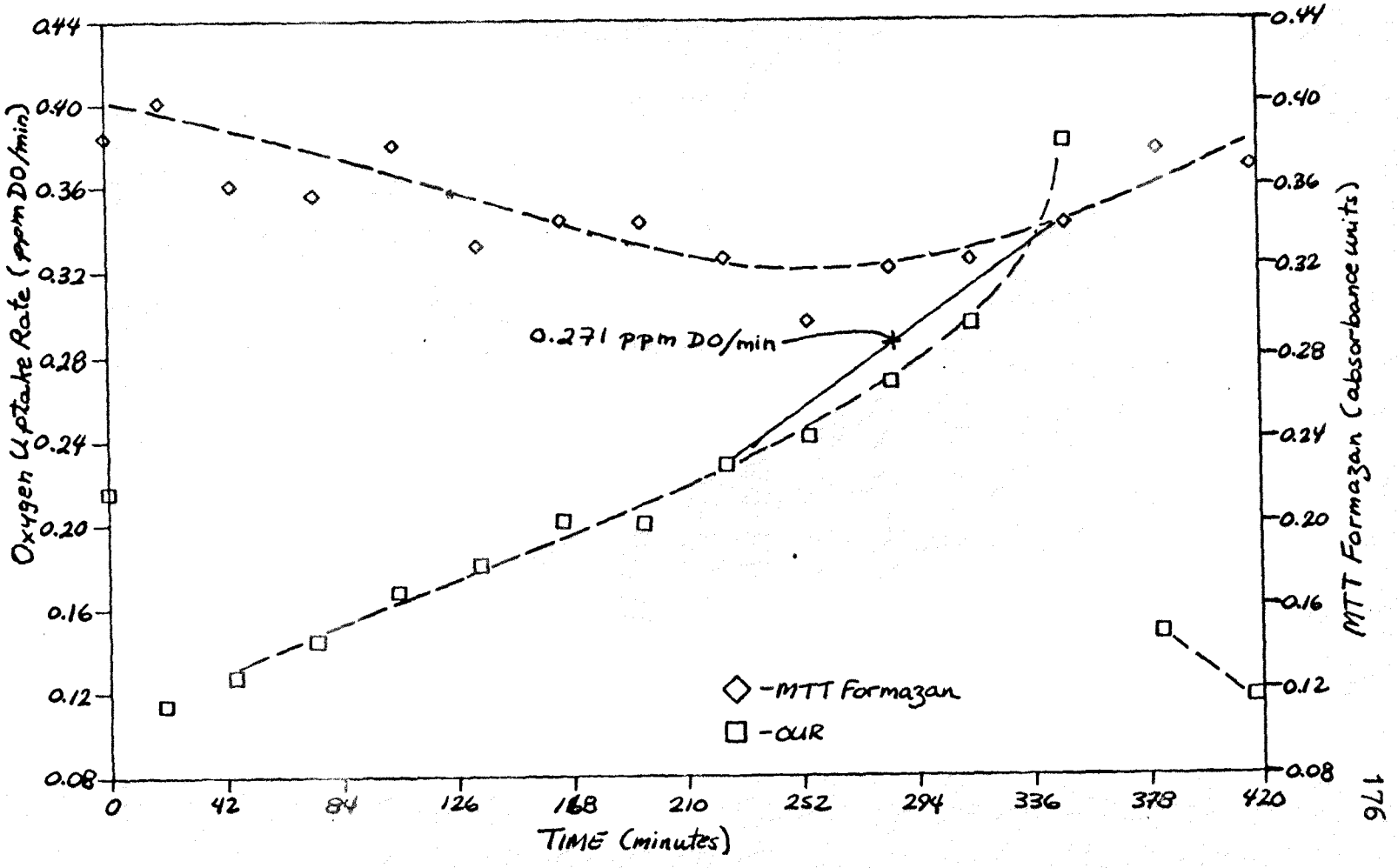


FIGURE B-20 PHENOL / HYDROBAC II - RUN 2
OXYGEN UPTAKE RATE, MTT FORMAZAN

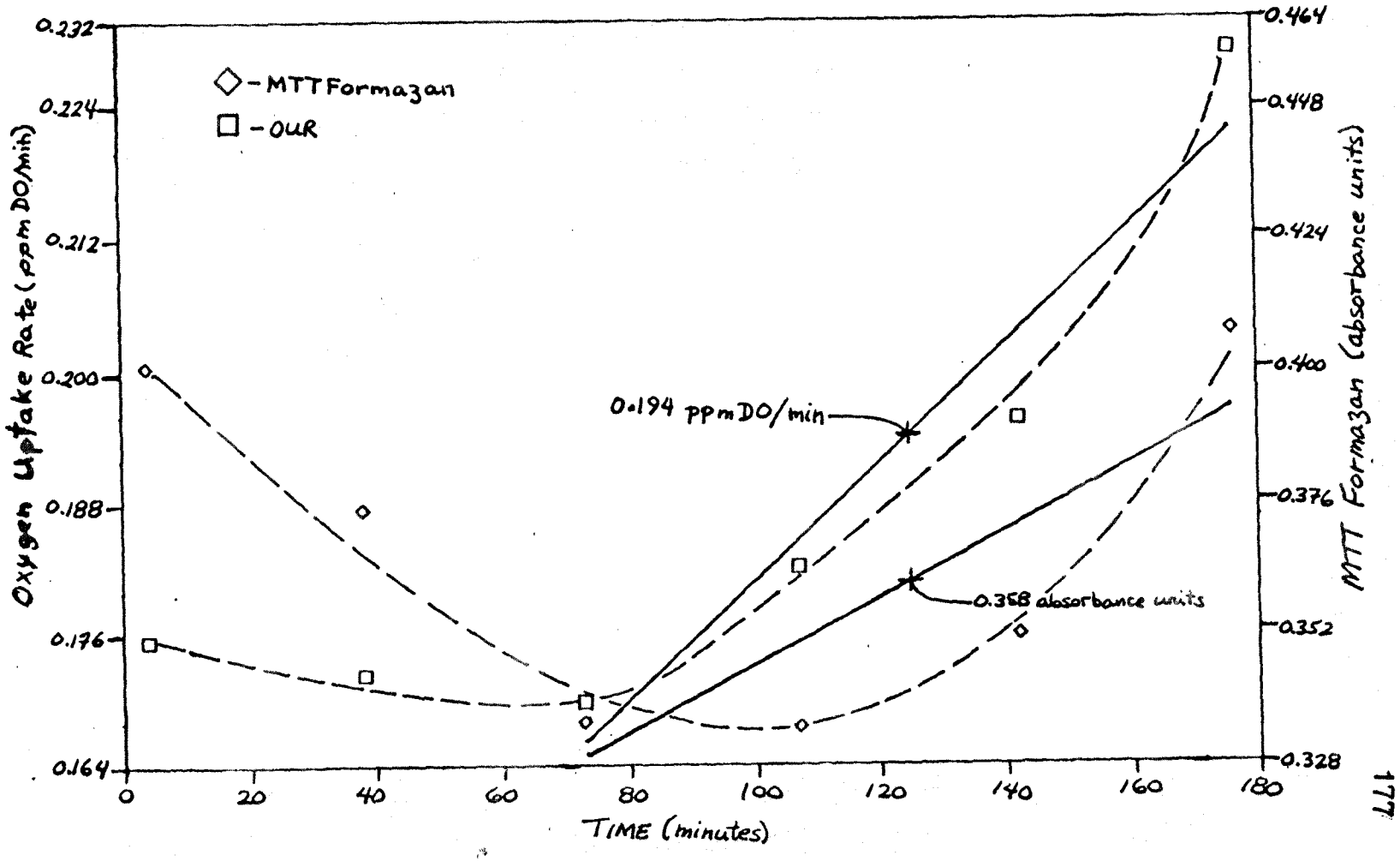


FIGURE B-21

PHENOL / LLMO - RUN 1
OXYGEN UPTAKE RATE, MTT FORMAZAN

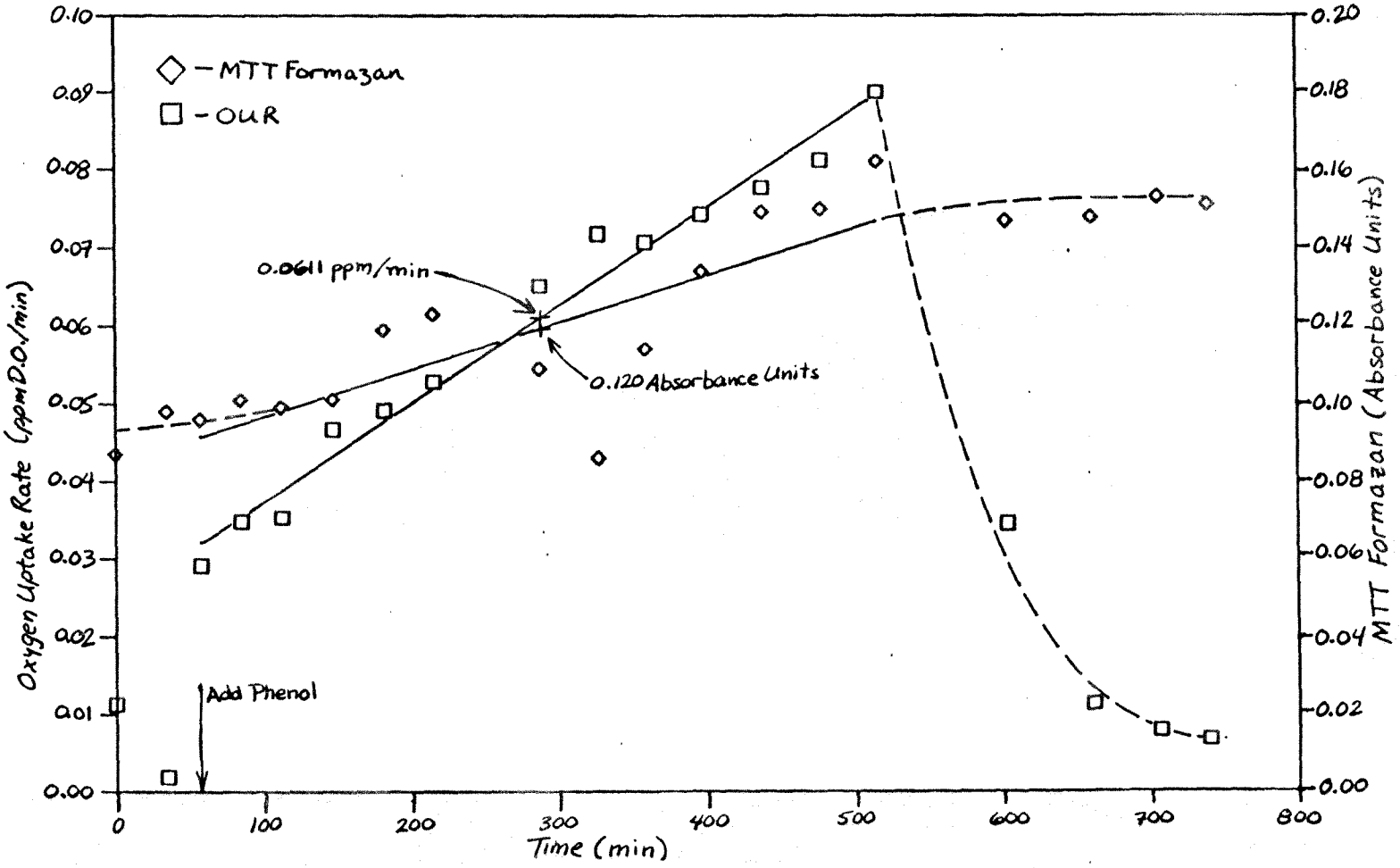


FIGURE B-22 o-CHLOROPHENOL / LIVINGSTON SLUDGE II - RUN 3
SUBSTRATE, COD, OXYGEN UPTAKE RATE

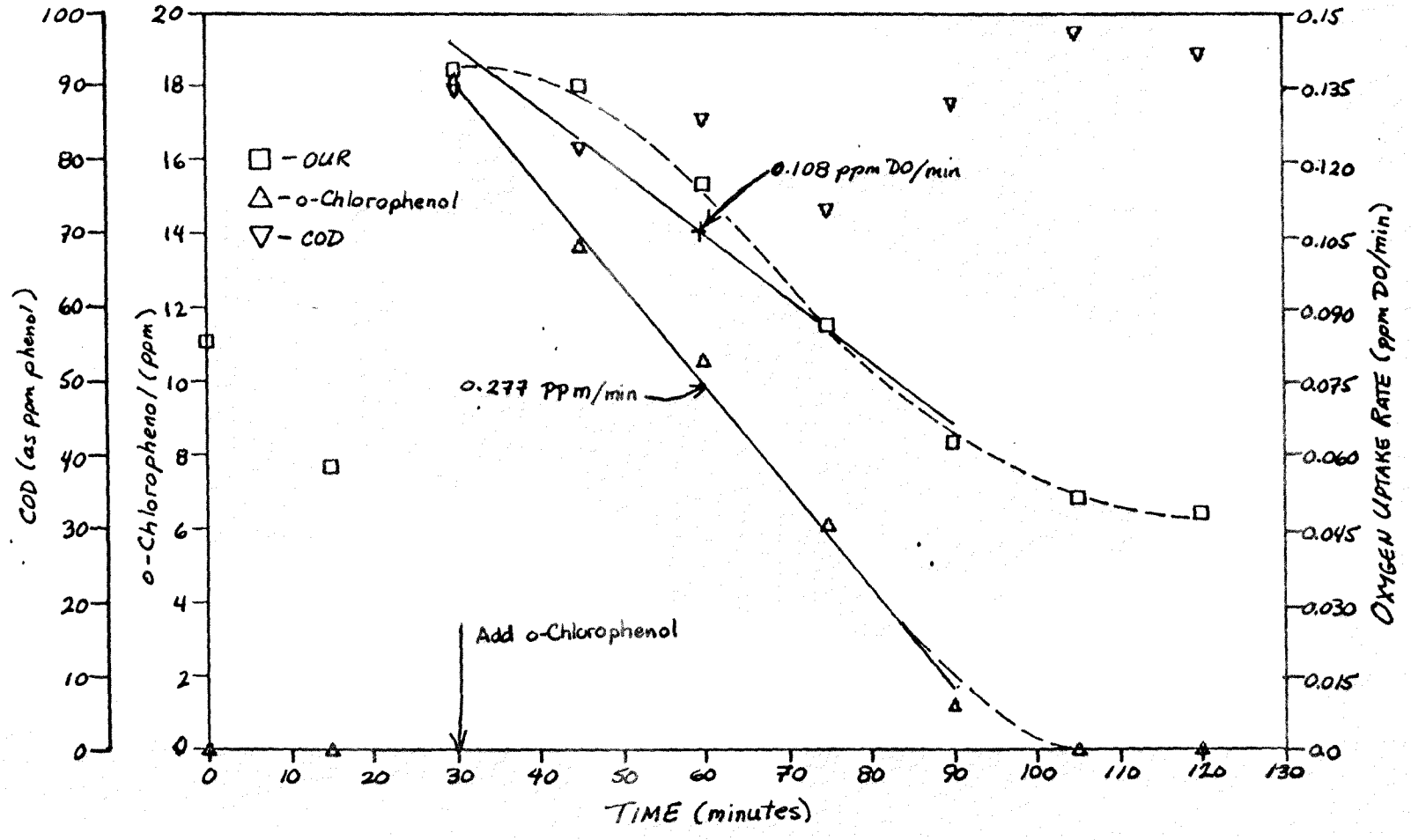


FIGURE B-23 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 1
SUBSTRATE, COD, OXYGEN UPTAKE RATE

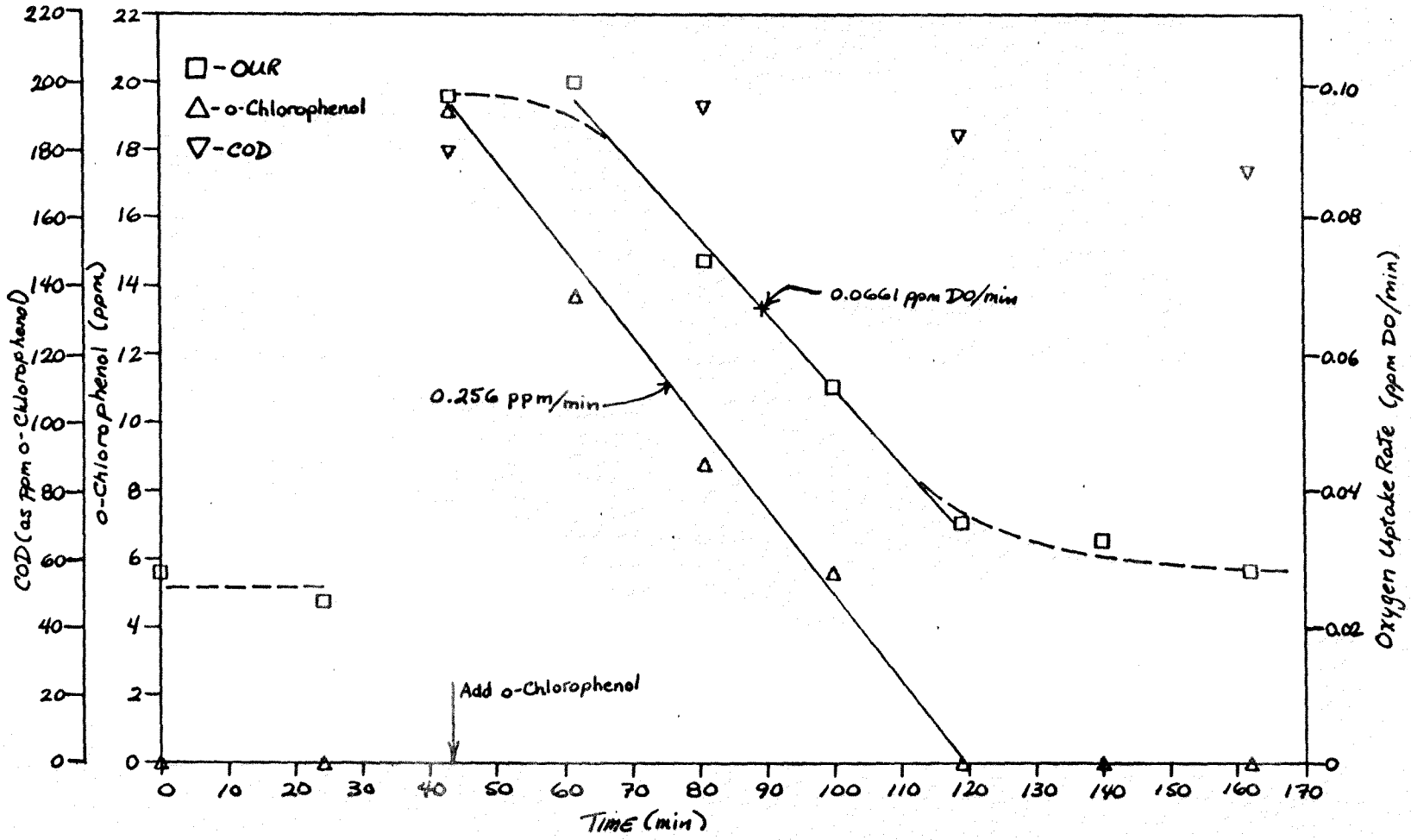


FIGURE B-24 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 2
SUBSTRATE, COD, OXYGEN UPTAKE RATE

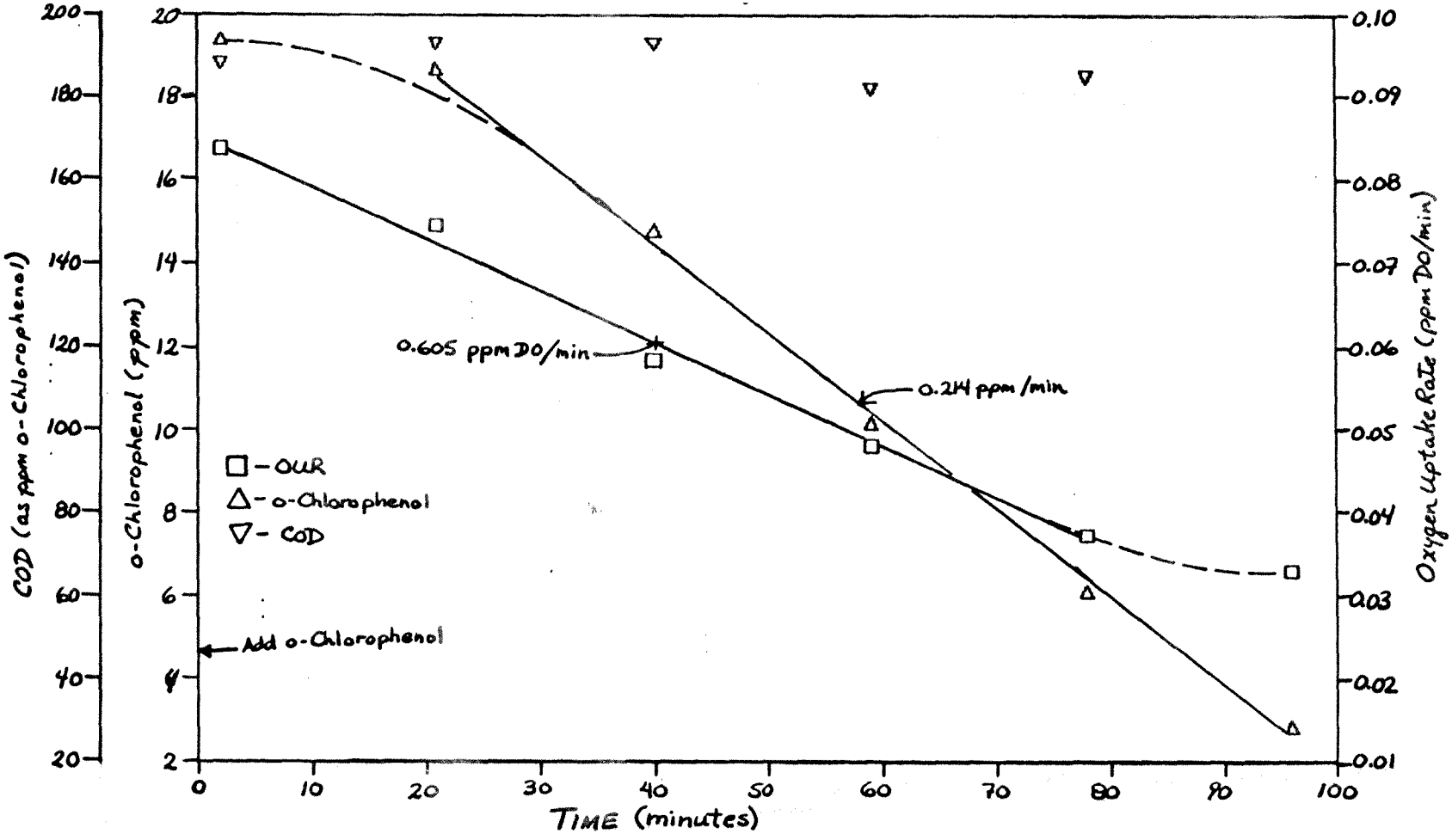


FIGURE B-25 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 3
SUBSTRATE, COD, OXYGEN UPTAKE RATE

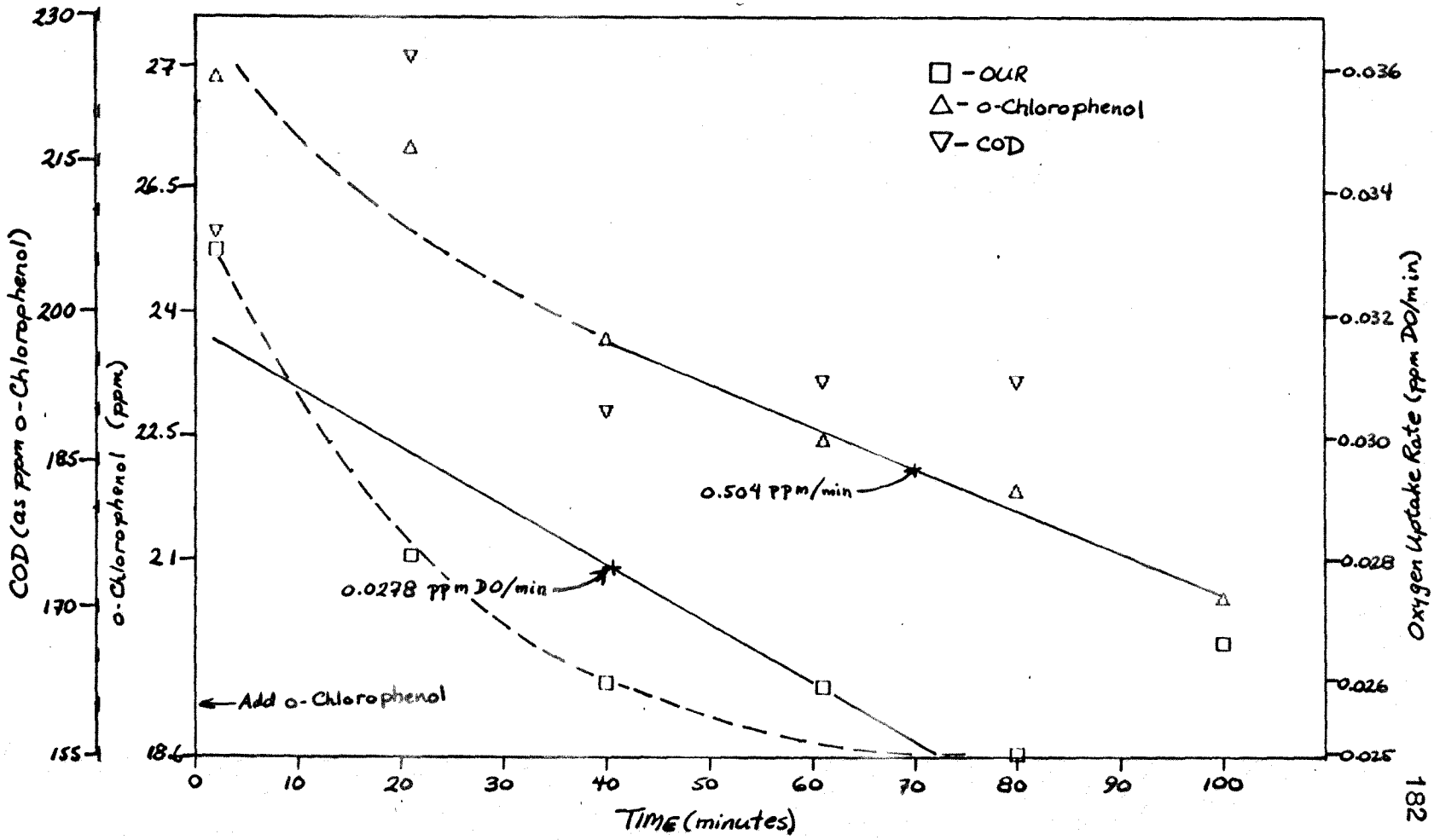


FIGURE B-26 PHENOL / HYDROBAC III - RUN 1
SUBSTRATE, COD, OXYGEN UPTAKE RATE

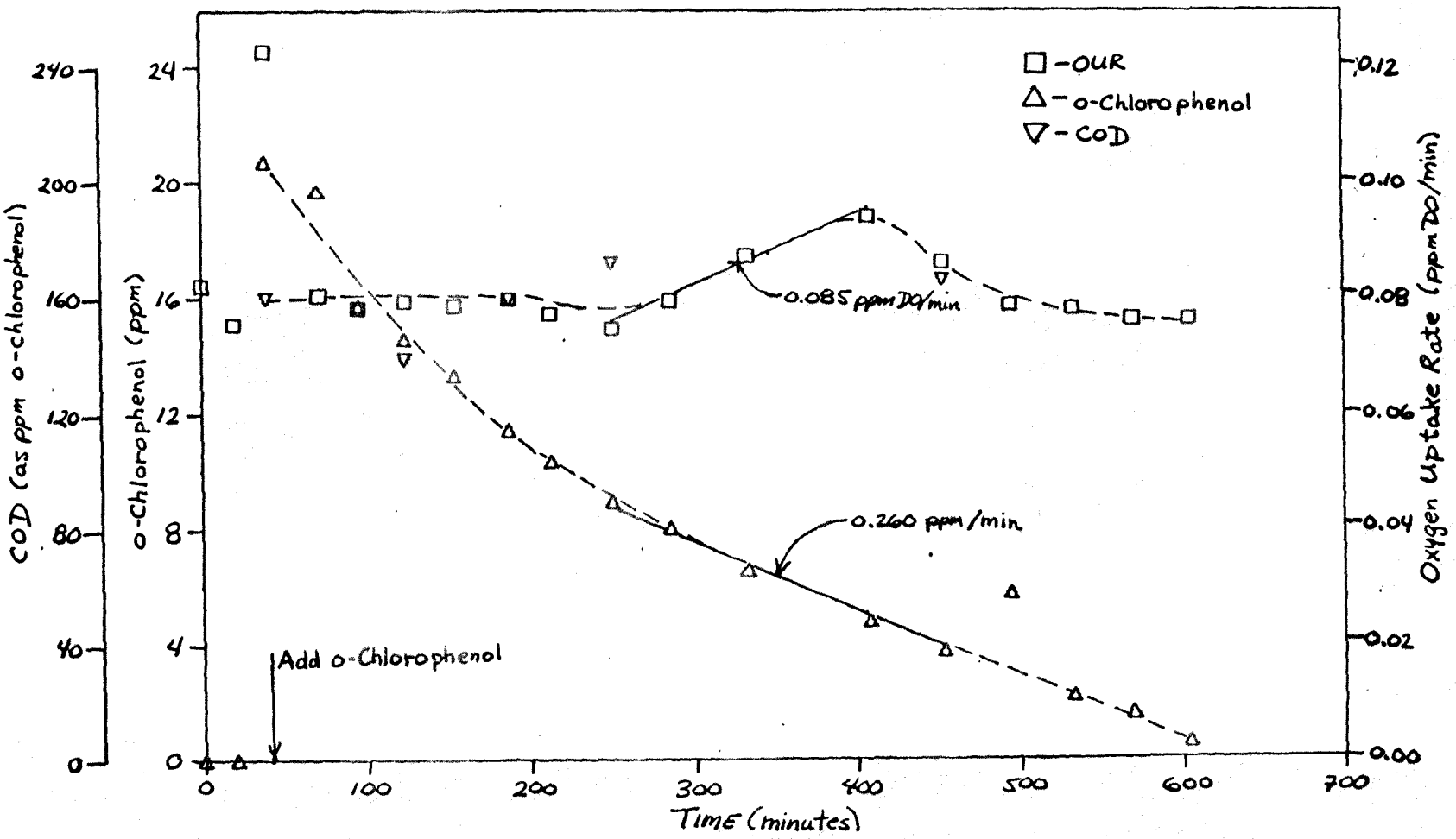


FIGURE B-27 o-CHLOROPHENOL / HYDROBAC III - RUN 2
SUBSTRATE, COD, OXYGEN UPTAKE RATE

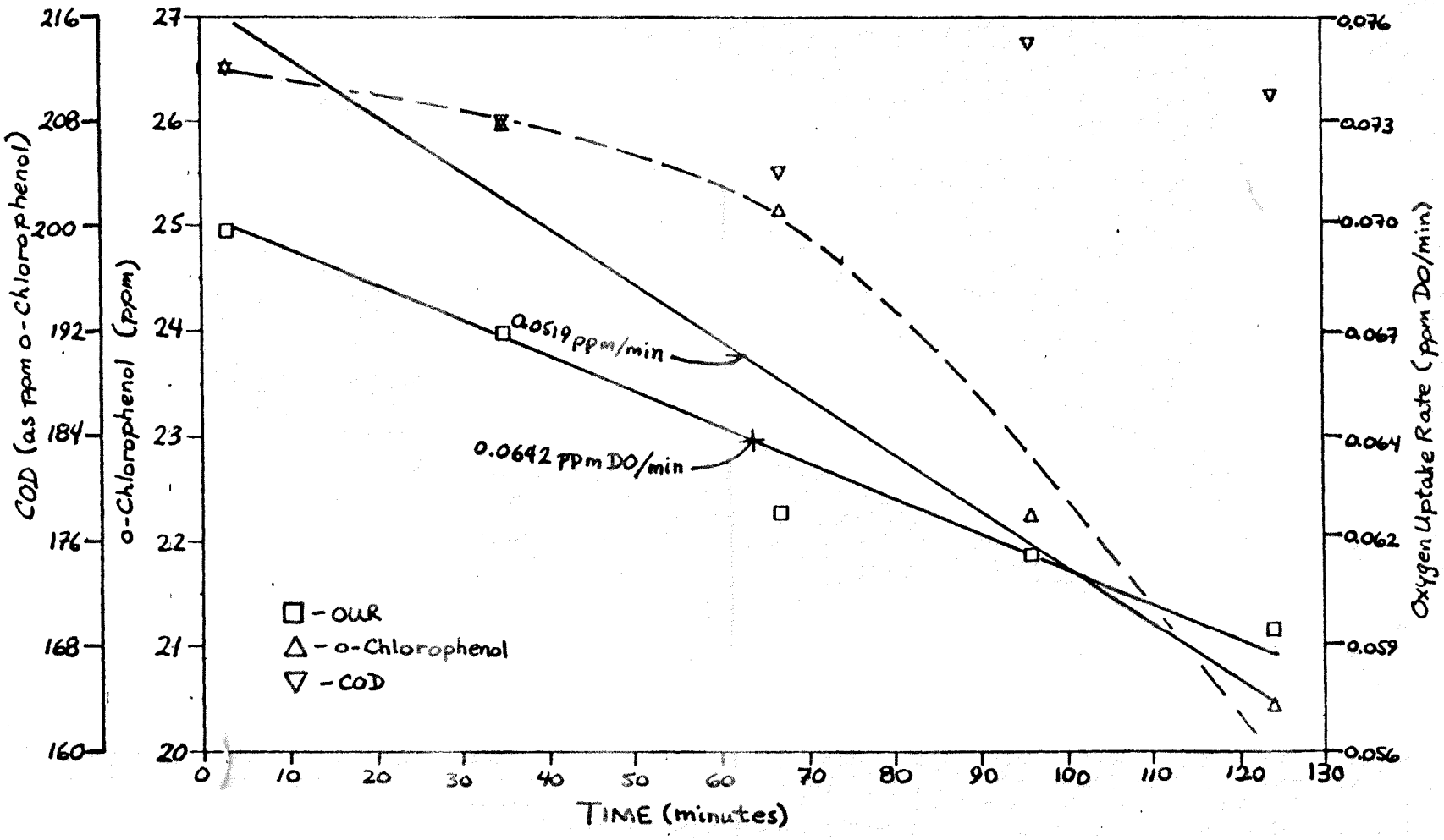


FIGURE B-28 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 1
SUBSTRATE, COD, MTT FORMAZAN

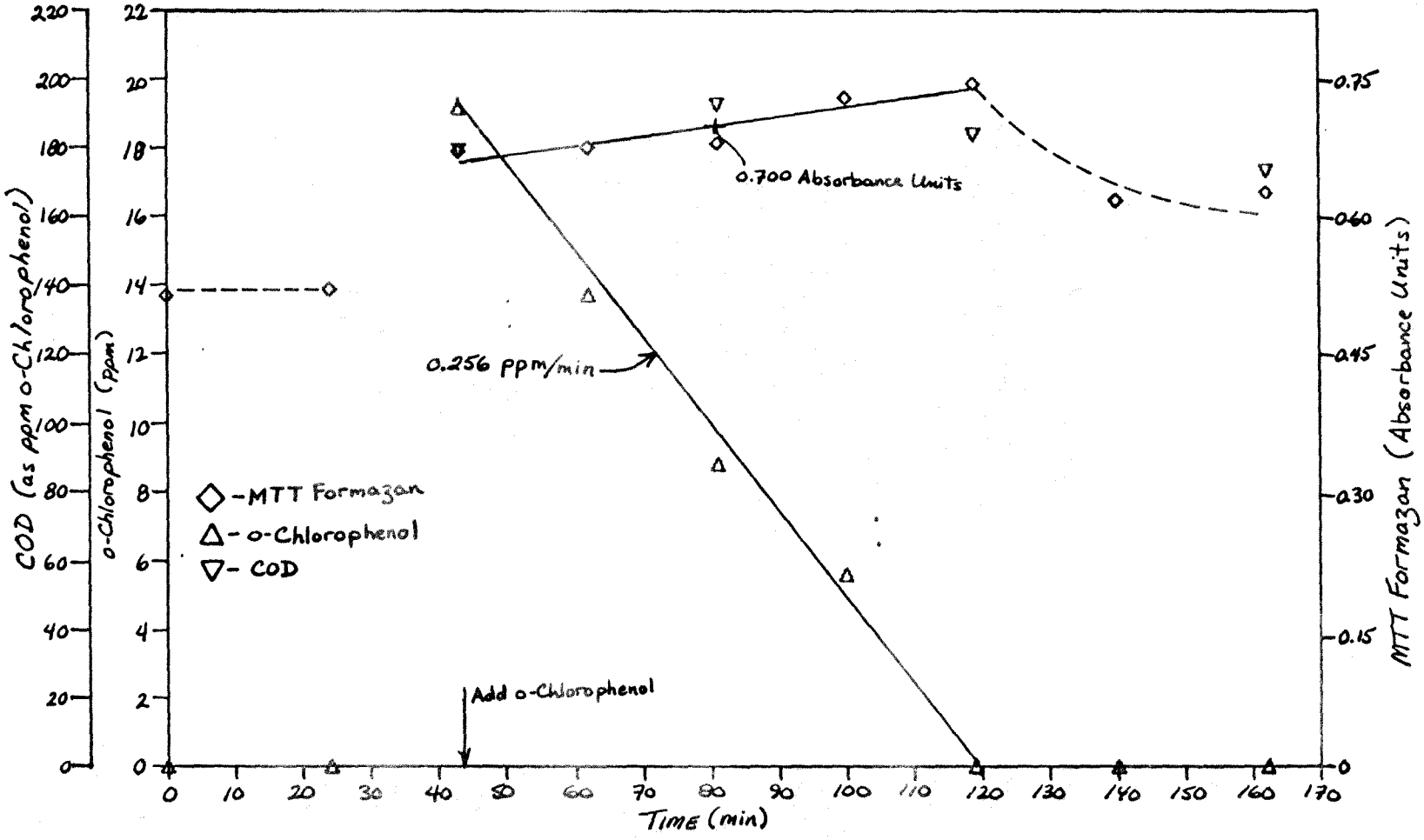


FIGURE B-29 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 2
SUBSTRATE, COD, MTT FORMAZAN

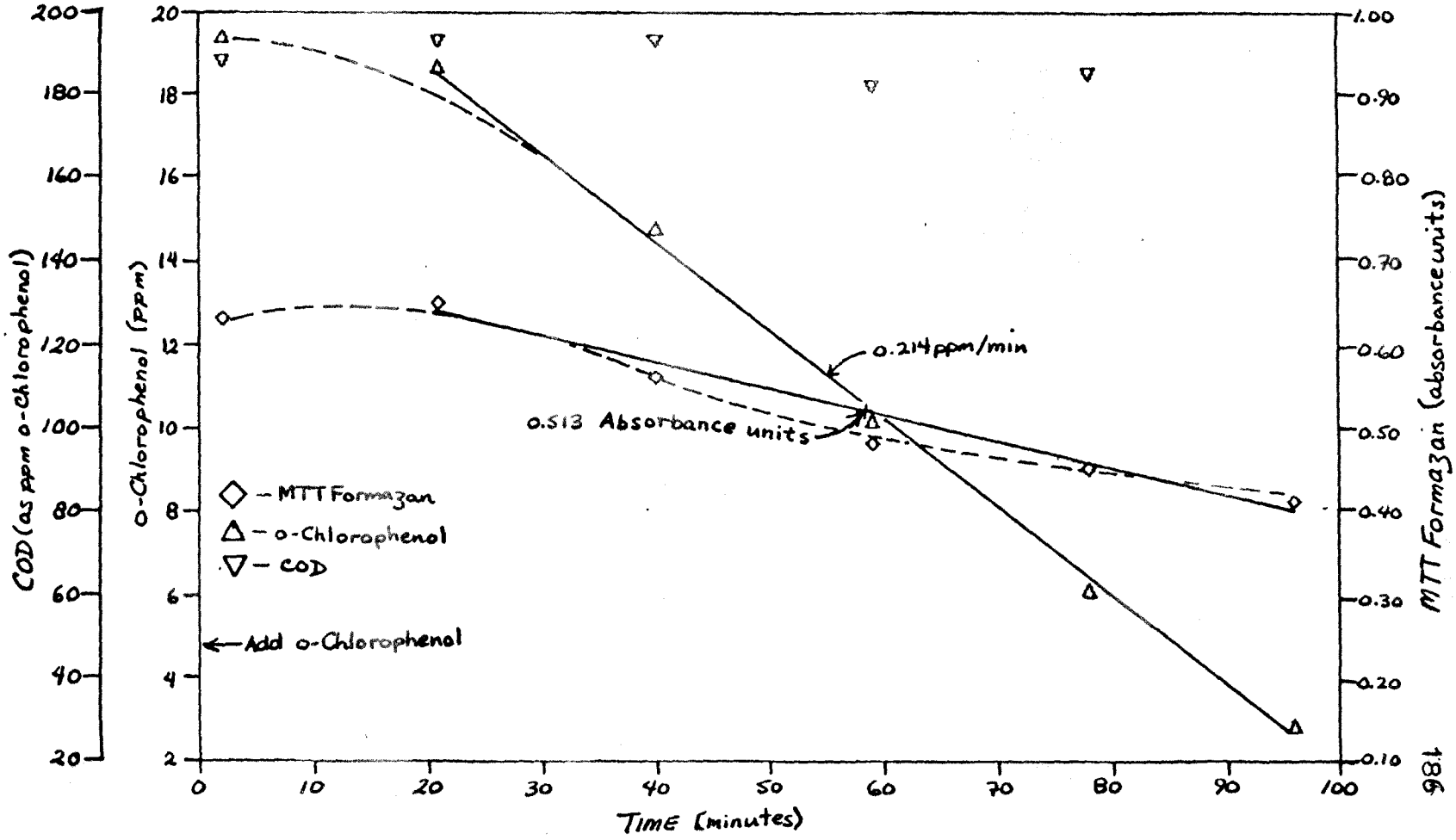


FIGURE B-30 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 3
SUBSTRATE, COD, MTT FORMAZAN

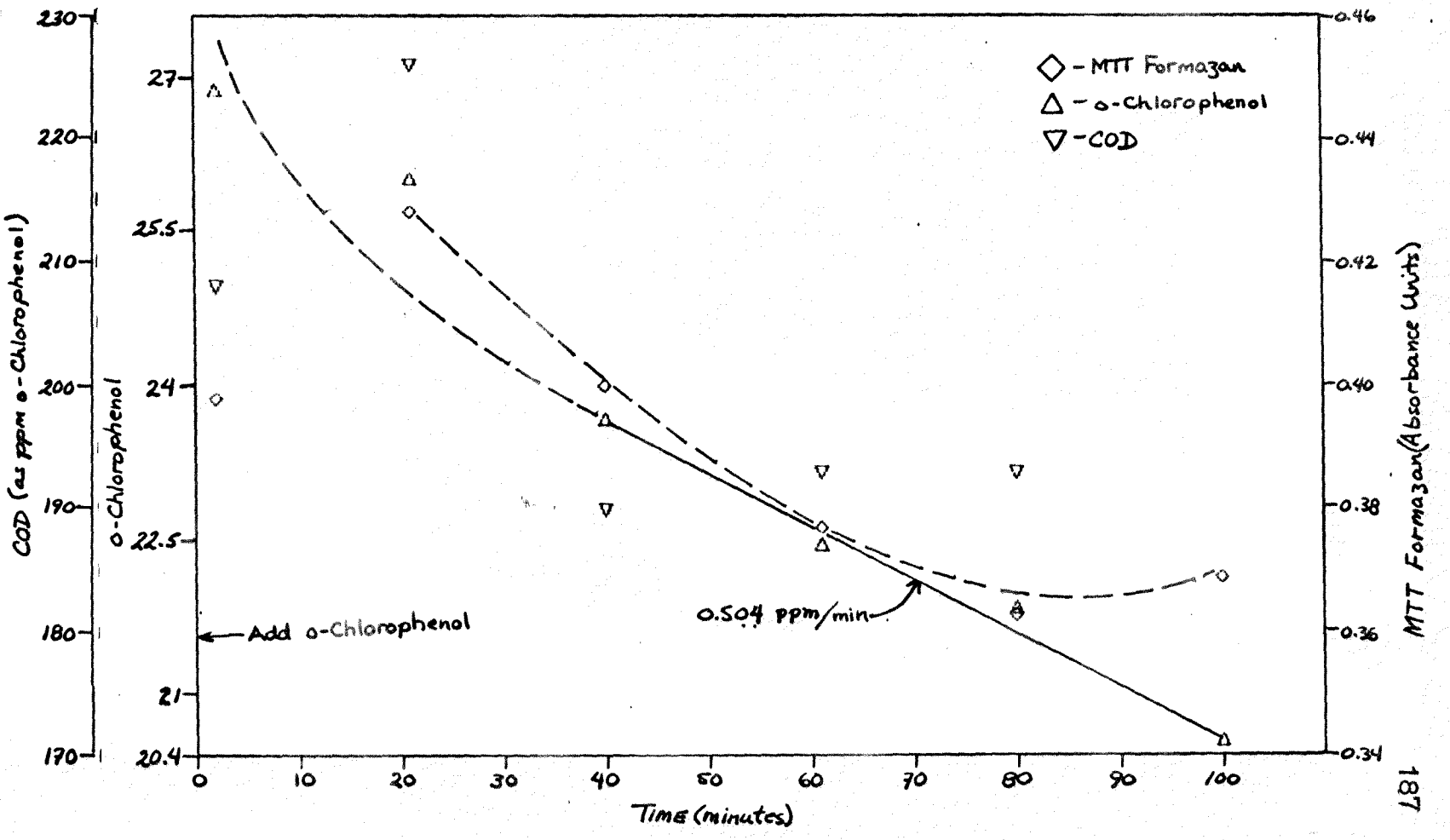


FIGURE B-31 o-CHLOROPHENOL/HYDROBAC III - RUN 1
SUBSTRATE, COD, MTT FORMAZAN

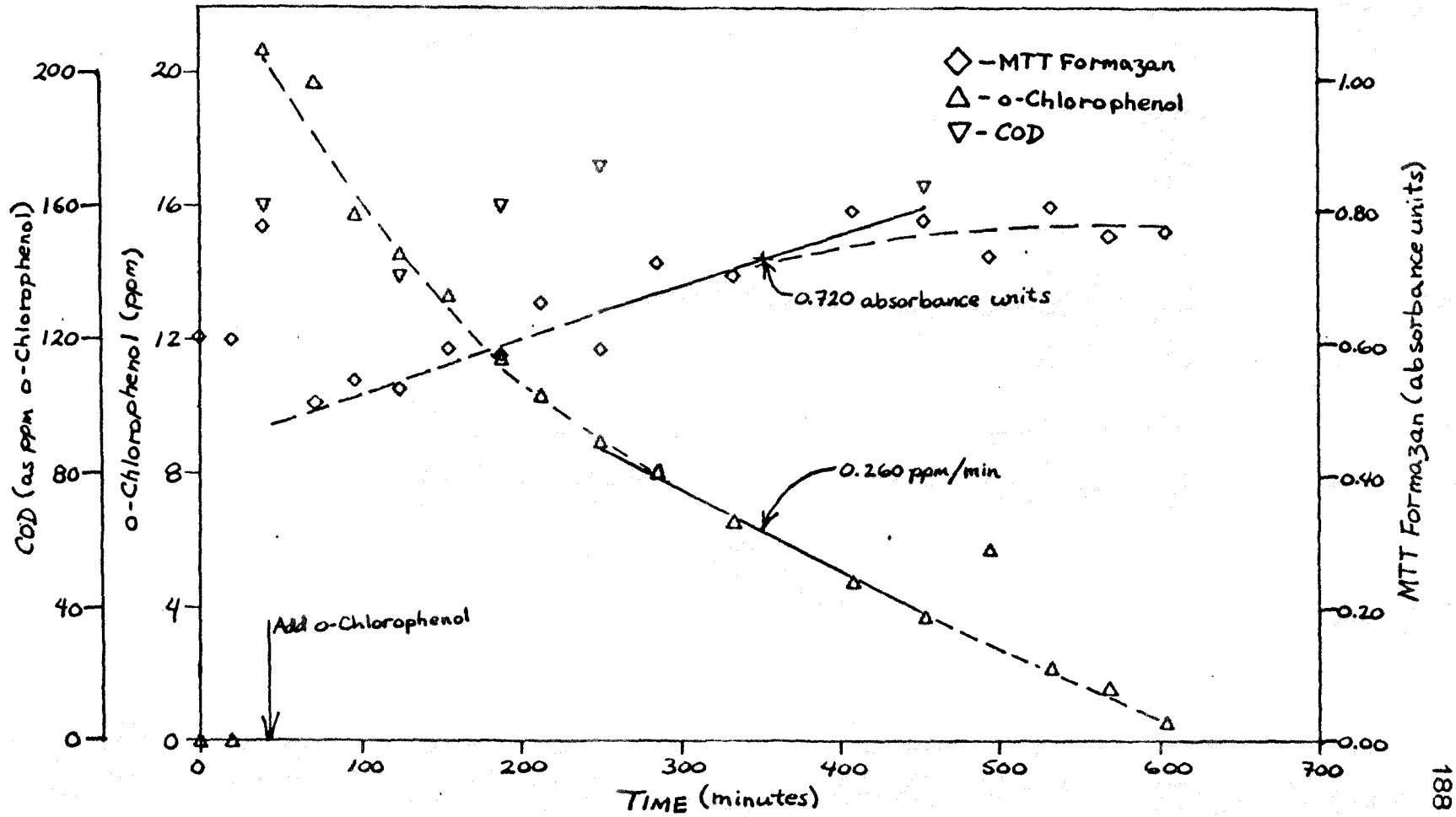


FIGURE B-32 o-CHLOROPHENOL / HYDROBAC III - RUN 2
SUBSTRATE, COD, MTT FORMAZAN

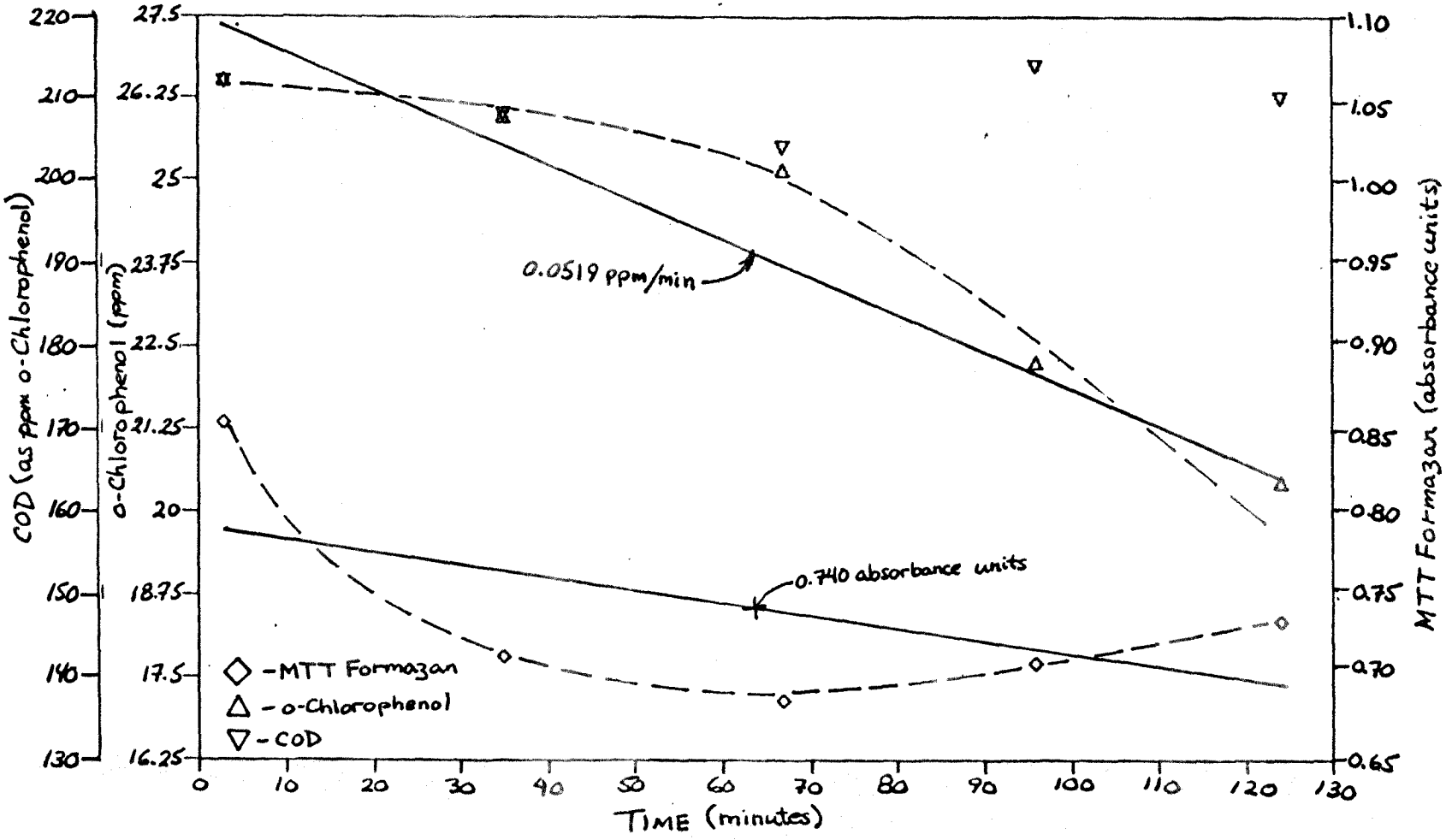


FIGURE B-33 o-CHLOROPHENOL / LIVINGSTON SLUDGE II - RUN 1
OXYGEN UPTAKE RATE, MTT FORMAZAN

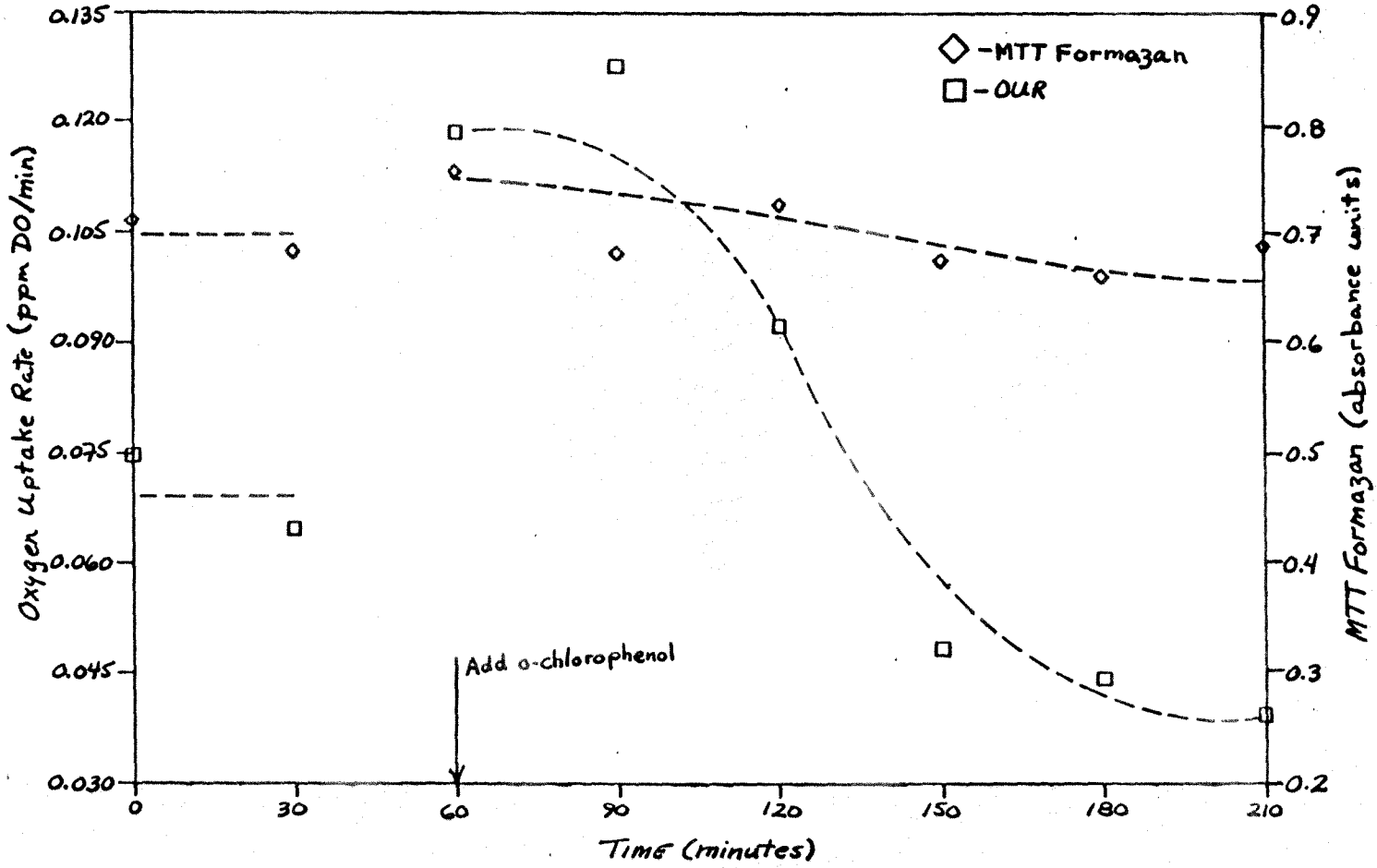


FIGURE B-34 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 1
OXYGEN UPTAKE RATE, MTT FORMAZAN

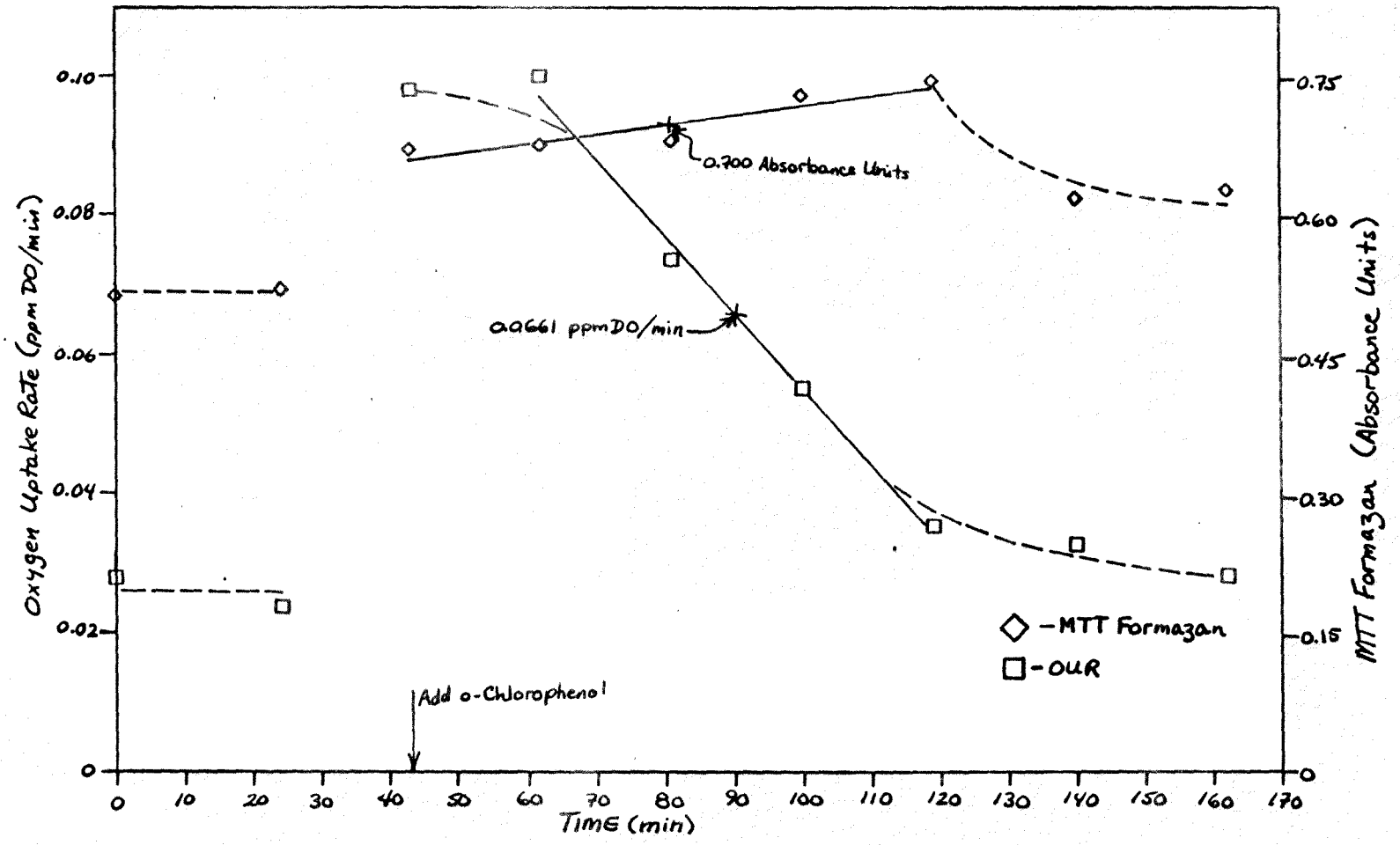


FIGURE B-35 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 2
OXYGEN UPTAKE RATE, MTT FORMAZAN

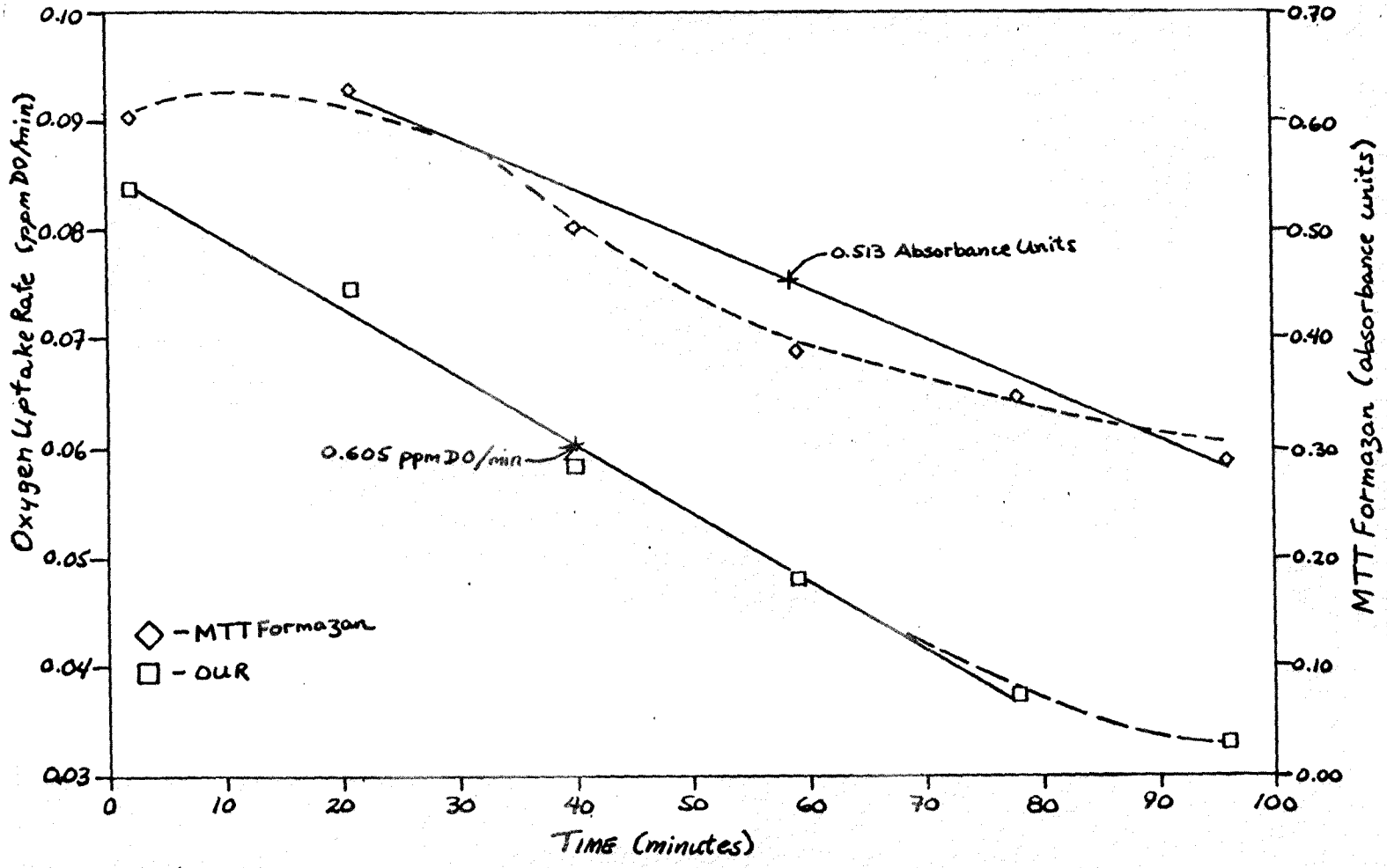


FIGURE B-36 o-CHLOROPHENOL / LIVINGSTON SLUDGE IV - RUN 3
OXYGEN UPTAKE RUN, MTT FORMAZAN

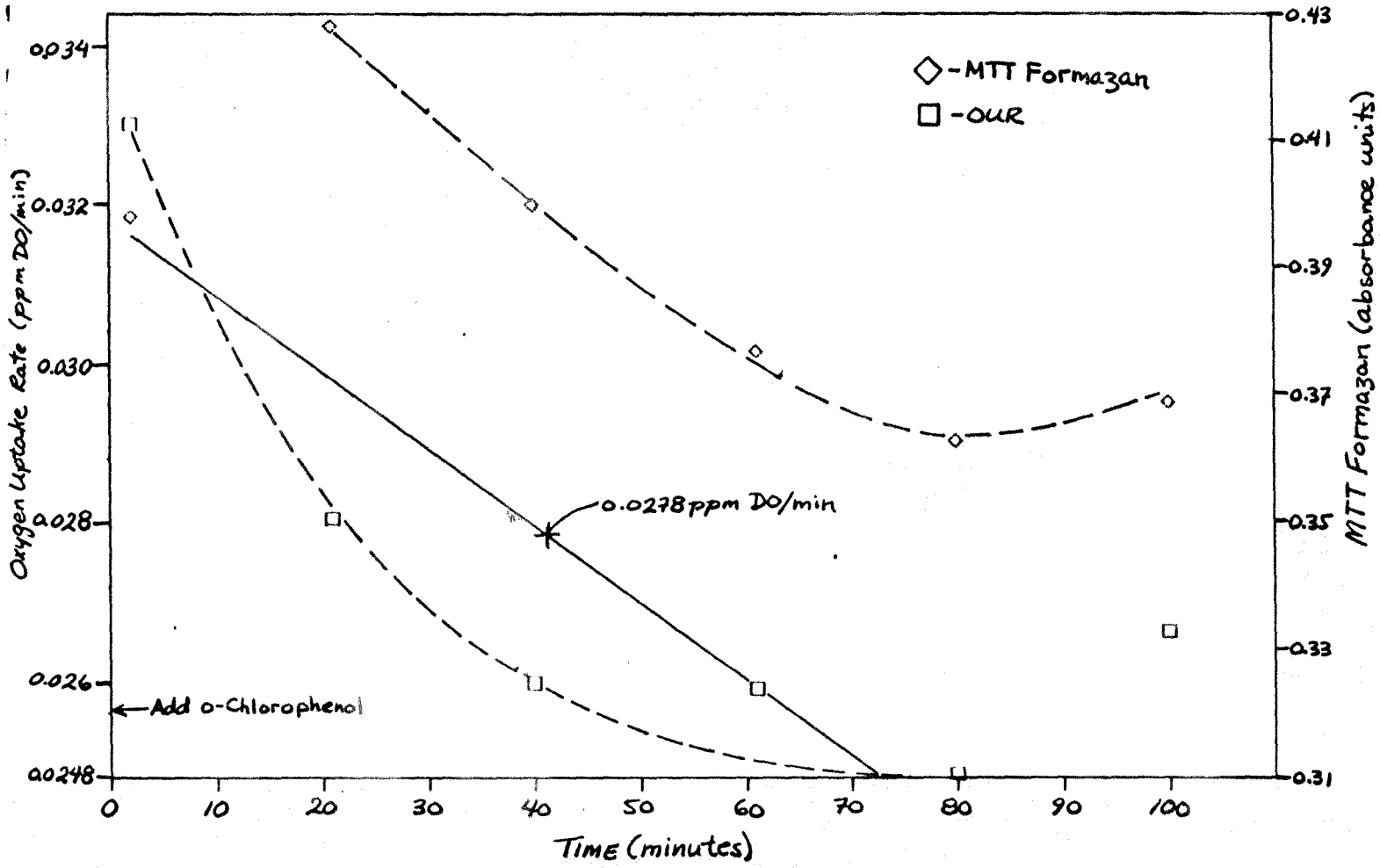


FIGURE B-37 o-CHLOROPHENOL / HYDROBAC III - RUN 1
OXYGEN UPTAKE RATE, MTT FORMAZAN

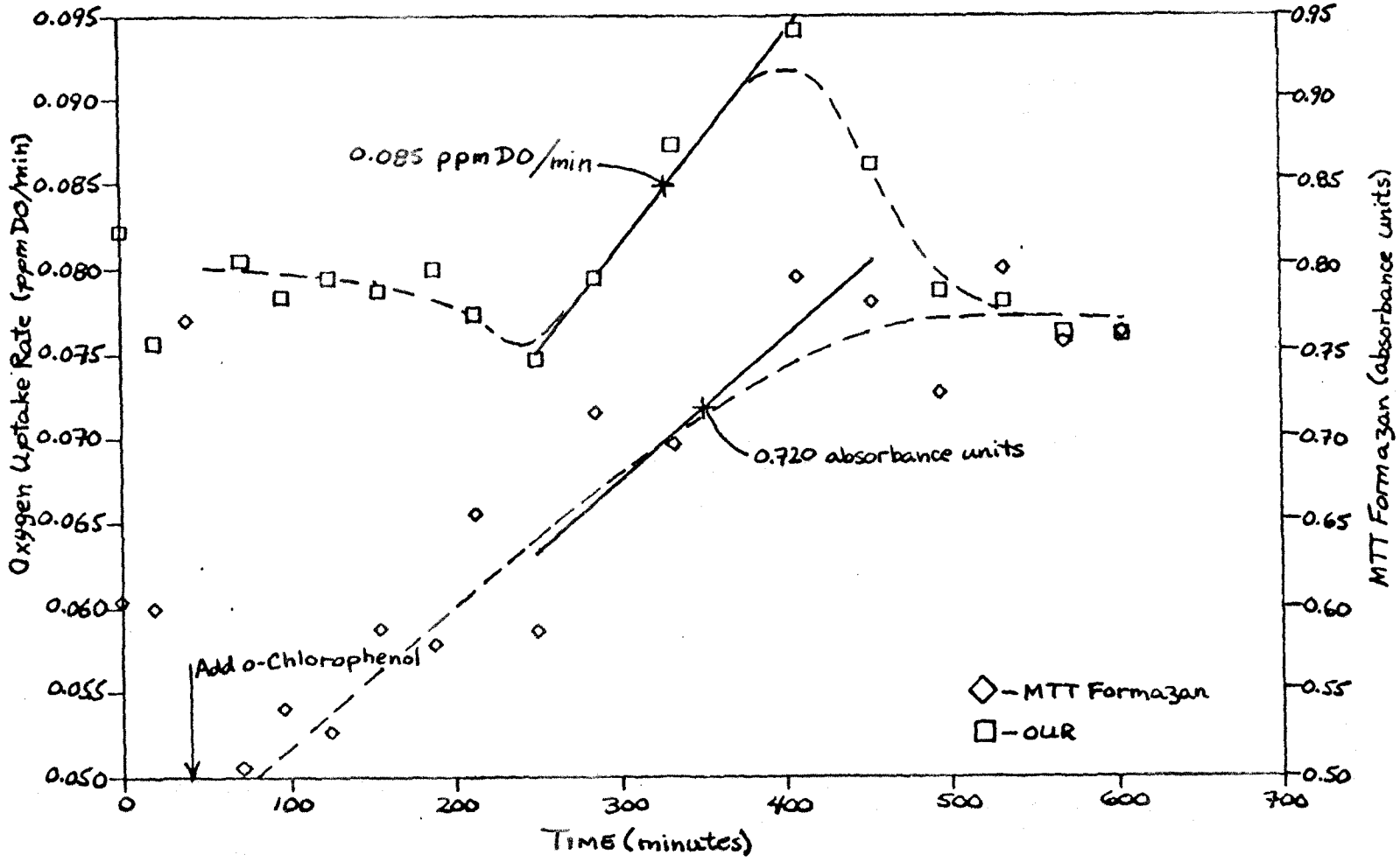
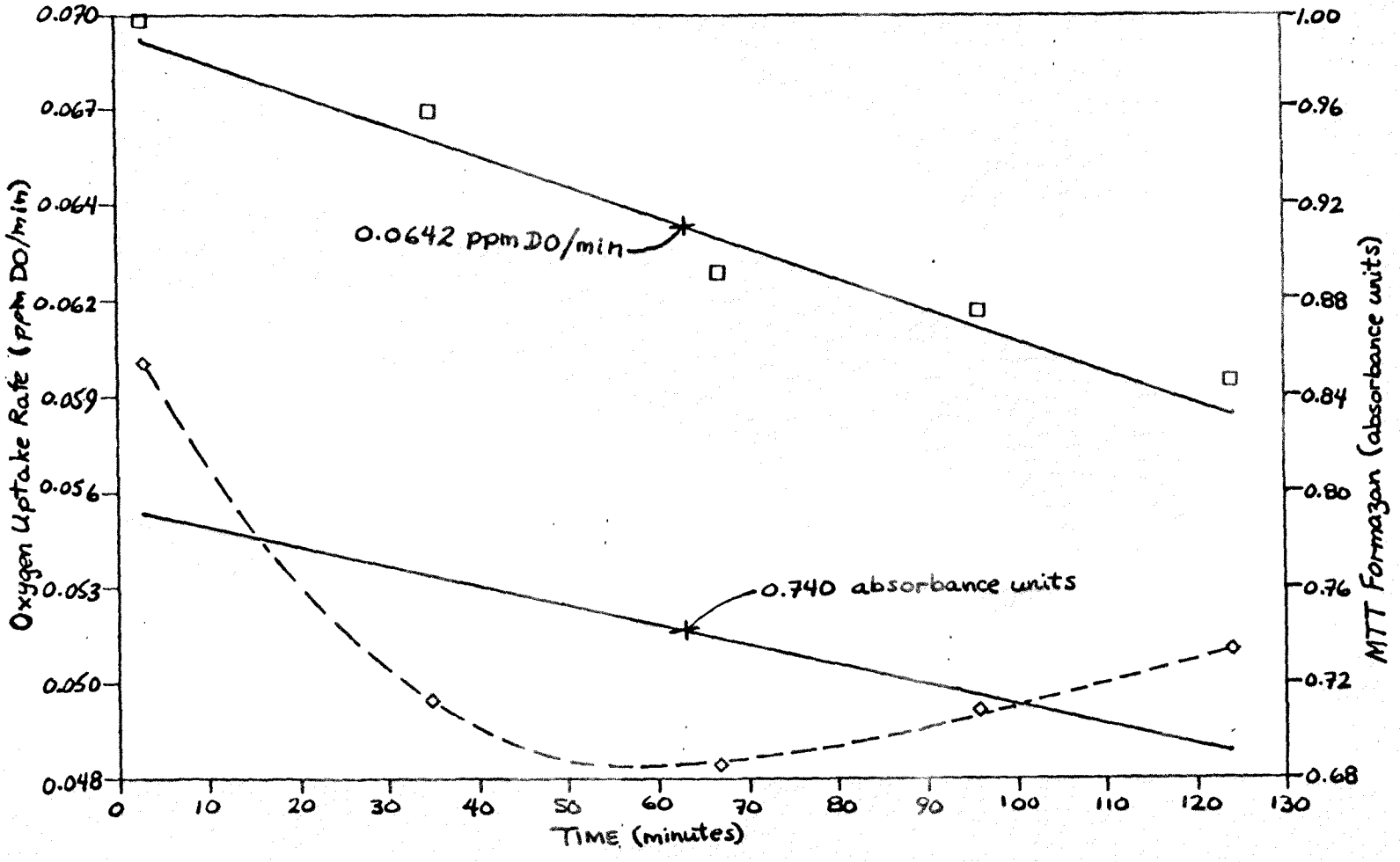


FIGURE B-38 o-CHLOROPHENOL/HYDROBAC III - RUN 2
OXYGEN UPTAKE RATE, MTT FORMAZAN



APPENDIX C

TECHNICAL BULLETINS
ON ENZYME ASSAY KITS
BY SIGMA CHEMICAL COMPANY

PHENYLALANINE — Fluorometric procedure, for serum, at 365 nm activation, 450-535 nm emission. Based on the McCaman and Robins method ..... No. 60-F

PHENYLALANINE — A microbiologic screening procedure for early detection of phenylketonuria based on the Guthrie inhibition assay ..... No. 180

PHOSPHATASE — Colorimetric procedure, for serum, at 400-420 nm, for alkaline, acid and prostatic acid phosphatase. Also urinary alkaline phosphatase. Based on the Bessey-Lowry-Brock procedure ..... No. 104

PHOSPHATASE, ACID — Histochemical procedure, for blood, bone marrow or tissue. Based on use of Naphthol AS-BI and Fast Garnet GBC. For research use only ..... No. 386

PHOSPHATASE, ALKALINE — Histochemical procedure; for blood or bone marrow smears ..... No. 85

PHOSPHATASE, ALKALINE — Kinetic colorimetric procedure; for serum or plasma, at 410 nm. Requires a narrow-bandwidth spectrophotometer. Based on measurement of liberated p-nitrophenol ..... No. 244

PHOSPHATASE ISOENZYMES, ALKALINE — Colorimetric procedure for detection of alkaline phosphatase isoenzymes following electrophoresis on agarose gels. Based on the formation of an indigo dye ..... No. 710-EP

PHOSPHATASE, MILK — Colorimetric procedure, for milk, at 400-420 nm. For research use only ..... No. 104M

PHOSPHOHEXOSE ISOMERASE — Colorimetric procedure; for serum, at 470-510 nm. Based on the Bodansky method ..... No. 650

PHOSPHOHEXOSE ISOMERASE — Ultraviolet kinetic procedure; for serum, at 340 nm ..... No. 355-UV

PROTEIN, TOTAL — Colorimetric procedure; for serum, at 540-545 nm. Based on the Biuret reaction ..... No. 540

PYRUVATE KINASE DEFICIENCY — Fluorescence screening procedure; for erythrocytes. Rate of NADH consumption is noted by loss in fluorescence under long-wave ultraviolet light. All reagents conveniently pre-standardized in a single vial ..... No. 205

PYRUVIC ACID — Ultraviolet enzymatic procedure, for blood, at 340 nm. ..... No. 726-UV

SORBITOL DEHYDROGENASE (SDH) — Ultraviolet kinetic procedure; for serum, at 340 nm. β-D-Fructose used as substrate ..... No. 50-UV

TRANSAMINASE (GOT and GPT) — Colorimetric procedure; for serum, plasma or cerebrospinal fluid, at 490-520 nm. Based on the Sigma-Frankel method ..... No. 505

TRANSAMINASE (GOT and GPT) — Ultraviolet kinetic procedure; for serum, plasma or cerebrospinal fluid, at 340 nm. Based on the Karmen et al. method. All reagents conveniently pre-standardized in a single vial ..... No. 56-UV

TRANSAMINASE (GOT and GPT) — Ultraviolet kinetic procedure; for serum, plasma or cerebrospinal fluid, at 340 nm. Based on the Karmen et al. method. A two-reagent system for the LKB Reaction Rate Analyzer or similar automated equipment ..... No. 155-UV

TRIGLYCERIDES — Colorimetric procedure; for serum or plasma, at 405-415 nm. Based on the Kessler-Lederer and Fletcher methods ..... No. 405

TRIGLYCERIDES — Ultraviolet semi-enzymatic procedure; for serum or plasma, at 340 nm. Based on assay of glycerol released from triglycerides after saponification ..... No. 320-UV

TRIZMA® BUFFERS — Widely used biologic buffers, pH range 7-9. For research use only ..... No. 108B

TYROSINE — Fluorometric procedure, for serum, at 460 nm excitation and 570 nm emission. Based on the reaction of tyrosine with 1-nitroso-2-naphthol ..... No. 70-F

UREA NITROGEN — Colorimetric procedure; for serum, plasma or urine, at 515-540 nm. Based on the urea-diacetyl monoxime reaction ..... No. 535

UREA NITROGEN — Colorimetric procedure; for serum, plasma or urine, at 500-650 nm. Ammonia formed by action of urease is determined by Berthelot reaction ..... No. 640

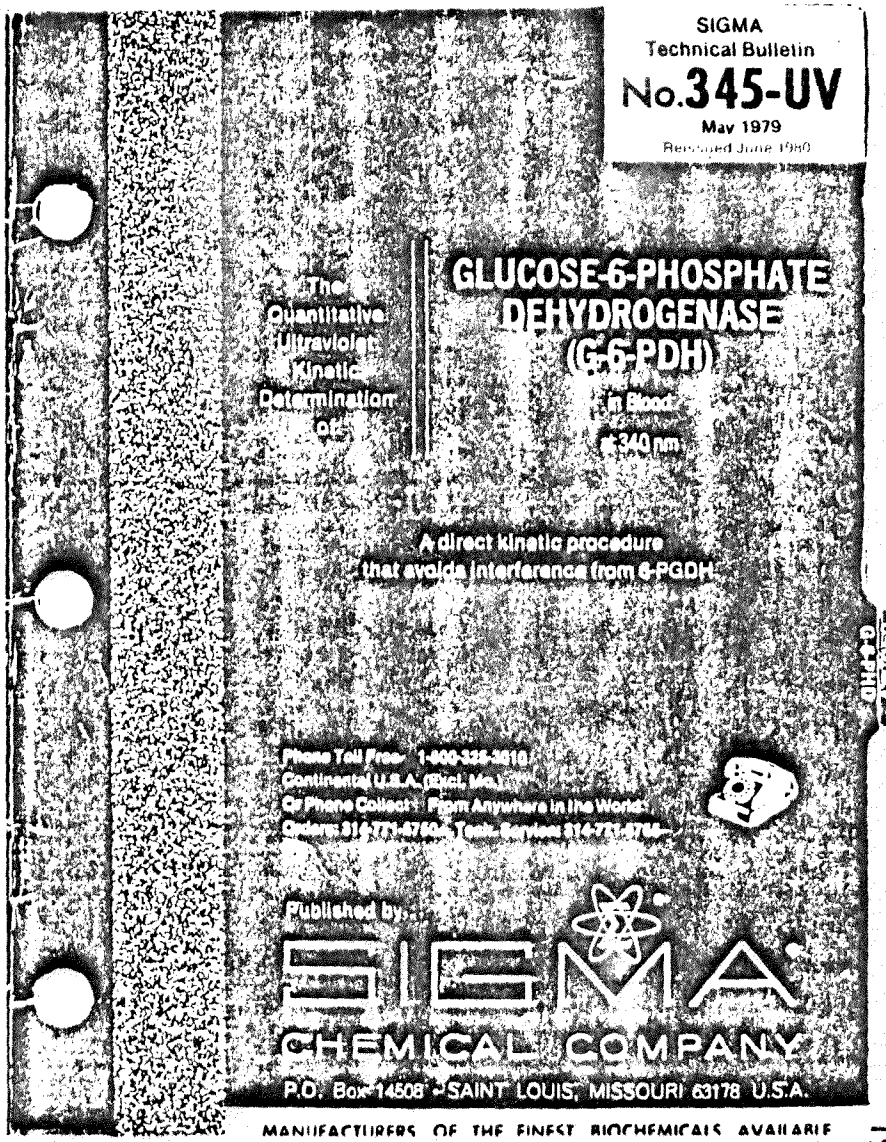
UREA NITROGEN — Ultraviolet enzymatic procedure; for serum, plasma or urine, at 340 nm. Employs urease/glutamate dehydrogenase coupled enzyme system. All reagents conveniently pre-standardized in a single vial ..... No. 65-UV

URIC ACID — Colorimetric procedure, for urine, at 650-750 nm. Combines the accuracy of the enzyme uricase with the simplicity of a colorimetric method ..... No. 680

URIC ACID — Ultraviolet procedure, for serum or urine, at 292 nm. Based on specific reaction of uric acid with uricase ..... No. 292-UV

VANILMANDELIC ACID (VMA) — Colorimetric procedure; for urine, at 470-510 nm. Based on method of Sunderman et al ..... No. 480

VANILMANDELIC ACID (VMA) — Ultraviolet procedure; for urine, at 360 nm. Simplification of VMA Procedure No. 480 if instrument is available with 360 nm reading capability ..... No. 481-UV



SUMMARY AND EXPLANATION OF TEST

Glucose-6-Phosphate Dehydrogenase (G-6-PDH, D-glucose-6-phosphate: oxidoreductase, EC 1.1.1.49) catalyzes the first step in the pentose phosphate shunt, oxidizing glucose-6-phosphate (G-6-P) to 6-phosphogluconic acid, and reducing NADP to NADPH, a requirement for a variety of bio-synthetic reductions. The enzyme is found in almost all mammalian cells. Although only very small amounts are present in serum, heart, kidney and skeletal muscle, high concentrations of G-6-PDH occur in the formed elements of blood.[1] The enzyme present in erythrocytes is of the greatest clinical interest.[1]

G-6-PDH deficiency occurs in erythrocytes as a genetically determined, sex-linked trait.[2,3] A number of variants of the enzyme have been identified, several of which are associated with an increased susceptability to hemolytic anemia.[3] In one form of the defect, the red cell appears to be sensitive to a number of drugs, most notably primaquine.[4,5] Acute hemolytic episodes may also be precipitated by a physiological crisis, such as diabetic acidosis or bacterial or viral infections.[1,4] Another variant (Mediterranean) causes severe intravascular hemolysis following ingestion of fava beans.[3] A non-drug-dependent form of the defect is manifested as a congenital non-spherocytic anemia in which hemolysis may occur spontaneously, even in the absence of exogenous stress.[3]

Since the red cell has no citric acid cycle, the pentose phosphate shunt, and particularly G-6-PDH, appears to be the sole means for NADP reduction available to the cell.[3] Oxidation of certain drugs or metabolites by the erythrocyte results in the accumulation of oxidized glutathione (GSSG).[1,6] Normally, this oxidized glutathione is then reduced to glutathione (GSH) by glutathione reductase, using NADPH as a hydrogen donor.[6] Red cells deficient in G-6-PDH are unable to maintain the pool of NADPH necessary for the reduction of GSSG to GSH. Failure to maintain adequate GSH concentrations results in defects in cell membrane structure, leading to early senescence of the erythrocyte and to hemolytic episodes following stress to the cell.[7]

Spectrophotometric procedures for the determination of G-6-PDH are in general based on the method of Kornberg and Horecker,[8] in which the NADPH formed by the oxidation of glucose-6-phosphate is determined. However, the amount of NADPH measured includes NADPH formed as the result of oxidation of 6-phosphogluconate (6-PG) by 6-phosphogluconate dehydrogenase (6-PGDH) as well as that resulting from the oxidation of G-6-P by G-6-PDH. Bishop[9] and Glock and McLean[10] have proposed correcting for the activity of 6-PGDH by performing two reactions, one with only 6-PG and the second with both 6-PG and G-6-P present. The activity due to G-6-PDH is then represented by the difference between the two activities. In another approach,[10,11] 6-PGDH is added to the reaction, so that all 6-PG formed by G-6-PDH is converted to ribulose-5-phosphate. Oxidation of one mole of G-6-P thus results in formation of two moles of NADPH, one half of which is due to G-6-PDH activity.

The procedure described in this bulletin (No. 345-UV) is based on the methods of Kornberg and Horecker[8], Bishop[9], and Lohr and Waller.[12] Maleimide is incorporated into the system to inhibit the 6-PGDH present, thus permitting direct measurement of the true G-6-PDH activity.[12]

Other methods for G-6-PDH determination have been published which are useful for enzyme deficiency screening purposes. These are visual tests which estimate changes in dye color or in fluorescence. Procedures based on these principles are described in Sigma Technical Bulletins No. 400 and 202, respectively.

PRINCIPLE

NADP is reduced by glucose-6-phosphate dehydrogenase in the presence of G-6-P as follows:

$$\text{G-6-P} + \text{NADP}^+ \xleftarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH} + \text{H}^+$$

The rate of formation of NADPH is proportional to the G-6-PDH activity, and may be determined spectrophotometrically as an increase in absorbance at 340 nm. Production of a second molar equivalent of NADPH by 6-phosphogluconate dehydrogenase, also present in erythrocytes, according to the reaction:

$$\text{6-PG} + \text{NADP}^+ \xleftarrow{\text{6-PGDH}} \text{Ribulose-5-Phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

is prevented by the presence of maleimide which inhibits the 6-PGDH.

Abbreviations:

G-6-PDH	·	Glucose-6-phosphate dehydrogenase
G-6-P	·	Glucose-6-phosphate
$NADP^+$	·	Nicotinamide adenine dinucleotide phosphate
NADPH	·	Nicotinamide adenine dinucleotide phosphate, reduced form
6-PG	·	6-Phosphogluconate
6-PGDH	·	6-Phosphogluconate dehydrogenase

REAGENTS

For In Vitro Diagnostic Use

A. G-6-PDH REAGENT

SINGLE-ASSAY VIAL, Stock No. 345-1

NADP,	1.5 μmol
Maleimide,	12 μmol

FIVE-ASSAY VIAL, Stock No. 345-5

NADP,	8.2 μmol
Maleimide,	66 μmol

Vials also contain buffer, stabilizer and lysing reagent.

Store dry vials in refrigerator.

Reconstituted reagent is stable for 8 hours at room temperature or 5 days in the refrigerator.

CAUTION: Maleimide is an irritant. Avoid ingestion, inhalation or contact with eyes or skin.

B. G-6-PDH SUBSTRATE SOLUTION, Stock No. 345-8

Contains Glucose-6-Phosphate, 1.05 mmol/L, buffer and magnesium salt. Also contains 0.1% Sodium Azide as a preservative.

Store in refrigerator.

C. POTASSIUM DICHROMATE, 3 mg Vial, Stock No. PD-3

Dissolve contents of vial in 45 mL of water.

The absorbance of the solution should be approximately 0.7 vs water as reference at 340 nm using a 1-cm lightpath.

Stable at room temperature.

The use of this solution is recommended over water as reference in the procedure, as it will bring the absorbance readings of the Test into a range which can be more accurately read on your instrument. The concentration of the Potassium Dichromate Solution may be increased or decreased to suit your requirements.

SPECIMEN COLLECTION AND PREPARATION

Blood may be collected in any of the common anticoagulants such as heparin or ACD[1] (Acid Citrate Dextrose solution, NIH Formula A or B), although ACD is preferred.[3] ACD Formula B may be prepared as follows: dissolve citric acid monophosphate, 0.48 g, trisodium citrate dihydrate, 1.32 g, and dextrose, 1.47 g, in distilled water and bring to a final volume of 100 mL. Use 1.0 mL for each 4.0 mL whole blood. Preliminary studies in our laboratory indicate that EDTA is also satisfactory for use as an anticoagulant.

When stored in the refrigerator, G-6-PDH is stable in the intact erythrocyte for 7 days in heparin,[13] and for 7 days to several weeks in ACD.[3,8] The enzyme is not stable in a hemolysate; thus freezing of the specimen must be avoided.[1]

Since activity is reported in terms of number of RBC's or grams of hemoglobin, the red cell count or hemoglobin concentration should be determined prior to performing the G-6-PDH assay. The integrity of erythrocytes collected in ACD is preserved even after prolonged storage so that obtaining reliable red cell counts generally presents no difficulty.[13] However, red cell counts on specimens collected in heparin become unreliable after about 2 days.[13] Thus, for heparinized samples, it may be preferable to report results in terms of hemoglobin concentration.

Interfering Substances: Both copper, which completely inhibits the enzyme at a concentration of 100 μmol/L, and sulfate ions (0.005 mol/L) interfere with the test and will result in decreased levels of G-6-PDH activity. For further information concerning interfering substances, consult the review article of Young, et al.[14]

INSTRUMENTS AND MATERIALS REQUIRED

Instrument: A narrow-bandwidth spectrophotometer, such as a Beckman DU, Gilford, Cary, Zeiss, etc., capable of measuring absorbance at 340 nm is required for this procedure.

Equipment and reagents for performing a red cell count or determining hemoglobin concentration are also required. Sigma offers procedure No. 525 for the Determination of Hemoglobin.

Materials:

Cuvets: Square, 1-cm pathlength

Pipets: 1, 5 and 10 mL, serologic
10 μL, micropipet

Centrifuge

PROCEDURE

The temperature of the reaction should be maintained at 30°C or some other constant temperature. Refer to "Temperature Correction Factors (TCF)" Table 1.

Using Single-Assay Vial	Using Five-Assay Vial
1. a) To: One vial G-6-PDH Reagent, Stock No. 345-1 Add: 1.0 mL water.	1. a) To: One vial G-6-PDH Reagent, Stock No. 345-5 Add: 5.5 mL water.
b) Swirl gently and invert several times to dissolve contents. Wait 2-3 minutes and mix again.	b) Swirl gently and invert several times to dissolve contents. Wait 2-3 minutes and mix again. Reagent is stable at least 5 days stored in the refrigerator or longer if frozen.
c) Add 10 µL Test blood. Mix thoroughly to **completely suspend** erythrocytes. Let stand 5-10 minutes at room temperature.	c) To cuvets marked Test 1, Test 2, etc. Add: 1.0 mL reconstituted G-6-PDH Reagent. d) Add: 10 µL Test blood. Mix thoroughly to **completely suspend** erythrocytes. Let stand 5-10 minutes at room temperature.
2. Add 2.0 mL G-6-PDH Substrate Solution, Stock No. 345-8. Mix well and pour contents into a cuvet marked Test.	2. Add 2.0 mL G-6-PDH Substrate Solution, Stock No. 345-8. Mix well by gently inverting cuvet several times.

3. Place cuvet in constant temperature cuvet compartment or water bath at 30°C or some other constant temperature for approximately 5 minutes to attain thermal equilibration.

4. Read and record absorbance (A) of Test at 340 nm vs a Dichromate Solution Blank, (See Reagent C, Reagent section.)

   This is the INITIAL A.

5. Exactly 5 minutes later, again read and record absorbance.

   This is the FINAL A.

CALCULATIONS:

A. Calculate the ΔA/min:

$$\Delta A/min = \frac{\text{FINAL A} - \text{INITIAL A}}{5}$$

B. Activity is expressed as U (Units)/10^{11} erythrocytes or, alternatively, as U/g hemoglobin

1. $U/10^{11}$ erythrocytes $= \frac{\Delta A/min \times 48390}{N^*} \times TCF$

   Where:

   N = Red cell count divided by 10^6
   (e.g., RBC count = 4.5×10^6, N = 4.5)

   TCF = Temperature Correction Factor (TCF = 1.0 at 30°C)

2. U/g hemoglobin $= \Delta A/min \times \frac{4839}{Hb(g/dL)^*} \times TCF$

   *Previously determined for each specimen.

   Note: If ΔA per minute is greater than 0.060, repeat determination using 5 µL blood and multiply results by 2.

For explanation of calculations see "Results" section.

CALIBRATION:

This procedure is standardized on the basis of the millimolar absorptivity (6.22 at 340 nm) of NADPH. Oxidation/conversion of substrate (G-6-P) by G-6-PDH leads to reduction of NADP to NADPH on a molar equivalent basis. Measurement of the rate of increase in absorbance (ΔA) at 340 nm serves to quantitate enzymatic activity. The maxium G-6-PDH activity which may be measured by this procedure is approximately 650 $U/10^{11}$ RBC or 19.5 U/g Hb.

The performance characteristics of the spectrophotometer should be monitored frequently to assure reliable readings. Calibration standards for this purpose are generally available from the instrument manufacturer or from the National Bureau of Standards. For discussions of spectrophotometric principles, the analyst is referred to any good basic clinical chemistry text.

TEMPERATURE CORRECTION:

If the temperature of the reaction is 30°C, no temperature correction is required in the calculations. If the assay is performed at a temperature other than 30°C, a Temperature Correction Factor (TCF) is used in calculating the enzyme activity.

Table 1. Temperature Correction Factors (TCF)

Cuvet Temp. °C	TCF	Cuvet Temp. °C	TCF
20	1.90	30	1.00
21	1.76	31	0.94
22	1.66	32	0.89
23	1.55	33	0.83
24	1.46	34	0.78
25	1.37	35	0.74
26	1.28	36	0.70
27	1.20	37	0.66
28	1.13	38	0.62
29	1.06	39	0.58

QUALITY CONTROL:

Commercially available quality control material of known activity should be assayed concurrently with patient specimens to assure proper performance of the procedure. Alternatively, blood from a known normal donor may be used for this purpose.

RESULTS

$$\text{G-6-PDH (U/}10^{14}\text{ RBC)} = \Delta A/\text{min} \times \frac{3.01 \times 10^{14}}{0.01 \times 6.22 \times N \times 10^{6} \times 1000} \times TCF$$

$$= \Delta A/\text{min} \times \frac{48{,}390}{N} \times TCF$$

Where:
- 3.01 = Total volume (mL) in cuvet
- 10^{14} = Factor for expressing activity in 10^{14} cells
- 0.01 = Volume (mL) of sample in cuvet
- 6.22 = Millimolar absorptivity of NADPH
- $N \times 10^{6}$ = Number of erythrocytes/mm³
- 1000 = Conversion of red cell count from count per mm³ to count per mL
- TCF = Temperature correction factor (1.0 at 30°C)

$$\text{G-6-PDH (U/g Hb)} = \Delta A/\text{min} \times \frac{100 \times 3.01}{0.01 \times 6.22 \times \text{Hb (g/dL)}} \times TCF$$

$$= \Delta A/\text{min} \times \frac{4839}{\text{Hb (g/dL)}} \times TCF$$

Where:
- 100 = Factor to convert activity to 100 mL
- 3.01 = Total volume (mL) in cuvet
- 0.01 = Volume (mL) of sample in cuvet
- 6.22 = Millimolar absorptivity of NADPH
- Hb (g/dL) = Hemoglobin concentration
- TCF = Temperature correction factor (1.0 at 30°C)

Example:

Assay of a specimen which had a red cell count of 4.6 × 10⁶/mm³ and a hemoglobin concentration of 15.2 g/dL resulted in a ΔA/min at 30°C of 0.028.

$$\text{G-6-PDH (U/}10^{14}\text{ RBC)} = 0.028 \times \frac{48{,}390}{4.6} = 295$$

$$\text{G-6-PDH (U/g Hb)} = 0.028 \times \frac{4839}{15.2} = 8.9$$

UNIT DEFINITION:

One International Unit (U) is that amount of G-6-PDH activity that will convert 1 micromole of substrate per minute under the conditions specified in this bulletin. Activity may be expressed in terms of either a standard number of cells or amount of hemoglobin. Since it is the G-6-PDH activity per cell that is important, the former convention is preferred, despite the fact that it is believed by some that red cell counts are subject to considerable uncertainty.[1] Hemoglobin concentration may be determined with greater accuracy, but the amount of hemoglobin contained in a cell is under separate genetic control and may vary independently of G-6-PDH activity.[1]

LIMITATIONS OF PROCEDURE

Refer to "Specimen Collection and Preparation" section for interfering substances.

EXPECTED VALUES

Blood specimens from 90 clinically healthy adult males and females were assayed for G-6-PDH activity at 30°C by the method described in this bulletin.

NORMAL RANGE

	U/10^{12} RBC	U/g Hb
Adults (Mixed Population)	146 - 376	4.6 - 13.5

It has been determined that G-6-PDH deficiency in red cells is the basis for certain drug-induced hemolytic anemias.[5] This type of susceptibility to drug-induced hemolysis is often called "primaquine sensitivity" because studies which led to its characterization were made during investigations of the hemolytic properties of this antimalarial compound.

Red cell G-6-PDH deficiency has been found in about 13% of American Negro males and in about 3% of American Negro females. The mode of inheritance is X-linked, but not transmitted as a simple autosomal or sex-linked dominant trait. Erythrocytes from males are either normal or deficient. Two cell populations may be present in the female heterozygote, resulting in an intermediate enzyme deficiency.

The incidence is also high among other racial and ethnic groups, such as Sardinians, Greeks, Sephardic Jews and others.[5,15,16] A very high frequency of G-6-PDH deficiency occurs among subjects with sickle cell trait.[17]

Tarlov et al.[5] points out the importance of identifying individuals with this biochemical defect as an aid in the selection of therapeutic agents. Severe hemolytic anemia may result in these individuals when they are given many commonly used drugs. This defect should be considered whenever an otherwise unexplained case of hemolytic anemia is encountered.

Among subjects of Mediterranean origin, it has been found that the degree of enzyme deficiency is more severe than in Negro subjects.[17] In some Caucasians, particularly among Sardinians and Italians with G-6-PDH deficiency, ingestion of fava beans results in a severe and sometimes fatal hemolytic reaction (favism).[1] The majority of subjects who have demonstrated G-6-PDH deficiency are clinically normal until exposed to one of numerous drugs (antimalarial drugs, sulfa drugs, ascorbic acid and others).[17]–The hemolytic episodes that follow may be self limiting. In other words, following an acute episode, essentially full recovery occurs until a new attack is brought about by an initiating agent.[1] In rarer instances, G-6-PDH deficiency may be a contributory factor to hemolysis observed in patients with hereditary nonspherocytic hemolytic anemia, in which cases individuals may undergo constant hemolytic crises.

SPECIFIC PERFORMANCE CHARACTERISTICS

Reproducibility: Ten replicate assays of both normal and abnormal blood samples yielded standard deviations of 9.4 and 7.2 U/L and coefficients of variation of 1.5 and 2.4%, respectively, with means of 617 and 303 U/L. Eleven replicate assays of a normal blood specimen over a 3-day period produced a mean of 298 U/10^{12} RBC with a standard deviation of 13.5 U/10^{12} RBC and a coefficient of variation of 4.5%.

Sensitivity: Assuming the limit of sensitivity to represent a change in absorbance at 340 nm of 0.001/minute, a G-6-PDH activity of 0.4 U/g Hb or 10.8 U/10^{12} RBC may be detected using this procedure (assuming a hemoglobin concentration of 12.0 g/dL and a red cell count of 4.5 x 10^6/mm^3).

Specificity: The oxidation of glucose-6-phosphate by G-6-PDH is specific. Any nonspecific formation of NADPH due to oxidation of other substrates by endogenous enzymes occurs during the preincubation period. 6-Phosphogluconate dehydrogenase is completely inhibited by maleimide in the reagent system,[12] preventing formation of additional NADPH which might otherwise occur through oxidation of the 6-phosphogluconate produced in the initial reaction.

Correlation: Results obtained by the method described in this bulletin were compared with those obtained when the same 27 specimens were assayed by a modification of the method of Marks.[18] Comparison of these data yielded a linear regression equation with $y = 1.47x - 0.26$ and a correlation coefficient of 0.9081.

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Sigma Chemical Company
P.O. Box 14508
St. Louis, Missouri 63178 U.S.A.

PRICE LIST No. 345-UV
as of June 1980
Reagents for the Determination of
GLUCOSE-6-PHOSPHATE DEHYDROGENASE
Per Sigma Technical Bulletin No. 345-UV

KITS

Kit No.	Max. Assays	PRICE U.S. $	PRICE U.K. £	Contents—Stock Numbers: G-6-PDH REAGENT Single Assay 345-1	G-6-PDH REAGENT Five Assay 345-5	G-6-PDH Substrate Solution 345-8	Potassium Dichromate PD-3
345-A	20	18.00	11.93	20 vials		50 ml	1 vial
345-B	50	26.00	17.23		10 vials	2 x 50 ml	1 vial

Stock Numbers 345-1, 345-5, and 345-8 are available in kits only and cannot be purchased separately.

INDIVIDUAL REAGENT

Stock No.	Item	Quantity	U.S. $	U.K. £
PD-3	Potassium Dichromate, 3 mg/vial	1 vial	0.50	0.33

TECHNICAL BULLETINS LISTED IN ALPHABETICAL ORDER

The following is a complete list of bulletins that are provided by SIGMA as of May, 1979.

ADENOSINE-5'-TRIPHOSPHATE (ATP) — Ultraviolet enzymatic procedure; for blood, at 340 nm. Employs phosphoglycerate kinase/glyceraldehyde phosphate dehydrogenase enzyme coupled system. ..... No. 366-UV

ALBUMIN — Colorimetric procedure; for serum, at 625-635 nm. Based on anion binding capacity of albumin for bromcresol green. ..... No. 630

ALCOHOL, ETHYL (ETHANOL) — Ultraviolet enzymatic procedure; for blood, serum or plasma, at 340 nm. ..... No. 331-UV

ALDOLASE — Colorimetric procedure; for serum, at 500-580 nm. Based on Sibley-Lehninger method which measures hydrazone derivatives of trioses formed from fructose-1,6-diphosphate ..... No. 750

ALDOLASE — Colorimetric procedure; for serum, at 520-600 nm. Modification of Aldolase Procedure No. 750, resulting in increased sensitivity and improved color stability ..... No. 752

AMMONIA — Ultraviolet enzymatic procedure; for plasma, at 340 nm. Based on the conversion of α-ketoglutarate and ammonia to glutamate in the presence of glutamate dehydrogenase. ..... No. 170-UV

AMYLASE — Colorimetric procedure, for serum or urine. Based on visual observation of the starch-iodine color change. ..... No. 700

ANTITHROMBIN III (AT-III) — Clotting time method for plasma employing ATHOXIN® (*Bothrops atrox* venom) for defibrination ..... No. 865

ATROXIN® — Clotting time method for plasma using *Bothrops atrox* snake venom. Useful for determining clottability of plasma from heparinized patients. ..... No. 845

BILIRUBIN — Two colorimetric procedures, for serum or plasma, at 600 nm. Method A, for narrow-bandwidth spectrophotometers, is based on millimolar extinction coefficient of alkaline azobilirubin. Method B, for wide-bandwidth spectrophotometers, requires use of a bilirubin reference for calibration purposes ..... No. 605

BIOLUMINESCENCE DEMONSTRATION PROCEDURE — Visible light is produced as a result of the reaction between luciferin, ATP and molecular oxygen, catalyzed by luciferase (uses desiccated firefly tails as source of luciferin and luciferase). *For research use only.* ..... No. 2-FF

CALCIUM — Colorimetric procedure; for serum or plasma, at 570-575 nm. Based on cresolphthalein complexone color reaction ..... No. 585

CALIBRATION PROCEDURE FOR WIDE BANDWIDTH SPECTROPHOTOMETERS — For reactions involving NADH and NADPH, at 340 nm. Describes a method for correcting wide bandwidth instrument readings for direct use in calculations. For research use only ..... No. 30-UV

CARBAMYL PHOSPHATE and/or ORNITHINE — Colorimetric procedure; at 490 nm. Measures citrulline formed by action of ornithine carbamyl transferase. For research use only. ..... No. 22

CHLORIDE — Titrimetric procedure, Schales and Schales; for serum, plasma, urine or cerebrospinal fluid. ..... No. 830

CHOLESTEROL, TOTAL — Enzymatic procedure; for serum or plasma, at 500 nm. Employs a cholesterol esterase/oxidase system. ..... No. 350

CHOLINESTERASE — Colorimetric procedure; for serum or plasma, at 400-440 nm. Measures acetic acid liberated from acetylcholine. ..... No. 420

CREATININE — Colorimetric procedure; for serum, plasma or urine, at 490-510 nm. Modified Jaffe reaction; avoids deproteinization. ..... No. 555

CREATINE PHOSPHOKINASE (CPK) — Colorimetric procedure; for serum, plasma or cerebrospinal fluid, at 620-700 nm. Measures inorganic phosphorus released from phosphocreatine ..... No. 661

CREATINE PHOSPHOKINASE (CPK) — Colorimetric procedure, for serum or plasma, at 500-540 nm. Creatine released from phosphocreatine is reacted with α-naphthol and diacetyl to form a red complex ..... No. 520

CREATINE PHOSPHOKINASE (CPK) — Ultraviolet kinetic procedure; for serum, plasma or cerebrospinal fluid, at 340 nm. Employs hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme system. All reagents conveniently pre-standardized in a single vial ..... No. 45-UV

CREATINE PHOSPHOKINASE (CPK) ISOENZYMES — Colorimetric procedure for detecting CPK isoenzymes, following electrophoresis on agarose gels. Based on reduction of tetranitroblue tetrazolium ..... No. 715-EP

CREATINE PHOSPHOKINASE (CPK) ISOENZYMES — Fluorometric procedure for detecting CPK isoenzymes following electrophoresis on agarose gels. Based on the fluorescence of reduced NADP. ..... No. 745-EP

CYTOCHROME OXIDASE — Histochemical procedure; for frozen tissue sections. ..... No. 185
Based on the Burstone procedure. For research use only.

2,3-DIPHOSPHOGLYCERIC ACID (2,3-DPG) — Ultraviolet enzymatic procedure; ..... No. 35-UV
for erythrocytes, at 340 nm. Employs phosphoglycerate mutase/phosphoglycerate phosphokinase/glyceraldehyde phosphate dehydrogenase enzyme coupled system.

2,3-DIPHOSPHOGLYCERIC ACID (2,3-DPG) — Colorimetric procedure; for ery- ..... No. 665
throcytes, at 620-700 nm. Measures phosphate released by action of phosphoglycerate mutase.

ENDOTOXINS — An agglutination reaction for the detection of gram-negative ..... No. 210
bacterial endotoxins. For research use only.

ESTERASES — Cytochemical demonstration, for blood, bone marrow or tissue. ..... No. 90
Based on use of naphthol AS-D chloroacetate or α-naphthyl acetate. For research use only.

FACTOR II (PROTHROMBIN) — One-stage clotting time assay for plasma employ- ..... No. 82-7D
ing Factors II and VII deficient plasma.

FACTORS II, VII AND X (PROTHROMBIN AND PROCONVERTIN) — One-stage ..... No. 82-7-10D
clotting time assay for plasma employing Factors II, VII and X deficient plasma.

FACTOR V — One-stage clotting time assay for plasma employing Factor V de- ..... No. 85D
ficient plasma.

FACTOR VII (PROCONVERTIN) — One-stage clotting time assay for plasma ..... No. 87D
employing Factor VII deficient plasma. For research use only.

FACTOR X (STUART-PROWER) — One-stage clotting time assay for plasma em- ..... No. 87-10D
ploying Factors VII and X deficient plasma.

FETAL HEMOGLOBIN — Histochemical acid elution procedure; for blood smears. ..... No. 285

FIBRIN DEGRADATION PRODUCTS (FDP) — A visual semi-quantitative estima- ..... No. 850
tion, in serum, by the staphylococcal cell suspension agglutination technique.

FORMIMINO-L-GLUTAMIC ACID (FIGLU) — Ultraviolet enzymatic procedure; ..... No. 385-UV
for urine, at approximately 385 nm.

GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE — Ultraviolet procedure; ..... No. 600-UV
for erythrocytes, at 340 nm. Measures rate of consumption of uridine diphosphoglucose (UDPG). For research use only.

GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE DEFICIENCY — Fluores- ..... No. 195
cence screening procedure, for erythrocytes. Rate of NADPH formation is noted by visual observation of increasing fluorescence under long-wave ultraviolet (black) light.

GLOBULIN, TOTAL — Colorimetric procedure; for serum, at 550-570 nm. Based ..... No. 560
on the modification of Hopkins-Cole reaction for tryptophan.

GLUCOSE — Colorimetric enzymatic procedure; for serum or plasma, at 520 nm. ..... No. 115
Based on the use of hexokinase and glucose-6-phosphate dehydrogenase enzymes, coupled with the reduction of iodonitrotetrazolium to produce a colored INT formazan.

GLUCOSE — Colorimetric procedure; for blood, plasma or serum, at 420-475 nm. ..... No. 510
Employs glucose oxidase and peroxidase with o-dianisidine as chromogen.

GLUCOSE — Colorimetric procedure; for blood, plasma or serum, at 620-650 nm. ..... No. 635
Based on o-toluidine color reaction.

GLUCOSE — Ultraviolet enzymatic procedure; for serum, plasma, cerebrospinal ..... No. 15-UV
fluid and urine, at 340 nm. Employs hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme system. All reagents conveniently pre-standardized in a single vial.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD) — Semiquantitative ..... No. 400
colorimetric procedure; for erythrocytes. Based on visual observation of dichlorophenol indophenol dye decolorization.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PDH) — Quantitative ultra- ..... No. 345-UV
violet kinetic procedure for blood at 340 nm. Interference from 6-phosphogluconate dehydrogenase (6-PGDH) is avoided by use of a special inhibitor.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD) DEFICIENCY — Fluor- ..... No. 202
escence screening procedure; for erythrocytes. Rate of NADPH formation is noted by visual observation of increase in fluorescence under long wave ultraviolet light.

β-GLUCURONIDASE — Colorimetric procedure; for serum or urine, at 550 nm. ..... No. 325
Based on measurement of phenolphthalein liberated from phenolphthalein mono-β-glucuronic acid.

γ-GLUTAMYL TRANSPEPTIDASE (GGTP) — Colorimetric procedure; for serum ..... No. 545
or plasma, at 545 nm. Based on measurement of p-nitroaniline liberated from γ-glutamyl-p-nitroanilide.

γ-GLUTAMYL TRANSPEPTIDASE (GGTP) — Kinetic procedure; for serum or ..... No. 415
plasma, at 405 nm. Based on rate of formation of p-nitroaniline from γ-glutamyl-p-nitroanilide.

GLUTATHIONE REDUCTASE DEFICIENCY — Fluorescence screening procedure, ... No. 190
for erythrocytes. Rate of NADPH consumption is noted by visual observation of loss in fluorescence under long wave ultraviolet light. All reagents conveniently pre-standardized in a single vial.

"GOOD" BUFFERS — A series of amine buffers with a pH range of 5.5-11.0. For ..... No. 107
research use only.

HDL CHOLESTEROL AND TOTAL CHOLESTEROL — Enzymatic procedure, for ..... No. 350-HDL
serum at 500 nm. Following selective precipitation of lipoproteins (LDL and VLDL) with Mg^{++}/phosphotungstate, cholesterol in HDL fraction is determined enzymatically.

HEMOGLOBIN — Colorimetric procedure; for whole blood, at 540 nm. Employs ..... No. 525
modified Drabkin's solution and a stable, freeze dried methemoglobin standard.

HEPARIN — A quantitative clotting procedure; for plasma. Based on heparin's ..... No. 870
effect on activated Factor X (Xa) inhibitors.

HISTOPAQUE®-1077 — A solution containing Ficoll and Sodium Diatrizoate. Facil- ..... No. 1077
itates the isolation and recovery of viable lymphocytes from blood.

α-HYDROXYBUTYRATE DEHYDROGENASE (α-HBD) — Ultraviolet kinetic pro- ..... No. 20-UV
cedure; for serum, at 340 nm.

α-HYDROXYBUTYRATE DEHYDROGENASE (α-HBD) — Colorimetric procedure; ..... No. 495
for serum, at 400-550 nm. Based on measurement of hydrazone derivative of residual substrate, α-ketobutyrate.

INORGANIC PHOSPHORUS — Colorimetric procedure; for serum or urine, at ..... No. 670
620-700 nm. Based on the Fiske and SubbaRow procedure.

IRON AND TOTAL IRON-BINDING CAPACITY — Colorimetric procedure; for ..... No. 585
serum, at 560 nm. Based on the reaction of ferrous ions with ferrozine to produce a magenta colored complex.

ISOCITRATE DEHYDROGENASE (ICD) — Colorimetric procedure; for serum, ... No. 176
at 380-400 nm. Based on measurement of hydrazone derivative of α-ketoglutarate formed from isocitrate.

ISOCITRATE DEHYDROGENASE (ICD) — Ultraviolet kinetic procedure; for serum, ... No. 153-UV
at 340 nm.

17-KETOSTEROIDS (17-KS) — Colorimetric procedure; for urine, at 520-550 nm. ..... No. 17
Based on the Zimmermann reaction.

17-KETOSTEROIDS (17-KS) — Colorimetric procedure; for urine, at 480, 520 and ..... No. 270
560 nm. Based on a simplified, rapid extraction and improved Zimmermann reaction.

L-(+)-LACTIC ACID — Ultraviolet enzymatic procedure; for blood, at 340 nm. ..... No. 826-UV

LACTATE DEHYDROGENASE (LDH) — Colorimetric procedure, for serum, urine ..... No. 500
or cerebrospinal fluid, at 400-550 nm. Based on the Berger-Brodia method.

LACTATE DEHYDROGENASE (LDH) — Ultraviolet kinetic procedure; for serum, ... No. 225-UV
at 340 nm. Based on method of Wacker et al.

LACTATE DEHYDROGENASE (LDH) — Ultraviolet kinetic procedure; for serum, ... No. 340-UV
at 340 nm. Based on the Wroblewski-LaDue method.

LACTATE DEHYDROGENASE (LDH) ISOENZYMES — Colorimetric procedure ..... No. 705-EP
following electrophoresis on cellulose acetate or agarose gels. Based on the reduction of tetranitroblue tetrazolium.

LEUCINE AMINOPEPTIDASE (LAP) — Colorimetric procedure for serum and ..... No. 251
urine, at 540-620 nm. Modification of the Goldberg and Rutenburg procedure.

LIPASE — Titrimetric procedure; for serum (Sigma-Tietz method). Based on titra- ..... No. 800
tion of liberated fatty acids from a standardized olive oil substrate.

MALATE DEHYDROGENASE (MDH) — Ultraviolet kinetic procedure; for serum, ... No. 340-UV
at 340 nm. Based on the Siegel-Bing method.

NBT BLOOD TEST — Histochemical procedure; for blood smears. Based on reduc- ..... No. 840
tion of NBT to highly colored formazan deposits by leukocytes in the presence of bacterial infection. For research use only.

5'-NUCLEOTIDASE — Colorimetric procedure, for serum, at 660 nm. Based on ..... No. 675
the Dixon and Purdom method.

ORNITHINE and/or CARBAMYL PHOSPHATE — Colorimetric procedure; at ..... No. 22
490 nm. Measures citrulline formed by the action of ornithine carbamyl transferase. For research use only.

ORNITHINE CARBAMYL TRANSFERASE (OCT) — Colorimetric procedure, for ..... No. 108
serum, at 400-420 nm. Nesslerization of ammonia formed from decomposition of carbamyl arsenate.

Discrete Analyzer Applications Included

SIGMA
Technical Bulletin

No. 226-UV

Revised August 1982

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SUMMARY AND EXPLANATION OF TEST

The dehydrogenases constitute a group of enzymes that catalyze biomolecular oxidation-reduction reactions between a metabolite and the pyridine nucleotide system. Lactate Dehydrogenase (LD, L-lactate: NAD oxidoreductase, EC 1.1.1.27) catalyzes the interconversion of lactate and pyruvate with NAD as the hydrogen acceptor.[1] The enzyme is widely distributed in animal tissues with the highest concentration present in the liver, heart, and skeletal muscles.[2] Acute destruction of tissue results in the release of LD into the blood. Serum LD levels begin to increase about 8 to 12 hours following myocardial infarction, and the level remains elevated for 7 to 12 days.[3] In myopathic diseases such as pseudohypertrophic, facio-scapular-humoral and limb-girdle muscular dystrophy, values 30 times normal may be present.[4] Elevated levels of serum LD activity are also observed in cirrhosis of the liver, hepatitis, hepatoma, hepatic metastases and following pulmonary embolism.[5,6] Leukemia, lymphomas, and various other neoplastic diseases contribute to the long list of pathologic conditions that are accompanied by increased serum LD levels.[7,8,9]

LD activity in serum is usually determined either colorimetrically, by complexing the pyruvic acid produced with a chromogen,[10] or by an ultraviolet (UV) kinetic method based on the rate of NADH formation or disappearance. Since LD catalyzes the interconversion of pyruvate and lactate, under controlled conditions either reaction may be used to assay LD activity kinetically. The first UV kinetic method for the determination of LD activity in serum, described by Wroblewski and LaDue,[11] was based on the classical assay of Kubowitz and Ott,[12] utilizing the pyruvate to lactate reaction. Although this reaction is kinetically more rapid than lactate to pyruvate there are favorable aspects of the latter which suggests it as the preferred method.[13] No preincubation of serum with reactants is required, and the reaction is linear over a wider range of enzyme levels, thereby minimizing the need for reassaying sera with high LD activities.

The method described in this bulletin is based on the lactate to pyruvate reaction described by Warburg et al., in 1935[1] and later by Wacker et al.[14] Reaction conditions have been optimized to provide greater sensitivity and linearity over a wider range of enzyme activity.

An electrophoretic technique for determination of LD isoenzymes is outlined in Sigma Technical Bulletin No. 705-EP. Also available are colorimetric and UV kinetic procedures based on the pyruvate to lactate reaction (Sigma Technical Bulletins No. 500 and 340-UV, respectively).

PRINCIPLE

Lactate Dehydrogeanse (LD) catalyzes the following reversible oxidation-reduction reaction:

$$\text{Lactate} + \text{NAD} \xleftrightarrow{\text{LD}} \text{Pyruvate} + \text{NADH}$$

Formation of NADH is accompanied by an increase in Absorbance at 340 nm, the rate of which is proportional to serum LD activity.

Abbreviations:

NAD — Nicotinamide adenine dinucleotide
NADH — Nicotinamide adenine dinucleotide, reduced form

REAGENTS

For In Vitro Diagnostic Use

See Price List section for reagent pricing and packaging configurations

A. LD-L REAGENT A

	Stock No. 226-2	Stock No. 226-10
Contains: β-NAD	32 μmol	153 μmol

See "Procedure" sections for reconstitution instructions.

Store in refrigerator at 0-5°C. Reagent label bears expiration date.

B. LD-L REAGENT B, Stock No. 226-1

Contains L-Lactate, 60 mmol/L in Tris Buffer, 106 mmol/L, pH 9.0, 30°C. Also contains Sodium Azide, 0.05%. See warning below.

Suitable for use in the absence of visible microbial growth.

Store in refrigerator at 0-5°C.

C. LD-L REAGENT B CONCENTRATE, Stock No. 226-3*

Contains L-Lactate, 74 mol/L and Tris Buffer, 132 mmol/L, pH 9.0, 30°C. Also contains Sodium Azide, 0.05%. See warning below.

Suitable for use in the absence of visible microbial growth.

Store in refrigerator at 0-5°C.

*For centrifugal analyzers

WARNING: Stock No. 226-1 and 226-3 contain Sodium Azide. Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide buildup.

OPTIONAL

SIGMA ENZYME CONTROL 2-N, Product No. S 2005

An assayed preparation containing several enzymes, including LD, at normal levels.

Store in refrigerator at 0-5°C.

SIGMA ENZYME CONTROL 2-E, Product No. S 1005

An assayed preparation containing several enzymes, including LD, at elevated levels.

Store in refrigerator at 0-5°C.

SPECIMEN COLLECTION AND PREPARATION[15,16,17]

The patient should be in the fasting state and, if possible, not have been engaged in strenuous exercise. Blood must be obtained with minimum stasis to prevent spurious elevation of serum LD originating from muscle cells. Prolonged clotting in plastic tubes can result in intracellular enzyme leakage from blood components. Therefore, blood should be allowed to coagulate in glass tubes.

Hemolysis must be avoided. Serum should be used rather than plasma to prevent possible inhibition of LD by anticoagulants. Heparin is not an inhibitor of LD, but plasma obtained with this anticoagulant may exhibit factitiously high or low LD activity depending upon the degree of platelet destruction. If heparinized plasma must be used, the sample should be centrifuged at 1200 x g for at least 10 minutes.

Stability:[18] When the test is not performed the same day, serum should be stored at room temperature and assayed within 10 days. Samples must not be frozen or exposed to temperatures above 37°C, since thermolabile LD isoenzymes will be inactivated.

Interfering Substances: Administration of certain drugs and medications has been shown to influence serum levels of LD activity. A comprehensive review has been prepared by Young, et al.[19] and should be consulted for further specific information.

INSTRUMENTS AND MATERIALS REQUIRED

Instrument: A narrow-bandwidth spectrophotometer capable of measuring the absorbance at 340 nm is required for this procedure.

Materials:

Cuvets: Square, 1-cm lightpath
Pipets: 0.1, 5, and 10 mL graduated
Graduated cylinder, 50 mL
Centrifuge
Timer

MANUAL PROCEDURE

The temperature of the reaction mixture should be maintained at 30°C or some other constant temperature. Refer to "Temperature Correction" section.

1. To:	LD-L Reagent A, Stock No. 226-2	To:	LD-L Reagent A, Stock No. 226-10
Add:	6.5 mL LD-L Reagent B, Stock No. 226-1	Add:	31 mL LD-L Reagent B, Stock No. 226-1

2. Cap vial and invert several times until reagent is completely dissolved.

   Reconstituted reagent is stable for 8 hours at room temperature, 5 days in the refrigerator at 0-5°C or at least 7 days stored below 0°C.

3. Pipet 3.0 mL of above reagent into cuvet and bring to reaction temperature.

4. Add 0.1 mL specimen. Mix by inversion.

5. Place cuvet in constant temperature cuvet compartment at 30°C.

6. Read and record the absorbance (A) at 340 nm vs water as reference.

   This is the INITIAL A.

7. Exactly 3 minutes later, again read and record the absorbance.

   This is the FINAL A.

CALCULATIONS:

ΔA per 3 min = FINAL A — INITIAL A

LD-L (U/L) = ΔA per 3 min x 1660 x TCF

NOTES:

1. If ΔA per 3 min is greater than 0.300, repeat the test using 0.05 mL of serum and multiply your results by 2.
2. You may demonstrate that the reaction is linear by taking readings at 1-minute intervals after the INITIAL A. The absorbance changes for each minute should be essentially the same.
3. If a recording spectrophotometer is available, the time required for the determination may be considerably reduced. Obtain the ΔA per minute directly from your curve. Then, multiply ΔA per min x 3.

CALIBRATION:

This procedure is standardized on the basis of the millimolar extinction coefficient of NADH, taken as 6.22 at 340 nm. Formation of pyruvate in the LD reaction is accompanied by the reduction of NAD to NADH at a rate proportional to the LD activity present. Measurement of the resulting increase in absorbance (A) serves to quantitate LD enzyme activity.

The performance characteristics of the instrument should be frequently monitored to assure reliable readings. Calibration standards for this purpose are generally available from the instrument manufacturer or from the National Bureau of Standards.

At 30°C, the lowest and maximum practical limits of LD activity that may be measured is approximately 10 U/L and 500 U/L, respectively.

TEMPERATURE CORRECTION:

If the temperature of the reaction is 30°C, no temperature correction is required in the calculations. If the assay is performed at a temperature other than 30°C, a Temperature Correction Factor (TCF) is used in calculating the enzyme activity.

Table 1: TEMPERATURE CORRECTION FACTORS (TCF)

Cuvet Temp. °C	TCF	Cuvet Temp. °C	TCF
20	2.25	30	1.00
21	2.07	31	0.92
22	1.91	32	0.86
23	1.76	33	0.79
24	1.62	34	0.73
25	1.49	35	0.68
26	1.37	36	0.63
27	1.27	37	0.59
28	1.17	38	0.54
29	1.08	39	0.50

QUALITY CONTROL:

Commercially available quality control material of known activity should be assayed concurrently with patient specimens to assure proper performance of the procedure. Sigma Enzyme Control, 2-N, Product No. S 2005, and Sigma Enzyme Control, 2-E, Product No. S 1005, are assayed preparations which contain several enzymes, including LD, in the normal and elevated ranges, respectively.

RESULTS, MANUAL PROCEDURE

$$\text{LD-L (U/L)} = \frac{\Delta A/3\text{ min} \times 3.1 \times 1000 \times \text{TCF}}{6.22 \times 0.1 \times 3} = \Delta A/3\text{ min} \times 1660 \times \text{TCF}$$

Where:

- $\Delta A/3$ min = FINAL A — INITIAL A
- 3.1 = Total reaction volume (mL)
- 1000 = Conversion of activity from U/mL to U/L
- TCF = Temperature Correction Factor (TCF = 1.00 at 30°C. For other temperatures see Table 1)
- 6.22 = Millimolar absorptivity of NADH at 340 nm
- 0.1 = Volume (mL) of serum in cuvet
- 3 = Conversion of $\Delta A/3$ min to ΔA/min

NOTE: If your cuvet does not have a 1-cm lightpath, divide your results by the actual lightpath (cm).

Example: The following absorbance values were obtained using a 1-cm lightpath at 30°C.

- INITIAL A = 0.320
- FINAL A = 0.429
- $\Delta A/3$ min = 0.109
- TCF = 1.00

LD-L (U/L) = 0.109 x 1660 x 1.00 = 181

UNIT DEFINITION:

One International Unit (U) of an enzyme will transform 1 micromole of substrate per minute. International Units obtained by this procedure cannot be compared directly with International Units obtained by another procedure unless methods and reaction conditions are the same.

LIMITATIONS OF PROCEDURE

Refer to "Specimen Collection and Preparation" section for interfering substances.

EXPECTED VALUES

Serum specimens from 130 clinically healthy adult males and females were assayed for LD activity by the method described in this bulletin.

NORMAL RANGE

Males	50 to 166 U/L (30°C) 80 to 285 U/L (37°C)
Females	60 to 132 U/L (30°C) 103 to 227 U/L (37°C)

ABBOT BICHROMATIC ANALYZER (ABA)-100

REAGENT PREPARATION

LD-L REAGENT A, Stock No. 226-2 (≈ 25 ABA Assays)

Reconstitute 1 vial with 6.5 mL LD-L Reagent B, Stock No. 226-1.

LD-L REAGENT A, Stock No. 226-10 (≈ 120 ABA Assays)

Reconstitute 1 vial with 31 mL LD-L Reagent B, Stock No. 226-1.

Reconstituted reagent is stable for 8 hours at room temperature, 5 days in the refrigerator at 0-5°C or at least 7 days stored below 0°C.

INSTRUMENT SETTINGS AND CONDITIONS

Filters	380/340 L
Temperature	30 or 37°C
Mode Selector	Rate
Reaction Direction	Down
Zero	+0800
Decimal	0000
Syringe Plate	1:51
Sample Size	5 µL
Carousel Revolution	3
Incubation Time	5 min
Calibration Factor	$\frac{5100}{FF}$

FF = Filter factor derived from the molar extinction of NADH with the specific filters used. The specific FF is recorded on each filter set.

PROCEDURE

1. Fill the syringe plate with reagent ensuring that there are no air bubbles.
2. Place approximately 50 µL of water in cup 01 and 50 µL control sera and samples in subsequent cups.
3. Attach reagent-sample probe to the end of the boom arm. Adjust carousel to position 00.
4. Depress the STOP and then the RUN button. LD results in U/L will be printed out during the 3rd revolution.

NOTE: Samples with values printed in red should be diluted with an equal volume of saline and reassayed. The printed results are multiplied by 2.

ABBOTT BICHROMATIC ANALYZER (ABA)-100 (Continued)

RESULTS

Explanation of Calibration Factor (CF): $CF = \frac{5100}{FF}$

The Factor 5100 is derived as follows: $\frac{0.5 \times 0.255 \times 1000}{5 \times 0.005} = 5100$

Where:

0.5 = Scaling factor used to obtain ΔA
0.255 = Total reaction volume (mL)
1000 = Conversion from U/mL to U/L
5 = Time intervals (min)
0.005 = Sample (mL)

For example, if the FF on your filter set is 4.61: $CF = \frac{5100}{4.61} = 1106$

Kit No.	Approximate ABA Assays
226-A	375
226-B	1200

REAGENT PREPARATION

LD-L REAGENT A, Stock No. 226-2, (≈ 25 VP Assays)

Reconstitute 1 vial with 6.5 mL LD-L Reagent B, Stock No. 226-1.

LD-L REAGENT A, Stock No. 226-10, (≈ 120 VP Assays)

Reconstitute 1 vial with 31 mL LD-L Reagent B, Stock No. 226-1.

Reconstituted reagent is stable for 8 hours at room temperature, 5 days in refrigerator at 0-5°C, or at least 7 days stored below 0°C.

INSTRUMENT SETTINGS AND CONDITIONS

Test Number	Enter a test number that does not currently exist in the VP's P-file.	Code xx
Test Name	LD	15
Temperature	30 or 37°C	30 or 37
Filter Value	340/380	43
Unit of Measurement	U/L	11
Dilution Ratio	1:101	101
Rev Time	2	2
Aux Disp	No	N
FRR?	Yes	Y
Reaction Up?	Yes	Y
Standards	No	N
Reagent Blank	Yes	Y
Assay Factor	50500	50500
End Point?	No	N
Begin Print Revolutions	3	3
No. Print Revolutions	1	1
Initial ABS	0.36	.36
Up Limit	Yes	Y
Substrate Depletion	0.4	.4
Reagent Degradation	50	50

PROCEDURE

1. Fill the syringe plate with reagent ensuring that there are no air bubbles.
2. Place approximately 50 µL of water in cup 01 and 50 µL control sera and samples in subsequent cups.
3. WASH/PRIME as described in operations manual.

NOTE: When LD activity exceeds 800 U/L, dilute sample with equal volume of saline, repeat assay and multiply results by 2.

Kit No.	Approximate VP Assays
226-A	375
226-B	1200

REAGENT PREPARATION

LD-L REAGENT A, Stock No. 226-2 (≈ 25 MCA Assays)

Reconstitute 1 vial Reagent A, Stock No. 226-2, with 5.2 mL LD-L Reagent B Concentrate, Stock No. 226-3. Cap vial and invert several times until reagent is completely dissolved.

Reconstituted reagent is stable for 8 hours at room temperature, 5 days in the refrigerator at 0-5°C or at least 7 days stored below 0°C.

INSTRUMENT SETTINGS

Loader:

	Setting, %	Volume
Sample Volume	5	5 µL
Total Volume	40	40 µL
Reagent Volume	70	175 µL
Total Volume	74	185 µL
2nd Reagent Volume	00	
2nd Reagent Switch	Off	
Ref. Cuvet Switch	Diluent	

Analyzer:

Enzyme Tape
Program Code 25
Change Program No. 4

1. Factor	14469
2. Lower Limit	50
3. Upper Limit	188
4. Filter Code	1
5. Temperature Code (30°C)	1
6. Delay Time (sec)	30
7. Interval Time (sec)	20
8. Maximum ΔA	0.15
9. Maximum A	Enter
10. Start Mode	0

DISCRETE ANALYZER APPLICATIONS

INSTRUMENTATION LABORATORY MULTISTAT III (MCA)

(Continued)

PROCEDURE

1. Fill the syringes with water ensuring that there are no air bubbles.
2. Place approximately 200 µL water in Cup 1 and 200 µL controls and samples in subsequent Cups.

NOTE: Samples with values greater than 800 U/L should be diluted 1:1 with saline and reassayed. Multiply your results by 2.

RESULTS

LD (U/L) = ΔA per min x Factor = ΔA per min x 14469

The Factor 14469 is derived as follows: $\text{Factor} = \frac{1000 \times 0.225}{6.22 \times 0.005 \times 0.5} = 14469$

Where:

1000 = Conversion from mL to L
0.225 = Total reaction volume, mL
6.22 = Millimolar absorptivity of NADH at 340 nm
0.005 = Sample volume, mL
0.5 = Length of lightpath, cm

The reagents listed under Reagent Preparation may be used to perform LD determination manually as follows:

1. To 1 vial LD-L Reagent A, Stock No. 226-2, add:

   5.2 mL LD-L Reagent B Concentrate, Stock No. 226-3 and 1.3 mL water. Cap vial and invert several times until reagent is completely dissolved.

2. Follow Manual Procedure starting with Step 3. Substitute reagent prepared in Step 1 above.

Kit No.	Approximate MCA Assays
226-C	375

REAGENT PREPARATION

LD-L REAGENT A, Stock No. 226-2 (= 20 Centrifichem Assays)

Reconstitute 1 vial Reagent A, Stock No. 226-2, with 5.2 mL LD-L Reagent B Concentrate, Stock No. 226-3. Cap vial and invert several times until reagent is completely dissolved.

Reconstituted reagent is stable for 8 hours at room temperature, 5 days in the refrigerator at 0-5°C or at least 7 days stored below 0°C.

INSTRUMENT SETTINGS

PIPETTOR:	
Reagent	250 µL
Sample	10 µL
Sample + Diluent	60 µL
ANALYZER:	
T_0 (sec)	.30
ΔT (min)	01:00
ABN ABS	1.5 U
Blank	Auto
Test Mode	Rate
Print Out	Conc
CF	4984
No. Prints	3
Test Code	00
Temperature	30°C
Filter	340 nm

NOTES:

1. When substrate depletion is indicated by an absorbance flag or when values are greater than 2100 U/L at 30°C, samples should be diluted (1 part sample plus 9 parts saline) and reassayed. Multiply your results by 10.

2. The 3 values printed out for each sample should be consistent. The allowable difference between the highest and lowest values should be established for each individual test by each laboratory.

RESULTS

$$LD\ (U/L) = \Delta A/min \times CF = \Delta A/min \times 4984$$

The CF 4984 is derived as follows: $CF = \frac{1000 \times 0.310}{6.22 \times 0.010} = 4984$

Where:

1000 = Conversion from mL to L
0.310 = Total reaction volume, mL
6.22 = Millimolar absorptivity of NADH at 340 nm
0.010 = Sample volume, mL

The reagents listed under Reagent Preparation may be used to perform LD determination manually as follows:

1. To 1 vial LD-L Reagent A, Stock No. 226-2, add:

   5.2 mL LD-L Reagent B Concentrate, Stock No. 226-3 and 1.3 mL water. Cap vial and invert several times until reagent is completely dissolved.

2. Follow Manual Procedure starting with Step 3. Substitute reagent prepared in Step 1 above.

Kit No.	Approximate Centrifichem Assays
226-C	300

REAGENT PREPARATION

LD-L REAGENT A, Stock No. 226-2 (≈6 Assays)

Reconstitute 1 vial with 6.5 mL LD-L Reagent B, Stock No. 226-1.

LD-L REAGENT A, Stock No. 226-10 (≈30 Assays)

Reconstitute 1 vial with 31 mL LD-L Reagent B, Stock No. 226-1.

Reconstituted reagent is stable for 8 hours at room temperature or 5 days in the refrigerator at 0-5°C or at least 7 days stored below 0°C.

GILFORD SYSTEM 3500 INSTRUMENT SETTINGS

A. Using Gilford LD-L Program Card (Part No. 19080x22H).

Wavelength	340 nm
Temperature	30°C
Movable Tower Setting	2
Dispenser A	
Sample Syringe Volume	100 µL/0.1 mL Syringe (NO STOP)
Reagent Syringe Volume	1.0 mL/2.5 mL Syringe (40% STOP)
Dispenser B (Not Used)	
Dispenser C (Not Used)	

B. Using Worthington/Gilford LDH (L-P) Program Card (Part No. 15831x43H)

Wavelength	340 nm
Temperature	30°C
Movable Tower Setting	4
Dispenser A	
Sample Syringe Volume	50 µL/0.1 mL Syringe (50% STOP)
Reagent Syringe Volume	0.7 mL/2.5 mL Syringe (28% STOP)
Dispenser B (Not Used)	
Dispenser C (Not Used)	

DISCRETE ANALYZER APPLICATIONS

GILFORD SYSTEM 3500 ANALYZER (Continued)

PROCEDURE

Refer to Operation Instructions Manual, Part No. 25038 x 113 for complete details.

1. Connect reagent vial to dispenser A and fill syringes ensuring that there are no air bubbles.
2. Place approximately 0.5 mL water in the first two sample cups and 0.5 mL control sera and samples in subsequent cups.
3. Load sample racks as illustrated on the transport island.
4. Place the End-of-Run Detector (EORD) over the first empty sample cup.
5. Connect reagent/sample dispenser probe to tower.
6. Insert LD Program Card, side 1, then side 2.
7. Depress RUN push button and program instrument as described on tape printout.
8. Depress RUN push button after each step in the printout has been completed.
9. LD values will be printed out in U/L at 30°C.

NOTE: Samples with values greater than 300 U/L should be diluted with an equal volume of saline and reassayed. The results are multiplied by 2.

Kit No.	Approximate Gilford System 3500 Assays
226-A	95
226-B	300

SPECIFIC PERFORMANCE CHARACTERISTICS

Reproducibility: Ten replicate assays of 3 serum pools yielded mean values of 50, 156 and 716 U/L, with standard deviations of 1.9, 4.3 and 21.5 U/L, and coefficients of variation of 3.8, 2.7 and 3.0%, respectively. Ten replicate assays of 3 serum pools over a 7 day period produced means of 57, 173 and 839 U/L, with standard deviations of 3.2, 4.2 and 25.0 U/L, and coefficients of variation of 3.1, 2.4 and 3.0%, respectively.

Sensitivity: Assuming the limit of sensitivity to represent a change in absorbance at 340 nm of 0.006 A/3 minutes, an LD activity of 10 U/L may be detected using this procedure.

Correlation: 112 sera ranging in activity from 55 to 685 U/L were assayed by the procedure described in this bulletin and by another commercially available procedure also based on the method of Wacker et al.[14] The resulting correlation coefficient was 0.9988 and the regression equation had a slope of 0.9732, and a y-intercept of −0.4 U/L. Mean values were 137 and 133 U/L, respectively, for the two procedures.

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PRICE LIST NO. 226-UV

as of August 1982

Reagents for the Ultraviolet Determination of

LACTATE DEHYDROGENASE

per Sigma Technical Bulletin No. 226-UV

KITS

Kit No.	Price	Contents — Stock Numbers			
		LD-L Reagent A		LD-L Reagent B	LD-L Reagent B Concentrate
		226-2	226-10	226-1	226-3*
226-A	$19.50	15 x 6.5 ml	—	110 ml	—
226-B	49.60	—	10 x 31 ml	3 x 110 ml	—
226-C*	19.50	15 x 5.2 ml	—	—	1 x 80 ml

Reagents are available in kits only and cannot be ordered separately.
*For centrifugal analyzers.

OPTIONAL REAGENTS

Product No.	Item	Quantity	Price
S 1005	SIGMA ENZYME CONTROL 2-E Assayed preparation containing elevated enzyme levels. Reconstitutes to 3 ml.	10 vials	$23.25
S 2005	SIGMA ENZYME CONTROL 2-N Assayed preparation containing normal enzyme levels. Reconstitutes to 3 ml.	10 vials	23.25

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Sigma Chemical Company
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St. Louis, MO 63178

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Revised July 1980
Reissued March 1982

The Quantitative Colorimetric Determination of

LACTATE DEHYDROGENASE (LDH)

at 400-550 nm

By
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SUMMARY AND EXPLANATION OF TEST

The colorimetric procedure for the determination of Lactate Dehydrogenase (LDH, Lactate dehydrogenase: EC 1.1.1.27) in serum was originally published in 1957 by Berger and Broida in Sigma Technical Bulletin No. 500. More than a year later, Cabaud and Wroblewski published the Sigma method in the American Journal of Clinical Pathology. As a result, this technique is frequently termed the "Cabaud-Wroblewski" procedure.[1] It is noteworthy that a U.S. patent and several foreign patents have been issued to Sigma covering this method.

In developing the procedure described in this bulletin, it was necessary to perform LDH determinations on a large number of sera with low, normal and abnormal levels according to the Sigma No. 340-UV kinetic procedure for LDH. These same sera were simultaneously assayed by the presented method to obtain Absorbance values for each sample. A curve was then constructed by plotting the LDH activity of each serum assayed by the ultraviolet method (Sigma Procedure No. 340-UV) vs the Absorbance obtained with each serum as assayed by the method described in this bulletin. It was then possible to relate LDH activity measured by the ultraviolet technique (Sigma Procedure No. 340-UV) to Absorbance values obtained colorimetrically (Sigma Procedure No. 500). LDH activities were, in turn, correlated with the amounts of Pyruvate Substrate, Stock No. 500L-1, which were used to prepare the calibration curve as illustrated in this bulletin.

Since the method requires only a small sample (0.1 ml of a 6-fold serum dilution) it is suitable for pediatric or veterinary purposes. While this colorimetric method will not provide an individual test result as rapidly as with Sigma Procedure No. 340-UV, it is well suited for assaying a large series of samples. As many as 20-30 tests can be performed per hour.

Unless otherwise specified, LDH refers to the total Lactate Dehydrogenase of serum, which is the sum of all fractions (LDH isoenzymes) known to exist in serum. Most of the literature relating to the diagnostic value of serum LDH is based upon the determination of total circulating enzyme activity. Fractionation procedures have been proposed that seem to enhance the diagnostic value by measuring LDH isoenzymes to delineate their tissue origin. Sigma now offers a method for the electrophoretic detection of LDH isoenzymes that is described in Technical Bulletin No. 705-EP (available on request).

Amador and coworkers[2] have raised some objections to the colorimetric assay of LDH that merit comment. According to these investigators, colorimetric methods ignore significant blanks. Studies in our laboratories do not confirm this observation.[3] Although Amador et al.[2] allude to the inhibitory concentrations of NADH that are used for colorimetric LDH measurement, no data demonstrating this effect are presented. As this group points out, the reaction does not follow zero-order kinetics and, therefore, it is not strictly proportional to LDH activity. However, the reaction does not have to be strictly linear as long as a fixed time is used to measure activity, which, in turn, is calibrated against a zero order reference method. Sigma also offers a kinetic ultraviolet method for LDH assay based on optimized forward reaction of Wacker et al. (N Engl J Med 255:449, 1956). For details of this technique request Sigma Technical Bulletin No. 226-UV.

PRINCIPLE

LDH is an enzyme which catalyzes the following reversible reaction:

$$\text{Pyruvic Acid} + \text{NADH} \underset{}{\overset{\text{LDH}}{\rightleftharpoons}} \text{Lactic Acid} + \text{NAD}$$

The reaction equilibrium strongly favors reduction of pyruvate to lactate. The rate of conversion is proportional to the amount of LDH. Pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form an intensely colored "hydrazone", which has peak Absorbance over the broad wavelength range of 400-550 nm. Lactic acid, NAD and NADH do not absorb significantly in this range. Therefore, by using Pyruvate Substrate, Sigma Standardized, Stock No. 500L-1, whose hydrazone always yields the same Absorbance (A), it is possible to accurately measure changes resulting from the conversion of pyruvic acid to lactate acid due to LDH activity. The amount of pyruvate remaining after incubation is inversely proportional to the amount of LDH activity in the sample.

REAGENTS

For In Vitro Diagnostic Use

A. PYRUVATE SUBSTRATE, SIGMA STANDARDIZED, Stock No. 500L-1

Contains buffered sodium pyruvate, 0.75 mmol/l, pH 7.5 at 25°C.

Chloroform added as preservative.

Store in refrigerator 0-5°C. Reagent label bears expiration date.

B. SIGMA COLOR REAGENT, Stock No. 505-2

Contains 2,4-dinitrophenylhydrazine, approximately 20 mg/100 ml, in 1 N Hydrochloric Acid.

Store dark in refrigerator at 0-5°C.

NOTES:
1. Solution is normally clear and light yellow in color. If plastic bottle is stained yellow (due to reaction with 2,4-dinitrophenylhydrazine) solution should be discarded.
2. This reagent has been standardized to yield reproducible calibration curves.
3. To check reagent, set up calibration Tube No. 1 and compare with previous absorbance.

C. NADH, Stock No. 340-101

Contains β-nicotinamide adenine dinucleotide, reduced form, disodium, 1 mg (1.28 μmol).

Store at room temperature. Reagent box label bears expiration date.

NOTES:
1. Store dry in the dark. If vial contents have absorbed water to the point of appearing yellow to brown, do not use.

2. Since NADH is very unstable after dissolving , we do not recommend storage of a stock solution. This is why Sigma offers the single-determination vial. If multiple assays are performed in a single day, various sizes of larger preweighed vials are recommended and will greatly reduce the average assay cost. For even greater savings, when large numbers of LDH assays are performed, the high purity Sigma NADH, which is available in various bulk packages, may be advantageous. However, because of possible inhibitor formation, even in the bottle, the likelihood of erroneous results is increased. Contact with moisture can be very destructive to NADH.
3. The simplest way to prepare incubation solution is to dissolve the NADH in Pyruvate Substrate, Sigma Standardized Stock No. 500L-1, to yield a solution of 1 mg NADH per ml. Place 1 ml of this solution into an incubation tube and proceed with the addition of sample in Step 3 of "Procedure (Total LDH, Serum/CSF)" section.

See Price List following "Bibliography" section for reagent pricing and packaging configurations.

REQUIRED BUT NOT PROVIDED:

D. SODIUM HYDROXIDE SOLUTION, 0.40 N

Prepare by dissolving 16 g of Sodium Hydroxide, Anhydrous in 1000 ml of CO_2-free water.

Accurately standardize the solution at 0.40 ± 0.01 N.

Store in small polyethylene or pyrex bottles to avoid frequent exposure to atmosphere.

WARNING: Causes severe burns to skin and eyes.

NOTE: Sigma Stock No. 505-8, 16 g Vial, is available if you do not have your own laboratory Sodium Hydroxide.

OPTIONAL:

SIGMA ENZYME CONTROL 2-E, Product No. S 1005

An assayed preparation containing LDH and other enzymes at elevated levels.

Store in refrigerator at 0-5°C.

SIGMA ENZYME CONTROL 2-N, Product No. S 2005

An assayed preparation containing LDH and other enzymes at normal levels.

Store in refrigerator at 0-5°C.

DIALYSIS SACKS, Stock No. 250-11

Washed to remove possible inhibitors and thio compounds.

Packed in 0.2% benzoic acid solution.

Store at room temperature.

NOTE: Rinse sacks in water or buffer before use.

SPECIMEN COLLECTION AND PREPARATION

SERUM:

Blood is drawn into a sample tube which does not contain anticoagulant and is allowed to clot. As soon as the clot is formed, the serum is separated. Hill and Levi[4], MacDonald et al.[5] and Snodgrass et al. [6], have reported that LDH in serum is essentially stable for several days at 4°C. However, study of isoenzymes [7,8] suggests marked variation in stability of the various fractions at low temperatures. Freezing might be worse than 4°C. LDH, of hepatic origin, is reported to be highly sensitive to freezing. Kreutzer and Fennis[7] found that all isoenzymes (total LDH activity) remain virtually unchanged for 7 days at room temperature (16-22°C). This is consistent with the earlier report of Lazaroni et al.[9] Therefore it is recommended that serum be stored at room temperature prior to assay. Hemolysis must be avoided since the concentration of LDH in erythrocytes is 100 times that in serum. Hyperbilirubinemia does not affect LDH assay, but may mask hemolysis. Mild hyperlipemia does not affect results.[4] Friedel and Mattenheimer[10] have reported an increase in LDH and other enzyme activities arising from platelets lysed during the clotting of blood. This increase is apparently more significant in blood from small animals than from human blood.

PLASMA:

The use of plasma in measuring LDH activity is not recommended .[10] There are a number of contradictions in the literature with regard to the effect of heparin and citrate on LDH activity. Oxalate has been reported to inhibit LDH.

URINE:

One or more inhibitors of LDH are normally found in urine. However, these substances may be removed by dialysis.[11,12] For details on collection and preparation refer to "Procedure (Urine)" section. The stability of urinary LDH has not been reported. However, it is suggested that the 8-hour specimen be processed with a minimum of delay to prevent bacterial growth which can cause false elevations or false low values caused by enzyme inactivation.[12]

CEREBROSPINAL FLUID (CSF):

LDH is reportedly stable in CSF for 4-6 hours at room temperature and 2 weeks at 4°C.[13]

INSTRUMENTS AND MATERIALS REQUIRED

Instrument:

Practically any photoelectric colorimeter that transmits light ranging from 400-550 nm can be used. The procedure can be performed satisfactorily on a Coleman Jr. II Model 6/20, Gilford, Beckman DU, Cary, Zeiss, etc.

Materials:

Pipets, 0.1, 1.0, 10.0 ml, serologic
Pipet safety control bulb
Water bath, 37°C
Timer
Centrifuge

PROCEDURE (Total LDH, Serum/CSF)

1. Dilute 1 part of serum with 5 parts of water (6-fold dilution).

   NOTE: Undiluted CSF can be substituted for diluted serum, but final results must be divided by 6.

2. Pipet 1.0 ml Pyruvate Substrate, Sigma Standardized, Stock No. 500L-1, into bottom of an NADH Vial, Stock No. 340-101.

   or

   Pipet 1.0 ml Pyruvate Substrate, Sigma Standardized, Stock No. 500L-1, containing 1 mg/ml NADH, into an incubation tube (see Reagent C under "Reagents" section).

   Place in water bath at 37°C for a few minutes.

3. Add 0.10 ml diluted serum or 0.10 ml undiluted CSF from Step 1.

   Mix gently, start timer and replace in 37°C water bath.

4. Exactly 30 minutes after adding sample, remove from water bath and add to each vial:

   1.0 ml Sigma Color Reagent, Stock No. 505-2.

   Mix well by swirling (stops reaction and starts color development).

   Allow to remain at room temperature (25 ± 5°C).

5. Twenty minutes after Step 4, add:

   10.0 ml 0.40 N Sodium Hydroxide Solution (Reagent D) to each vial.

   Cap and mix well by inversion.

   Transfer to cuvets.

6. Wait at least 5 minutes after adding the Sodium Hydroxide Solution (Reagent D), but not more than 30 minutes, then read and record Absorbance (A) or % Transmission (%T) vs water as reference. Use same wavelength and same instrument employed in preparing your calibration curve.

   NOTE: Pyruvate is not usually present in serum in sufficient amount to affect the results significantly. While it is still possible that some pathologic condition may have an effect, the routine use of a specimen BLANK is not warranted.

CALCULATIONS:

Serum LDH values are obtained directly from your calibration curve. Results for CSF must be divided by 6.

NOTE: If a value greater than 2000 Berger-Broida (B-B) Units/ml is obtained, further dilute an aliquot of diluted serum with 4 parts of water and repeat the test. The result from your calibration curve must then be multiplied by 5.

PROCEDURE (Urine)

Dialysis

1. Obtain an 8-hour or overnight urine specimen.

   a) Measure and record volume (ml).

      NOTE: Reject specimens containing blood (may yield falsely elevated values).

   b) Centrifuge a 12-15 ml sample for 5 minutes to separate the sediment.

      Examine sediment microscopically for bacteria. If more than 10 bacteria per high-power field are present, a falsely high LDH value may be obtained.

2. Prepare a Dialysis Sack, Stock No. 250-11, by thorough rinsing in water or buffer to remove benzoic acid solution. Squeeze out excess liquid, but do not dry.

   a) Pipet 10 ml urine supernatant into sack.

   b) Twist down excess tubing to expel air and tie a secure knot about 3 inches above fluid level.

3. Place sack in a 250-ml Erlenmeyer flask.

   a) Fasten length of soft rubber tubing to cold water tap.

      Adjust water to a flow of at least 50 ml per minute.

   b) Insert rubber tube into flask so that end is at bottom.

      Set flask so that overflow runs down drain.

4. Remove sack from flask and blot excess water from outside sack with paper towel.

   a) Cut off top knot and transfer all urine from sack into a 25-ml graduated cylinder.

   b) Record volume to nearest 0.5 ml. Divide volume (ml) by 10 and record result as the dialysis dilution factor.

Assay:

Use 0.2 ml of the dialyzed urine in Step 3 of "Procedure (Total LDH, Serum/CSF)" section.

NOTE: Do not make a 6-fold dilution of urine as is done for serum.

CALCULATIONS:

1. Determine LDH activity of dialyzed urine from your serum calibration curve, but divide the result by 12. This value will be the number of B-B Units of LDH per ml of dialyzed urine.

   NOTE: Values greater than 2000 B-B Units/ml should be repeated using 0.05 ml of dialyzed urine. The result obtained from the calibration curve is divided by 3 instead of 12.

2. Urine Total LDH Activity (B-B Units/8 hr) = (B-B Units/ml) x 8-hr Urine Volume (ml) x Dialysis Dilution Factor.

CALIBRATION:

1. Label 6 test tubes or cuvets 1-6 and prepare mixture as shown in columns 2 and 3.

1	2	3	4	5
Tube No.	Pyruvate Substrate, Stock No. 500L-1	Water (ml)	Absorbance or %T	LDH Activity (B-B Units/ml)
1	1.0	0.1		0
2	0.8	0.3		280
3	0.6	0.5		640
4	0.4	0.7		1,040
5	0.2	0.9		1,530
6	0.1	1.0		2,000

Record wavelength or filter used_____

Values in Column 5 apply only when procedure is performed using 0.1 ml of diluted serum (1 part serum and 5 parts water or buffer). Since NADH does not contribute any appreciable Absorbance, it is omitted from the calibration procedure.

2. Add to each tube:

   1.0 ml Sigma Color Reagent, Stock No. 505-2.

   Mix gently and allow to remain at room temperature (25 ± 5°C).

3. After 20 minutes, add to each tube:

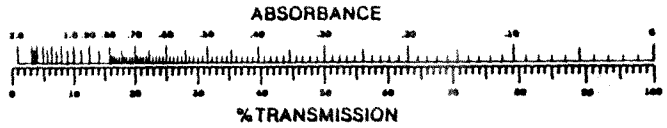
   10.0 ml 0.40 N Sodium Hydroxide Solution (Reagent D).

   Mix well by inversion.

4 Wait at least 5 minutes after adding Sodium Hydroxide Solution (Reagent D), but not more than 30 minutes, then read Absorbance (A) or % Transmission (%T) of each tube at your preferred wavelength (see Note 1 below) vs water as reference. Plot your A or %T values vs the corresponding units of LDH (Column 5) starting with your reading for tube 1 vs zero units, etc. It will probably not be a straight line throughout. Refer to Figure 1.

NOTES:

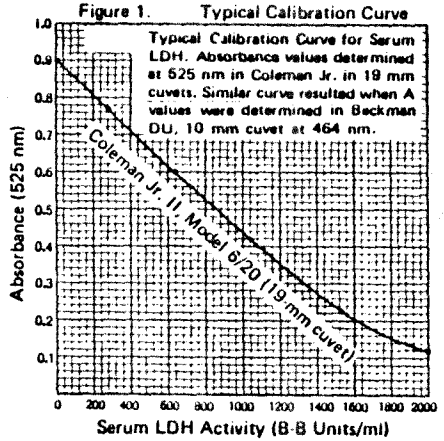
1. Selection of preferred wavelength: After developing color of the 6-calibration tubes, select a wavelength on your instrument which will result in an Absorbance of approximately 0.9 (12 %T) for tube 1, using water as reference. This will place the significant readings into an optimal Absorbance range. The wavelength may be increased or decreased to a convenient setting, but once selected, exercise care to set instrument at this identical wavelength for the entire calibration and for future determinations. The same procedure applies to the selection of a suitable filter for a Leitz, Evelyn, Klett and other similar instruments. The Klett reading should be as close to 450 as possible.



A convenient scale for converting %Transmission to Absorbance or vice-versa

2. Be sure to plot the highest Absorbance obtained (tube 1) vs zero B-B units of LDH. Similarly, the lowest Absorbance reading is plotted vs 2000 B-B units of LDH. The higher the Absorbance the lower the LDH activity. If your colorimeter reads %Transmission, be sure to plot the lowest reading (tube 1) vs zero units LDH, and the highest reading vs 2000 units LDH. If semi-log paper is not available for plotting %T readings, regular graph paper may be used.

Figure 1. Typical Calibration Curve



The typical calibration curve depicted in this bulletin (Figure 1) cannot be used to derive laboratory test results. Each laboratory must prepare its own calibration curve.

QUALITY CONTROL:

Frozen serum pools are not recommended as controls due to instability of some LDH isoenzymes at low temperatures. Refer to explanation under "Specimen Collection and Preparation" section. Commercial lyophilized controls are readily available. Sigma currently offers two controls for this purpose. Sigma Enzyme Control 2-F, Product No. S 1005, and Sigma Enzyme Control 2-N, Product No. S 2005, are assayed preparations which contain several enzymes including LDH in the elevated and normal ranges, respectively.

RESULTS

Results are expressed in terms of Berger-Broida Units per ml (B-B Units/ml) and read directly from your calibration curve. Examples given below are based on use of a Coleman Jr. II spectrophotometer (19-mm cuvet) and "Typical Calibration Curve" depicted in Figure 1.

EXAMPLE 1: A 6-fold dilution of a serum assayed according to the method described yielded an Absorbance (A) of 0.70. This A value is equivalent to a serum LDH activity of 420 B-B Units/ml.

EXAMPLE 2: A 0.2-ml aliquot of dialyzed urine (having a DDF of 1.2), assayed according to the method described, yielded an A value of 0.56. Reading from the Typical Calibration Curve (Figure 1) this A value is equivalent to an LDH activity of 720 B-B Units/ml. To obtain the urine LDH value, 720 is divided by 12, equaling 60 B-B Units/ml. The 8-hr urine volume was 850 ml.
Therefore,
Urine Total LDH Activity (B-B Units/8-hr) =
(B-B Units/ml) x 8-hr Urine Volume (ml) x DDF =
60 x 850 x 1.2 = 61,200

UNIT DEFINITION

One B-B Unit is defined as that amount of LDH that will reduce 4.8×10^{-4} µmol of pyruvate per minute at 25°C. This is equivalent to a decrease in Absorbance (at 340 nm) of 0.001 per minute in a 3-ml volume reaction mixture as performed by the Wroblewski procedure presented in Sigma Technical Bulletin 340-UV.

One International Unit (IU) of an enzyme is defined as that amount of enzyme that will convert 1 µmol of substrate per minute under the specified conditions of the procedure. The conventional units of LDH as used in this bulletin may be converted to IU by multiplying by 0.48.

INTERNATIONAL UNITS

Some enzyme authorities (The International Union of Pure and Applied Chemistry) have been recommending the universal use of a so-called "International Unit" which they say would facilitate exchange of data between laboratories. Sigma has always strongly opposed this proposal because it has actually caused far more confusion than it was intended to prevent. It should be recognized that the activity of an enzyme cannot be expressed in "units" alone without at the same time defining the precise assay conditions. While we are in favor of defining enzyme activities in terms of micromoles of substrate utilized per minute, we must caution against any impression that a "micromolar unit" or an International Unit obtained by a given procedure is in any way comparable to a "micromolar unit" or an International Unit obtained by a different procedure. For example, alkaline phosphatase measured by the Bodansky procedure and expressed in "IU" will not yield results comparable to "IU" values obtained by the Bressey-Lowry-Brock procedure. Use of an author's name, or a trademark, to define a unit is currently the simplest method available to identify both the procedure and test conditions so that no one will inadvertently believe the numeric results can be directly compared.

EXPECTED VALUES

Normal Ranges		
Serum Total LDH		
Normal	100-350	B-B Units/ml
Borderline	350-500	B-B Units/ml
Cerebrospinal Fluid LDH	8-40	B-B Units/ml
Urine LDH		
Normal	<8000	B-B Units/8 hr
Boderline	8000-15,000	B-B Units/8 hr

Normal ranges stated in the bulletin were taken from the literature. The cited methods used to obtain these values are similar to that described in this bulletin and results should be applicable. Copeland[14] suggests that each laboratory determine its own normal range. Attention should be given to the fact that certain measurements in clinically healthy individuals are influenced by diet, sex, age, diurnal variation, physical activity, menstrual cycle, pregnancy and environmental factors.[15]

Serum Total LDH

Wroblewski[16] orginally reported normal serum values, assayed by his ultraviolet procedure, to range from 200-680 units/ml. In a subsequent publication[1] he lowered the upper limit to 500 units/ml. MacDonald et al.[5] reported normal values of 165-332 units/ml based on 20 normal males and 20 normal females aged 20-64 years. Rapp and Bell[17] observed a normal range of 150-350 B-B units/ml in sera from 74 normal persons. The normals suggested in this bulletin are derived from the above as well as from personal communications.

Serum Total LDH values in normal infants and children may exceed those observed in adults. McCoord et al.[18] reported values of 470-1810 units/ml for 10 infants up to 8 days old and 250-700 units/ml for 22 children aged 7 months to 14 years. This confirms an earlier report by Birbeck and Stewart.[19] McCoord et al.[18] reported serum LDH activities up to 1810 units/ml in normal newborn infants and up to 6650 units/ml in some cases of erythroblastosis fetalis.

Hill and Levi[4] were among the first investigators to study serum LDH. They reported marked elevations in many patients with leukemia. Haleh and Blumenthal,[20] Wroblewski and LaDue,[21] White[22] and MacDonald et al. [5] and others have individually reported elevated serum total LDH in cases of myocardial infarction. The latter authors report that of 44 proven cases of myocardial infarction, all but one had maximum values between 340 and 4000 units/ml. The one exception expired before reaching a maximum. Ninety percent of these patients had values which were over 500 units/ml. Maximum values were reached on the second or third day following the attack and slowly declined to normal by about the eighth day. Snodgrass et al.[6] make the statement that all infarctions must be confirmed by a determination of serum LDH. Serum values ranging from 350-1000 units/ml were reported in hepatocellular necrosis, metastatic carcinoma, diabetic ketosis, infectious mononucleosis and cerebral infarction.[5]

Stewart and Warburton[23] recommend the colorimetric procedure for serum LDH described in this bulletin as a "simple and rapid method" of great accuracy with which they readily confirmed published values involving ultraviolet methods. These workers[23] believe serum LDH is superior to serum glutamic oxalacetic transaminase (SGOT) for diagnosing myocardial infarction. In most cases, serum LDH remains elevated much longer, permitting serial determinations over an extended period of time.

An elevated serum LDH is reported to be an important part of a "Triad" useful in the differentiation of pulmonary vs myocardial infarctions, as follows.[11]

Type of Infarction	Serum GOT	Serum LDH	Serum Bilirubin
Pulmonary	Normal	Elevated (up to 7 days)	Elevated
Myocardial	Elevated (up to 48 hr)	Elevated (up to 7 days)	Normal

Manzoli and Pennys[24] determined the effect of acute severe ischemia of the dog limb upon serum LDH, GOT and ICD (isocitric dehydrogenase) by embolization of the femoral artery with lycopodium spores. All the activities rose quickly and remained elevated for many days. These workers suggest that the determination of these serum enzymes might be of value in the management of patients with similar lesions.

Dinman et al.[25] report serum LDH elevation of 1000% in carbon tetrachloride hepatotoxicity. Maximum was reached approximately 24 hours after animals were exposed to vapors of this solvent (200 ppm) for 6 hours. The activity returned to normal 36-72 hours after exposure. An excellent review of various papers on the clinical significance of serum LDH has been prepared by Erickson and Morales.[26]

Cerebrospinal Fluid

Using a different procedure, Fleisher et al.[27] reported that CSF values of LDH are approximately one-fifth that of normal serum. It is, therefore, suggested that spinal fluid determinations be made with undiluted CSF by the procedure described in this bulletin. Using undiluted spinal fluid, the values obtained from the calibration curve must be divided by 6 to obtain corrected values.

Fleisher et al.[27] noted that CSF levels of LDH increased in cases of head injury, degenerative disease of the central nervous system and convulsive disorders, while serum LDH remained normal. Wroblewski et al.[28] observed normal CSF values of 8-40 Wroblewski units/ml (equivalent to 8-40 B-B units/ml) and increased values (up to 480 units/ml) in cases of acute meningitis and involvement of the central nervous system by metastatic carcinoma, lymphoma or leukemia lesions.

Gastric Juice

Schenker[29] and Smyrniotis et al.[30] reported a possible correlation between LDH in gastric juice and gastric cancer. Faulk et al.[31] reported the use of the Sigma Procedure No. 500 in determining LDH in gastric juice as a diagnostic adjunct in stomach cancer.

Urine LDH

Publications by Wacker and Dorfman[11] and Dorfman et al.[12] have evoked considerable interest in the possible clinical significance of urine LDH. Elevated values were reported in 18 out of 19 cases involving carcinoma of the kidney or bladder. Significant elevations were also reported in several other diseases involving the urinary system, including malignant hypertension, glomerulonephritis, lupus nephritis, acute tubular necrosis and possibly pyelonephritis. However, it is said that these diseases are easily differentiated from malignant lesions.[11] Urine LDH increases as much as 500% in cases of carcinoma of the kidney or bladder. Amador et al.[32] report that urine alkaline phosphatase is of help in this differentiation (refer to Sigma Technical Bulletin No. 104).

Wacker and Dorfman[11] used an ultraviolet procedure which yields much lower "normal" values ranging up to 2020 units. These workers concluded that two standard deviations above the mean yields an upper limit of normal of 1880 units. However, the activity of LDH is roughly 4 times as great according to Sigma Procedure No. 500. Preliminary assays[3] have confirmed the tentative normal range of up to 8000 B-B units/8 hour specimen. It is suggested that each laboratory assay as many samples as feasible in the presumably normal range to determine what might be considered the normal range for that laboratory. The suggested "borderline" range of up to 15,000 is based upon personal communications with various investigators that indicated apparently healthy individuals had values up to this level. Thus far, all reports of values above 15,000 B-B units/8 hour specimen were associated with diseases of the urinary tact. Diagnostic procedures of the urinary tract, especially cystoscopy with retrograde pyelography, have been reported to occasionally cause elevation of urine LDH activity, lasting as long as a week.[12]

SPECIFIC PERFORMANCE CHARACTERISTICS

Reproducibility Studies were conducted by performing assays on 20 separate occasions using each of 3 serum pools by means of the LDH method described in this bulletin. The following data were obtained:

Pool	Statistic	LDH Values
Low	Mean*	172.0
	SD*	13.3
	CV (%)	7.8
Medium	Mean*	329.5
	SD*	17.1
	CV (%)	5.2
High	Mean*	645.8
	SD*	18.3
	CV (%)	2.8

*Expressed in B-B Units/ml

Correlation Studies were conducted using sera from 40 patients by means of the colorimetric method described, as well as by the ultraviolet procedure presented by Henry et al.[33] The 2 methods yielded values that were in favorable agreement, as evidenced by the correlation coefficient of .99.

Sensitivity: The lowest limit of LDH activity that can be reliably measured is considered to be about 40 B-B Units/ml. This is a function of such factors as the degree of sophistication of the instrumentation, materials, technique, etc.

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Sigma Chemical Co.
P.O. Box 14508
St. Louis, Missouri 63178

Date of previous revision August 1974

TECHNICAL BULLETINS LISTED IN ALPHABETICAL ORDER

The following is a complete list of bulletins that are provided by SIGMA as of July 1980.

ADENOSINE-5'-TRIPHOSPHATE (ATP) — Ultraviolet enzymatic procedure; for blood, at 340 nm. Employs phosphoglycerate kinase/glyceraldehyde phosphate dehydrogenase enzyme coupled system. .. No. 366-UV

ALANINE AMINOTRANSFERASE (ALT) — See Transaminases .. No. 57-UV

ALBUMIN — Colorimetric procedure; for serum, at 625-635 nm. Based on anion binding capacity of albumin for bromocresol green. .. No. 630

ALCOHOL, ETHYL (ETHANOL) — Ultraviolet enzymatic procedure; for blood, serum or plasma, at 340 nm. .. No. 331-UV

ALDOLASE — Colorimetric procedure; for serum, at 500-580 nm. Based on Sibley-Lehninger method which measures hydrazone derivatives of trioses formed from fructose-1,6-diphosphate. .. No. 750

ALDOLASE — Colorimetric procedure, for serum, at 520-600 nm. Modification of Aldolase Procedure No. 750, resulting in increased sensitivity and improved color stability. .. No. 752

AMMONIA — Ultraviolet enzymatic procedure; for plasma, at 340 nm. Based on the conversion of α-ketoglutarate and ammonia to glutamate in the presence of glutamate dehydrogenase. .. No. 170-UV

AMYLASE — Colorimetric procedure; for serum or urine. Based on visual observation of the starch-iodine color change. .. No. 700

ANTITHROMBIN III (AT-III) — Clotting time method for plasma employing ATROXIN® (*Bothrops atrox* venom) for defibrination. .. No. 855

ASPARTATE AMINOTRANSFERASE (AST) — See Transaminases .. No. 56-UV

ATROXIN® — Clotting time method for plasma using *Bothrops atrox* snake venom. Useful for determining clottability of plasma from heparinized patients. .. No. 845

BILIRUBIN — Two colorimetric procedures; for serum or plasma, at 600 nm. Method A, for narrow-bandwidth spectrophotometers, is based on millimolar extinction coefficient of alkaline azobilirubin. Method B, for wide-bandwidth spectrophotometers, requires use of a bilirubin reference for calibration purposes. .. No. 606

BIOLUMINESCENCE DEMONSTRATION PROCEDURE — Visible light is produced as a result of the reaction between luciferin, ATP and molecular oxygen, catalyzed by luciferase (uses desiccated firefly tails as source of luciferin and luciferase). For research use only. .. No. 2-FF

CALCIUM — Colorimetric procedure; for serum or plasma, at 570-575 nm. Based on cresolphthalein complexone color reaction. .. No. 585

CALIBRATION PROCEDURE FOR WIDE-BANDWIDTH SPECTROPHOTOMETERS — For reactions involving NADH and NADPH, at 340 nm. Describes a method for correcting wide-bandwidth instrument readings for direct use in calculations. .. No. 30-UV

CHLORIDE — Titrimetric procedure. Schales and Schales; for serum, plasma, urine or cerebrospinal fluid. .. No. 830

CHOLESTEROL, TOTAL — Enzymatic procedure; for serum or plasma, at 500 nm. Employs a cholesterol esterase/oxidase system. .. No. 350

CHOLINESTERASE — Colorimetric procedure, for serum or plasma, at 400-440 nm. Measures acetic acid liberated from acetylcholine. .. No. 420

CREATINE PHOSPHOKINASE (CPK) — Colorimetric procedure; for serum, plasma or cerebrospinal fluid, at 620-700 nm. Measures inorganic phosphorous released from phosphocreatine. .. No. 661

CREATINE PHOSPHOKINASE (CPK) — Colorimetric procedure, for serum or plasma, at 500-540 nm. Creatine released from phosphocreatine is reacted with α-naphthol and diacetyl to form a red complex. .. No. 520

CREATINE PHOSPHOKINASE (CPK) — Ultraviolet kinetic procedure for serum, plasma or cerebrospinal fluid, at 340 nm. Employs hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme system. All reagents conveniently pre-standardized in a single vial. .. No. 45-UV

CREATINE KINASE (CK) — Ultraviolet procedure; for serum or plasma, at 340 nm. Based on optimized method recommended by the Scandinavian Society for Clinical Chemistry and Clinical Physiology, Scand J Clin Lab Invest 39:1, 1979. .. No. 48-UV

CREATINE PHOSPHOKINASE (CPK) ISOENZYMES — Colorimetric procedure for detecting CPK isoenzymes, following electrophoresis on agarose gels. Based on reduction of tetranitroblue tetrazolium. .. No. 715-EP

CREATINE PHOSPHOKINASE (CPK) ISOENZYMES — Fluorometric procedure for detecting CPK isoenzymes following electrophoresis on agarose gels. Based on the fluorescence of reduced NADP. .. No. 745-EP

CREATININE — Colorimetric procedure; for serum, plasma or urine, at 490-510 nm. Modified Jaffe reaction, avoids deproteinization. .. No. 555

CYTOCHROME OXIDASE — Histochemical procedure; for frozen tissue sections. Based on the Burstone procedure. For research use only. .. No. 185

2,3-DIPHOSPHOGLYCERIC ACID (2,3-DPG) — Ultraviolet enzymatic procedure; for erythrocytes, at 340 nm. Employs phosphoglycerate mutase/phosphoglycerate .. No. 36-UV

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GLOBULIN, TOTAL — Colorimetric procedure; for serum, at 550-570 nm. Based on the modification of Hopkins-Cole reaction for tryptophan. .. **No. 560**

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β-GLUCURONIDASE — Colorimetric procedure; for serum or urine, at 550 nm. Based on measurement of phenolphthalein liberated from phenolphthalein mono-d-glucuronic acid. .. **No. 325**

γ-GLUTAMYL TRANSPEPTIDASE (GGTP) — Colorimetric procedure; for serum or plasma, at 545 nm. Based on measurement of p-nitroaniline liberated from γ-glutamyl-p-nitroanilide. .. **No. 545**

γ-GLUTAMYL TRANSPEPTIDASE (GGTP) — Kinetic procedure; for serum or plasma, at 405 nm. Based on rate of formation of p-nitroanilide from γ-glutamyl-p-nitroanilide. .. **No. 415**

γ-GLUTAMYL TRANSPEPTIDASE (GGTP) — Kinetic procedure, for serum or plasma, at 405 nm. Based on optimized method of Szasz, Clin Chem 15 124, 1969. .. **No. 416**

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α-HYDROXYBUTYRATE DEHYDROGENASE (α-HBD) — Colorimetric procedure; for serum, at 400-550 nm. Based on measurement of hydrazone derivative of residual substrate, α-ketobutyrate. .. No. 495

INORGANIC PHOSPHORUS — Colorimetric procedure; for serum or urine, at 620-700 nm. Based on the Fiske and SubbaRow procedure. .. No. 670

IRON AND TOTAL IRON-BINDING CAPACITY — Colorimetric procedure; for serum, at 560 nm. Based on the reaction of ferrous ions with ferrozine to produce a magenta colored complex. .. No. 565

ISOCITRATE DEHYDROGENASE (ICD) — Colorimetric procedure; for serum, at 380-400 nm. Based on measurement of hydrazone derivative of α-ketoglutarate formed from isocitrate. .. No. 178

ISOCITRATE DEHYDROGENASE (ICD) — Ultraviolet kinetic procedure; for serum, at 340 nm. .. No. 153-UV

17-KETOSTEROIDS (17-KS) — Colorimetric procedure; for urine, at 480, 520 and 560 nm. Based on a simplified, rapid extraction and improved Zimmermann reaction. .. No. 270

LACTATE DEHYDROGENASE (LDH) — Colorimetric procedure; for serum, urine or cerebrospinal fluid, at 400-550 nm. Based on the Berger-Broida method. .. No. 500

LACTATE DEHYDROGENASE (LDH) — Ultraviolet kinetic procedure; for serum or plasma, at 340 nm. Based on optimized (Lactate → Pyruvate) method of Wacker et al., N Engl J Med 255:449, 1956. Replaces Bulletin No. 225-UV. .. No. 226-UV

LACTATE DEHYDROGENASE (LDH) — Ultraviolet kinetic procedure; for serum, at 340 nm. Based on the Wroblewski-LaDue method. .. No. 340-UV

LACTATE DEHYDROGENASE (LDH) ISOENZYMES — Colorimetric procedure following electrophoresis on cellulose acetate or agarose gels. Based on the reduction of tetranitroblue tetrazolium. .. No. 705-EP

L(+)-LACTIC ACID — Ultraviolet enzymatic procedure; for blood, at 340 nm. .. No. 826-UV

LEUCINE AMINOPEPTIDASE (LAP) — Colorimetric procedure; for serum or urine, at 540-620 nm. Modification of the Goldberg and Rutenburg procedure. .. No. 251

LIPASE — Titrimetric procedure; for serum (Sigma-Tietz method). Based on titration of liberated fatty acids from a standardized olive oil substrate. .. No. 800

MALATE DEHYDROGENASE (MDH) — Ultraviolet kinetic procedure; for serum, at 340 nm. Based on the Siegel-Bing method. .. No. 340-UV

NBT BLOOD TEST — Histochemical procedure; for blood smears. Based on reduction of NBT to highly colored formazan deposits by leukocytes in the presence of bacterial infection. For research use only. .. No. 840

5'-NUCLEOTIDASE — Colorimetric procedure; for serum, at 660 nm. Based on the Dixon and Purdom method. .. No. 675

ORNITHINE CARBAMYL TRANSFERASE (OCT) — Colorimetric procedure; for serum, at 400-420 nm. Nesslerization of ammonia formed from decomposition of carbamyl arsenate. .. No. 108

PERIODIC ACID-SCHIFF (PAS) — Histochemical method; for fresh whole or heparinized blood. Based on use of Schiff's Reagent (pararosaniline and sodium metabisulfite) after treatment of films with periodic acid solution. For research use only. .. No. 395

PEROXIDASE, LEUKOCYTE — Benzidine-free histochemical method; for blood or bone marrow films. Based on use of p-phenylenediamine, pyrocatechol and hydrogen peroxide. For research use only. .. No. 390

PHENYLALANINE — Fluorometric procedure; for serum, at 365 nm activation, 450-535 nm emission. Based on the McCaman and Robins method. .. No. 60-F

PHENYLALANINE — A microbiologic screening procedure for early detection of phenylketonuria based on the Guthrie inhibition assay. .. No. 160

PHOSPHATASE — Colorimetric procedure; for serum, at 400-420 nm, for alkaline, acid and prostatic acid phosphatase. Also urinary alkaline phosphatase. Based on the Bessey-Lowry-Brock procedure. .. No. 104

PHOSPHATASE, ACID — Histochemical procedure; for blood, bone marrow or tissue. Based on use of Naphthol AS-BI and Fast Garnet GBC. For research use only. .. No. 386

PHOSPHATASE, ALKALINE — Histochemical procedure; for blood or bone marrow smears. .. No. 85

PHOSPHATASE, ALKALINE — Kinetic colorimetric procedure; for serum or plasma, at 405 nm. Based on optimized method of Bowers and McComb, Clin Chem 12:70, 1966 and the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology, Scand J Clin Lab Invest 32:291, 1974. Replaces Bulletin No. 244. .. No. 245

PHOSPHATASE ISOENZYMES, ALKALINE — Colorimetric procedure for detection of alkaline phosphatase isoenzymes following electrophoresis on agarose gels. Based on the formation of an indigo dye. .. No. 710-EP

PHOSPHOHEXOSE ISOMERASE — Ultraviolet kinetic procedure; for serum, at 340 nm. .. No. 356-UV

PROTEIN, TOTAL — Colorimetric procedure; for serum, at 540-545 nm. Based on the Biuret reaction. .. No. 540

PYRUVATE KINASE DEFICIENCY — Fluorescence screening procedure; for erythrocytes. Rate of NADH consumption is noted by loss in fluorescence under long-wave ultraviolet light. All reagents conveniently prestandardized in a single vial. .. No. 205

PYRUVIC ACID — Ultraviolet enzymatic procedure; for blood, at 340 nm. .. No. 726-UV

SORBITOL DEHYDROGENASE (SDH) — Ultraviolet kinetic procedure; for serum, at 340 nm. β-D-Fructose used as substrate. .. No. 50-UV

SUDAN BLACK B — Histochemical method; for blood or bone marrow films. Based on use of buffered Sudan Black B and counterstaining with Mayer's Hematoxylin solution. For research use only. .. No. 380

TRANSAMINASES

For over 20 years, the serum enzymes Glutamic-Oxalacetic and Glutamic-Pyruvic Transaminases have been commonly referred to as GOT and GPT, respectively. Recently, the commission on Biochemical Nomenclature has suggested "Aminotransferase" as alternative nomenclature for "Transaminase." It further recommended the use of Aspartate Aminotransaminase and Alanine Aminotransaminase as new names for the individual enzymes, GOT and GPT. We will continue to employ the established abbreviations, GOT and GPT, in product literature developed prior to the time of the new recommendations. Product literature developed subsequently will incorporate the new nomenclature, Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT). We are continuing to list these enzymes under the general heading — Transaminases.

TRANSAMINASE (GOT and GPT) — Colorimetric procedure; for serum, plasma or cerebrospinal fluid, at 490-520 nm. Based on the Sigma-Frankel method. .. No. 505

TRANSAMINASE (GOT and GPT) — Ultraviolet kinetic procedure; for serum, plasma or cerebrospinal fluid, at 340 nm. Based on the Karmen et al., method. All reagents conveniently prestandardized in a single vial. .. No. 55-UV

ASPARTATE AMINOTRANSFERASE (AST/GOT) — Ultraviolet procedure; for serum or plasma, at 340 nm. Based on optimized method as recommended by the International Federation of Clinical Chemists, Clin Chem 24:720, 1978. .. No. 58-UV

ALANINE AMINOTRANSFERASE (ALT/GPT) — Ultraviolet procedure; for serum or plasma, at 340 nm. Based on optimized method as recommended by the Scandinavian Society for Clinical Chemistry and Clinical Physiology, Scand J Clin Lab Invest 32:291, 1974. .. No. 57-UV

TRANSAMINASE (GOT and GPT) — Ultraviolet kinetic procedure; for serum, plasma or cerebrospinal fluid, at 340 nm. Based on the Karmen et al., method. A two-reagent system for the LKB Reaction Rate Analyzer or similar automated equipment. .. No. 155-UV

TRIGLYCERIDES — Colorimetric procedure; for serum or plasma, at 405-415 nm. Based on the Kessler-Lederer and Fletcher methods. .. No. 405

TRIGLYCERIDES — Ultraviolet, totally enzymatic procedure; for serum or plasma, at 340 nm. Single reagent system. .. No. 335-UV

TRIGLYCERIDES — Ultraviolet semi-enzymatic procedure; for serum or plasma, at 340 nm. Based on assay of glycerol released from triglycerides after saponification. .. No. 320-UV

TRIZMA® BUFFERS — Widely used biologic buffers, pH range 7-9. For research use only. .. No. 1068

TYROSINE — Fluorometric procedure; for serum, at 460 nm excitation and 570 nm emission. Based on the reaction of tyrosine with 1-nitroso-2-naphthol. .. No. 70-F

UREA NITROGEN — Colorimetric procedure; for serum, plasma or urine, at 515-540 nm. Based on the urea-diacetyl monoxime reaction. .. No. 535

UREA NITROGEN — Colorimetric procedure; for serum, plasma or urine, at 500-650 nm. Ammonia formed by action of urease is determined by Berthelot reaction. .. No. 640

UREA NITROGEN — Ultraviolet enzymatic procedure; for serum, plasma or urine, at 340 nm. Employs urease/glutamate dehydrogenase coupled enzyme system. All reagents conveniently prestandardized in a single vial. .. No. 65-UV

URIC ACID — Colorimetric procedure; for urine, at 650-750 nm. Combines the accuracy of the enzyme uricase with the simplicity of a colorimetric method. .. No. 680

URIC ACID — Ultraviolet procedure; for serum or urine, at 292 nm. Based on specific reaction of uric acid with uricase. .. No. 292-UV

VANILMANDELIC ACID (VMA) — Colorimetric procedure; for urine, at 470-510 nm. Based on the method of Sunderman et al. .. No. 460

VANILMANDELIC ACID (VMA) — Ultraviolet procedure; for urine, at 360 nm. Simplification of VMA Procedure No. 460 if instrument is available with 360 nm reading capability. .. No. 461-UV