E.coli CITRATE SYNTHASE. A STUDY OF ITS QUATERNARY STRUCTURE AND NADH BINDING PROPERTIES

by

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ABSTRACT

Citrate synthase from acetate grown E.coli (strain K12 3000) after being treated with 0.1% SDS, yielded a major band in SDS gel electrophoresis, corresponding to a molecular weight of 44,500±5,000. If first cross-linked with dimethyl suberimidate, the enzyme gave rise to six bands in SDS gel electrophoresis whose molecular weights were almost integral multiples of about 46,000. The material corresponding to the dimer (91,000) was the most abundant of the six bands.

High speed sedimentation equilibrium experiments were performed on the enzyme in the presence of guanidine-HCl, KCl, and at pH 7.0, 7.8, 9.0 and 10.0. The native enzyme in 6M guanidine-HCl showed a molecular weight of 43,500. In 0.02M tris-HCl buffer pH 7.0 and 7.8, the enzyme was in equilibrium which involved species with molecular weights ranging from monomer (46,000) to those higher than a decamer; 0.05M KCl shifted the equilibrium at pH 7.8 to those between the dimer and hexamer (270,000). Addition of 0.1M KCl at pH 7.8 entirely shifted the enzyme to the hexameric state. In the same buffer at pH 9.0, only dimer was present, while at pH 10.0 aggregates with molecular weights above the decamer were present.

Methionine was the only N-terminal amino acid detectable by the dansylation method. This fact, together with the peptide mapping results from Wright & Sanwal (1971) and our amino acid analysis suggested that the citrate synthase subunits are identical.

The binding of the allosteric inhibitor NADH to citrate synthase, and the effect of pH, substrates (acetyl-CoA and oxaloacetate), the other inhibitor α-ketoglutarate and KCl

have on this binding was studied spectrofluorometrically. The complex NADH-citrate synthase enhances free NADH fluorescence by about 11 fold. In 0.02M tris-HCl buffer at pH 7.8, NADH binds to citrate synthase with a K_D of 2.2μM. Acetyl-CoA and KCl weakened this binding while α-ketoglutarate and oxaloacetate strengthened it. At 0.1M KCl, a concentration of KCl enough to eliminate all NADH inhibition, NADH binding was still observable. The estimated K_D for this binding is 4.7μM. NADH was able to bind citrate synthase from pH 6.2 to 10.0, the binding was stronger at lower pH, but weakened rapidly as pH increased. An average of 1.3 proton was found to be associated with each NADH bound.

The binding of NADH was shown to be a half site reaction. Under all conditions studied (except at very high pH or at 1M KCl whence binding is too weak to be measured), only 3 NADH sites per hexamer were observed, even at a very large excess of free NADH.

Simultaneous tryptophan fluorescence quenching measurements showed that the quenching ability of the NADH-enzyme complex was significantly changed only in the presence of acetyl-CoA. The fraction of tryptophan fluorescence quenched in the presence of acetyl-CoA was 64%, while with the NADH-enzyme complex alone it was 52%. This difference may reflect the involvement of a conformational change in the enzyme induced by acetyl-CoA.

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AMP	Adenosine 5' monophosphate
ATP	Adenosine 5' triphosphate
lnc	natural logarithm of concentration as measured by fringe displaced
dansyl	5-dimethylamino-1-naphthalenesulfonyl
$E^{1\%}_{1cm}$	Absorbance of a 1% solution over an optical path length of 1 cm.
E.coli	Escherichia coli
EDTA	Ethylene diaminetetra-acetate
g, mg, μg	gram, milligram, microgram
K_D	dissociation constant
M, mM, μM	molar, millimolar, micromolar
$\bar{M}_n$, $\bar{M}_w$, $\bar{M}_z$, $\bar{M}_{z+1}$	number, weight, z- and (z+1)-average molecular weights
$\tilde{n}$	saturation function, number of moles NADH bound divided by concentration of enzyme
nm	nanometer
N_f	concentration of free NADH
NADH	nicotinamide adenine dinucleotide
OD	optical density
r, R	radial distance from axis of rotation
rpm	revolution per minute
R_f	relative mobility
σ_n, σ_w, σ_z, σ_{z+1}	reduce molecular weight averages: number-, weight-, z- and (z+1)-
S	Svedberg unit of sedimentation velocity (10^{-13} sec)
$S_{20,w}$	sedimentation coefficient corrected to the viscosity and density of water at 20°C
SDS	sodium dodecasulfate

tris	tris (hydroxymethyl)aminomethane
UV	ultraviolet
$\bar{v}$	partial specific volume

INTRODUCTION

Since the discovery of citrate synthase in E.coli, work has been done on this enzyme consistently. Published papers have covered areas such as purification of the enzyme (Weitzman, 1967; Faloona & Srere, 1969; Wright & Sanwal, 1971), kinetic properties (Faloona & Srere, 1969; Wright & Sanwal, 1971; Johansson et al, 1974), effect of KCl on the activation of the enzyme (Faloona & Srere, 1969), NADH as an allosteric inhibitor (Weitzman, 1966a; Weitzman, 1966b), and α-ketoglutarate as an allosteric inhibitor (Wright, Maeba & Sanwal, 1967). Some physical properties of citrate synthase have also been reported. These include molecular weight information, subunit structure, sedimentation coefficient, amino acid composition, and the stability of the enzyme.

Wright and Sanwal (1971) have suggested in their paper that E.coli citrate synthase is composed of subunits which can undergo association and dissociation according to the following pattern :

$$M_1 \rightleftharpoons M_4 \rightleftharpoons M_8$$

where M represents citrate synthase and the subscripts refer to the state of aggregation of the enzyme. This aroused our interest in E.coli citrate synthase, since this association and dissociation behaviour may explain the allosteric properties of the enzyme. However, so far there is not too much direct evidence in support of the model of such an equilibrium, apart from the series of sedimentation experiments reported by Wright and Sanwal (1971).

Our interest in E.coli citrate synthase is in its allosteric properties. The purpose of the present work is an attempt to find out more about the equilibrium between the different forms of the enzyme and to study in more detail the relationship between these forms under different conditions. We have also carried out a study on the NADH binding behavior of E.coli citrate synthase. High speed sedimentation equilibrium (Yphantis, 1964) has provided us with a method for studying the molecular weight distribution of citrate synthase under various conditions. Fluorescence, on the other hand, has proven to be a sensitive and convenient method for studying NADH binding.

BACKGROUND

Citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating) EC 4.1.3.7] catalyzes the reaction through which the acetyl carbons of acetyl coenzyme A enter the Krebs cycle :

$$\text{Acetyl-CoA} + \text{Oxaloacetate}^{2-} + H_2O = \text{Citrate}^{3-} + \text{CoA} + H^+$$

In eukaryotic cells where the Krebs cycle is a major energy producing pathway, citrate synthase occurs chiefly in the mitochondria. The acetyl carbons of acetyl-CoA condense with oxaloacetate to give citrate. Citrate is then broken down stepwise to give back oxaloacetate, yielding 12 molecules of ATP on its way. In cells which contain glyoxysomes, citrate synthase is also present in mitochondria; the enzyme here functions in the glyoxylate shunt in which 2 acetyl units are converted to succinate. In bacteria, especially anaerobes, the series of reactions beginning with the one catalyzed by citrate synthase leads to the synthesis of glutamic acid. Thus citrate synthase occupies a key position in the metabolism of most organisms; and the control of its activity has been postulated as important in various regulatory mechanisms.

In 1950, Ochoa's group isolated some soluble enzymes from E.coli. They found that in the presence of pyruvate and oxaloacetate, this enzyme fraction catalyzed a reaction which resulted in citric acid. Ochoa et al. (1951) crystallized from pig heart the condensing enzyme which was responsible for the reaction between oxaloacetate and acetyl coenzyme A to yield citrate. This condensing enzyme is of course citrate synthase.

Srere & Kosicki (1961) developed a procedure which yielded far more condensing enzyme from pig heart. The development of a new assay procedure by Srere, Brazil & Gonen (1963) further facilitated the research work on citrate synthase. This procedure makes use of the reaction between Ellman's reagent (1959) and coenzyme A, a product of the citrate synthase catalyzed reaction, to yield a colored product which absorbs light at 412nm. This technique is more sensitive and more convenient than the original method, which involves first coupling citrate synthase to malate dehydrogenase then following the appearance of NADH at 340nm.

Wieland and Weiss (1963) found that pig heart citrate synthase activity was inhibited by palmityl CoA, and suggested that palmityl CoA was a regulator of the pig heart enzyme. However, Srere (1965) showed that sixteen moles of palmityl CoA were needed to inhibit one mole of this pig heart enzyme to an extent of 27%. Thus the effect of palmityl CoA appeared non-specific and was probably due to its detergent effect. Srere & Whissen (1967) found that the E.coli citrate synthase was more sensitive to palmityl CoA inhibition, and only 5% of the amount needed to inhibit the pig heart enzyme was enough to inhibit this bacterial enzyme.

Hathaway and Atkinson (1965) were the first to show that ATP is a good inhibitor of citrate synthase. This discovery was of interest because ATP is an energy charged molecule produced mainly by the Krebs cycle in higher organisms, and its ability to inhibit citrate synthase activity would mean that it exerts a feedback control over citrate synthase, and hence the Krebs cycle. However not all citrate synthases are affected by ATP. Among those that

are affected are citrate synthases from eukaryotic cells and Gram-positive bacteria. The findings of Kosicki & Lee (1966) that physiological levels of Mg^{2+} can erase the ATP effect, however showed that ATP inhibition may not be significant under *in vivo* conditions.

In 1966, Weitzman showed that NADH inhibits citrate synthase from bacteria sources, but was ineffective towards yeast or mammalian citrate synthase. He also noted that K^+ reversed this NADH inhibition. The allosteric nature of NADH inhibition emerged from the fact that desensitization of the enzyme to NADH can be carried out without loss of enzyme activity. The same author later (1968) reported that all citrate synthase from Gram-negative bacteria showed inhibition by NADH, and inhibition can be divided into two types depending on whether or not AMP can relieve this inhibition.

Wright, Maeba & Sanwal (1967) showed that α-ketoglutarate is an inhibitor of *E.coli* citrate synthase. Since the Krebs cycle can be divided into three units each one controlled independently of one another (Amarsinghan & Davis, 1965) the authors postulated that the first sequence of reactions leading from citrate to α-ketoglutarate was controlled by a feedback mechanism in which α-ketoglutarate was the feedback inhibitor.

Faloona & Srere (1969) investigated the effect of K^+ on *E.coli* citrate synthase. They found that K^+ activated the enzyme some twenty fold; reversed NADH and α-ketoglutarate inhibition; protected the enzyme from urea denaturation and normalized sigmoidal reciprocal plots of velocity as a function of concentration of acetyl-CoA.

Rowe & Weitzman (1969) examined citrate synthase purified from Acinetobacter Lwoffi under the electron microscope and claimed that the subunit molecular weight of the enzyme is 57,000 and predicted a tetrameric structure for the enzyme with a molecular weight of 232,000.

Wright & Sanwal (1971) did a more thorough study on E.coli citrate synthase. They noted that the enzyme undergoes association-dissociation at different pHs and proposed that the enzyme is equilibrating between the forms :

Monomer $\rightleftharpoons$ Tetramer $\rightleftharpoons$ Octomer

with a monomer molecular weight of 62,000. Their binding study revealed that there was little NADH binding to the enzyme in the absence of substrates. This finding is quite unexpected, since NADH is a very efficient inhibitor of E.coli citrate synthase.

Danson & Weitzman (1973) confirmed Wright & Sanwal's observation on the association-dissociation pattern of E.coli citrate synthase under the influence of pH. There are some disagreements between these two groups of workers; at pH 7.0, Danson & Weitzman reported they observed mainly tetramer whereas Wright & Sanwal reported the presence of mainly octamer; the tryptophan and total cysteine content as determined by both groups agreed closely, however the amino acid content of the rest of the amino acids reported by Danson & Weitzman are about a third higher than those reported by the former workers.

Johansson et al (1973) studied the mechanism of the citrate synthase catalyzed reaction. They interpreted their kinetic data in terms of a rapid equilibrium random order mechanism involving the formation of a tertiary complex.

Werdman, Drysdale & Mildvan (1973) worked on pig heart citrate synthase which exists as a dimer with molecular weight of about 100,000. They found that a spin labelled acetyl-CoA analogue binds to pig heart citrate synthase with approximately the same K_D as acetyl-CoA at a ratio of 2.2 moles/enzyme dimer. With EPR and proton relaxation techniques they showed the formation of the ternary complex acetyl-CoA analogue-enzyme-oxaloacetate. The analogue could bind a little tighter to the enzyme if oxaloacetate was incorporated first onto the enzyme.

Johansson & Petersson (1974) observed from their gel equilibrium diffusion experiment that acetyl-CoA was able to bind to two indistinguishable sites in the pig heart citrate synthase. The K_D measured was 0.13mM, exceeding Km by a factor of about 20. They concluded that this observation is inconsistent with a rapid equilibrium random order mechanism, and proposed a compulsory ordered mechanism in which oxaloacetate adds first to the enzyme.

Studies on citrate synthase have been carried out on extracts from various aerobic and facultative anaerobic bacteria (Weitzman & Jones, 1968), from strictly anaerobic bacteria (Gottschalk, 1968), higher plants (Sarkissian, 1970), fungi (Flavell & Fincham, 1968; Kobr, Vanderhaeghe & Combepine,

1969) and animal tissues (Broder & Srere, 1963; Jangaard, Unkeless & Atkinson, 1968). Citrate synthase has been purified from many of these sources. Among these citrate synthases, there are apparent variations. The enzymes obtained from animals seemed to have similar molecular weights of around 100,000 and are composed of two subunits. The enzymes from bacterial sources are much bigger. Molecular weights of 210,000 to 280,000 have been reported for E.coli citrate synthase. From Azotobacter the enzyme is 300,000 (Srere, 1972) with six subunits. From mango the enzyme is 65,000 (Srere, 1971).

The behavior of citrate synthase towards various effectors is also vastly different in different organisms. Weitzman and Jones (1968) attempted to classify the behavior of citrate synthase with relation to taxonomy of the organism from which it is isolated. Thus citrate synthase was classified into three groups: (a) NADH inhibited, (b) NADH inhibited, but inhibition reversed by AMP, (c) ATP inhibited. All Gram-positive bacteria and eukaryotic organisms have citrate synthase belonging to (c); of the Gram-negative bacteria, only the facultative anaerobes have citrate synthases that belong to (a), while the strict aerobes have citrate synthases that belong to (b). However, exceptions to these rules have been reported by various workers (Eidels & Preiss, 1970; Srere, 1972).

The effect of potassium ions on different groups of citrate synthases are also different. The animal enzymes are activated by increasing concentrations of potassium ions. The effect appears to be due to ionic strength, and the activity of the enzymes increases with potassium ion concentration, reaches a maximum and then decreases with

further increases of potassium ion. For rat heart citrate synthase, the activity maximizes at 60mM potassium (Srere, 1970). With bacterial citrate synthase, potassium ion appears to have a specific ion effect on the enzyme. It protects the enzyme from NADH and α-ketoglutarate inhibition and activates the enzyme. The Azotobacter citrate synthase activity has been reported to be absolutely dependent on potassium (Srere, 1970). ATP inhibits almost all citrate synthases, the inhibition is eliminated by potassium in most organisms but E.coli, in which case ATP inhibition is in fact strengthened, rather than weakened.

Dunmore & Weitzman (1969) did a study on the relationships between citrate synthase molecular sizes and NADH inhibitory pattern. They chose a total of twelve citrate synthases: three from Gram-positive bacteria, three from eukaryotic cells and six from Gram negative bacteria. Only the latter group was said to be inhibited by NADH; and of these six, three are AMP sensitive and three are AMP insensitive. E.coli citrate synthase falls in the last group in which NADH inhibition is not sensitive and therefore not reversed by AMP. These twelve partially purified citrate synthases were chromatographed individually with standards on a Sephadex G-200 column which had been preequilibrated in tris buffer pH 8.0 in the presence of 0.1M KCl. The citrate synthases examined fell into two groups; the large enzymes which came out before bovine liver catalase (250,000) and the small enzymes which came out after rabbit muscle dehydrogenase (140,000). Only the 'large' enzymes were found to be inhibited by NADH. Thus E.coli citrate synthase, being in the 'large' group would have a molecular weight of greater than 250,000.

Rowe & Weitzman (1969) purified a sample of citrate synthase from Acinetobacter lwoffi, a Gram-negative bacterium, and examined it under the electron microscope. In tris-EDTA buffer, the native enzyme has a mean diameter of 99Å. Assuming $\bar{v}$ = 0.73, this diameter corresponds to approximately 400,000 if the protein is a closely packed sphere. Closer examination revealed that about 10% of the enzyme showed a definite 3-fold symmetry. The diameter of each of the 3 substructures measured 51Å, each substructure occupies a vertex of a triangle thereby forming a 3-fold symmetry. Assuming a spherical structure, substructure molecular weight becomes 57,200 ($\bar{v}$=0.73), and if the actual stoichiometry is tetrahedral as the authors suggested, the molecular weight of the native enzyme would be 230,000. This approximation is inconsistent with the data they obtained by metal shadowing technique. Using this technique, they calculated out the molecular height to be 46Å; which means the substructure molecular weight would be less than 57,000, and considerably less if the subunits have to be arranged tetrahedrally to yield those dimensions.

The fact that only 10% of the molecules they examined showed a 3-fold symmetry structure may be, as they pointed out, because either the orientation or the degree of penetration by stain favored visualization of these substructures, or more probably, because they are in fact looking at one of several possible structures which are in equilibrium with each other under those experimental conditions.

Faloona & Srere (1969) purified the enzyme from E.coli

to apparent homogeneity, and subjected this enzyme to gel filtration on a precalibrated column of Bio-Gel A. The estimated molecular weight for the native enzyme was 280,000. Eluant used was 0.05M tris-HCl buffer pH 8.1, with 0.1M KCl.

Wright & Sanwal (1971) also purified the enzyme from E.coli. They reported the subunit molecular weight to be 62,000 from SDS gel electrophoresis. At pH 7 and 11, they obtained single symmetrical sedimentation peaks in the analytical ultracentrifuge, and estimated the molecular weights of the species to be 488,000 and 61,000 respectively. In zone sedimentation in sucrose gradients, they obtained three protein peaks corresponding to 60,000; 245,000; and 486,000. These pieces of evidence together led them to conclude that E.coli citrate synthase exists predominantly as monomers, tetramers and octamers.

Srere (1972) reported the molecular weight of native E.coli citrate synthase to be 210,000 from sedimentation equilibrium analysis.

Danson & Weitzman (1973) obtained a subunit molecular weight for the E.coli enzyme from SDS gel electrophoresis; the value was 55,000±4,000. The molecular weight calculated for their two sedimenting species in the ultracentrifuge were 57,000 and 230,000. It appears that these numbers come close to the numbers reported by Wright & Sanwal, but it should be noted that these workers have used the same diffusion coefficients used by Wright & Sanwal in calculating the molecular weights of the sedimenting species.

It is quite obvious from the above account that there are disagreements among workers concerning the molecular weight of this enzyme. The native enzyme has been reported to be: greater than 250,000 by Weitzman & Dunmore, 280,000 by Faloona & Srere and 210,000 by Srere. Wright & Sanwal and Danson & Weitzman have reported the presence of a species with molecular weight approximately 240,000, which is enzymatically active. This species was identified as a tetramer whose subunit has a molecular weight of 55,000-62,000. Well then what is the actual molecular weight of the native enzyme? And what other evidence is there to support the idea of having a tetramer? These are interesting and important questions whose answers will provide a useful basis for further work to be done on this enzyme. In our present study, we are not able to confirm the presence of E.coli citrate synthase tetramer as a distinct species. It will be shown that the enzyme we obtained from E.coli in fact showed a monomer molecular weight of approximately 46,000, a size similar to that of the eukaryotic enzyme monomer; and the prominent species appeared to be dimers (~90,000) and hexamers (~270,000).

There is no doubt that binding studies can lead to invaluable information regarding allosteric mechanism: aspartate transcarbamylase is a classical example. So far, the only attempt for such studies on E.coli citrate synthase was made by Wright & Sanwal (1971). Through equilibrium dialysis they have looked at the binding of the substrate acetyl-CoA and effectors α-ketoglutarate and NADH to the enzyme. They reported that binding of acetyl-CoA was co-operative, and binding was seen in both the catalytically active, and inactive species. As for

the effectors, while there was considerable binding of α-ketoglutarate, binding of NADH was reported to be surprisingly low. Since NADH is such a potent inhibitor of E.coli citrate synthase, normally we would expect very tight binding between the two. This fact prompted us to re-examine NADH binding to E.coli citrate synthase. Indeed, using a more sensitive technique, we were able to see more binding of NADH.

MATERIALS AND METHODS

MATERIALS

Dansyl chloride, standard L. dansyl amino acids, α-ketoglutarate, protamine sulfate, trizma (Tris), bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase and catalase were obtained from Sigma; Oxaloacetate and NADH were from Boehringer; acetyl-CoA, trilithium salt was from PL Biochemicals; Ellman's reagent (DTNB) was from Aldrich; guanidine-HCl, ultrapure, and ammonium sulfate, enzyme grade were from Schwarz/Mann; Sephadex G-200 was from Pharmacia Fine Chemicals; DEAE-cellulose, preswollen microgranule (DE52) was from Whatman. All other chemicals were reagent grade. Polyamide thin-layer plates for chromatography of dansyl amino acids were obtained from Gallard-Schlesinger. Kodak Type IN and type IIG spectroscopic plates were used for photographing Schlieren patterns and Rayleigh interference patterns respectively.

ORGANISM

E.coli strain Kl2 3000 was used throughout this investigation.

MEDIA

Stock LB medium is a solution containing:

Bacto-tryptone	1%
Yeast extract	0.5%
NaCl	1%
lN NaOH	1.0ml/liter
water to volume	

this solution is autoclaved at 121 degrees centigrades for 20 minutes before used.

Minimal medium is a solution containing:

K_2HPO_4	10.5 gm/liter
KH_2PO_4	4.5 gm/liter
$(NH_4)_2SO_4$	1.5 gm/liter
$MgSO_4.7H_2O$	0.1 gm/liter

The minimal acetate medium A contains in addition 1 % of sodium acetate, and 0.001 % thiamine.

GROWTH CONDITION

Growth of E.coli was initiated in 2x10 ml LB medium, this was used to inoculate 2x100 ml minimal acetate medium, which was used to inoculate a further 1x15 liters of minimal acetate medium.

At each stage the culture was allowed to grow overnight at 37 degrees centigrade with agitation to ensure good aeration. The final 15 liters of culture was inoculated into 4 carboys, each containing 15 liters of the same medium and allowed to grow for 8 hours before harvesting. Typical yield is about 140 gm wet of E.coli from the final 4 carboys.

ENZYME PURIFICATION

The method used was similar to that of Wright & Sanwal (1971) with the following changes:

Protamine sulfate was added at the rate of 1 mg/10 mg protein as determined by Lowry (1951) in the crude extract.

The reverse ammonium sulfate step was omitted.

The protein fraction that precipitated out between 55-70% ammonium sulfate was dissolved in a minimum amount of 0.02M tris-HCl buffer, pH 7.8 containing 1mM EDTA. This protein solution was dialysed against 4 liters of the same, standard buffer for 6 hours with one change of buffer.

Typical yield at this stage was 50-60% with about 4 gm of protein and a specific activity of about 4. This enzyme solution was put onto a pre-equilibriated DEAE cellulose column which is made up of 50 gm of cellulose in a 16x2.4cm column, and washed thoroughly with standard tris-HCl buffer containing 0.05M KCl. A linear gradient of KCl from 0.05M to 0.3M in standard buffer, total volume 2 liters, was applied to elute the column at a flow rate of 60 ml/hour. The fractions containing citrate synthase with specific activity greater than 40 were pooled and concentrated by ultrafiltration down to a few mls. This concentrated solution was applied onto a Sephadex G-200 column (2.8x85cm) and eluted with standard buffer containing 0.05M KCl. 5ml fractions were collected and fractions containing enzyme with a constant specific activity of about 40 again pooled and concentrated by ultrafiltration. The yield of enzyme after this final step was approximately 200 mg of protein; the enzyme thus obtained had a concentration of about 180mg/ml and an $E^{1\%}_{1cm}$ value of 9.76. It gave one symmetrical Schlieren peak in the ultracentrifuge, a single band in the SDS gel electrophoresis and 3 bands in the disc gel electrophoresis.

ENZYME ASSAY

A unit of enzyme is defined as the amount of enzyme which is necessary to catalyse the reaction:

acetyl-CoA + oxaloacetate = citrate + CoA

at the rate of 1μmole product formed per minute under

the following assay conditions: the assay mixture contains 0.1mM acetyl-CoA, 0.1mM oxaloacetate, 0.025mM DTNB, assay buffer, and up to 30 milliunits of citrate synthase in a total volume of 1.00ml. 0.1M KCl was included in ordinary assays. In inhibition studies, 0.05mM NADH was included in the assay mixture while KCl was omitted. The assay buffer was 0.02M tris-HCl at pH 8.0. The reaction was followed as described in Srere, Brazil and Gonen (1963).

PROTEIN DETERMINATION

During purification, protein concentrations were determined according to Lowry et al (1951) with bovine serum albumin as standard.

Pure citrate synthase concentration was determined spectrophotometrically on the Cary 14 spectrophotometer, using a value of $E^{1\%}_{1cm} = 9.76$ at 278nm obtained in this laboratory from a pure sample of citrate synthase by amino acid analysis. This value agrees well with the value obtained by Wright (1970), which is 9.79 at 280nm from dry weight determination.

POLYACRYLAMICE GEL ELECTROPHORESIS

Disc gel electrophoresis when used to determine the degree of purity of the enzyme preparation was performed according to Ornstein and Davis (1964). A gel concentration of 7% was used.

Disc polyacrylamide gel electrophoresis was also employed to study the relationship of the 3 bands obtained for citrate synthase under the normal conditions as described above. In such runs, the buffer system described in Ornstein and Davis (1964) was retained, and the method used by Hedrick and Smith (1968) was followed.

Stock gel solution was 48% in acrylamide monomer. Final gel concentration varied from 6-10%.

Each sample to be run contained 30μg protein, a trace of bromphenol blue and a drop of 10% sucrose in a total volume of 30μl. 6 gels of the same concentration were prepared and run each time and the average R_f value taken.

SDS GELS

SDS polyacrylamide gel electrophoresis was performed exactly as described by Weber and Osborn (1969). Gel concentration was 7%. Standards included bovine serum albumin, catalase, ovalbumin, glyceraldehyde 3-phosphate dehydrogenase and lysozyme.

The preparation of dimethyl suberimidate cross-linked protein samples was the same as described by Davies and Stark (1970). Dimethyl suberimidate/protein ratio was 3/1. The dimethyl suberimidate protein mixture after overnight incubation at room temperature was treated as a protein sample in the SDS gel experiments. The method in Weber and Osborn (1969) was followed. SDS gel concentration was 3.5%. Proteins used as standard for calibrating the

log molecular weight versus R_f plot include: bovine serum albumin, glyceraldehyde 3-phosphate dehydrogenase and catalase.

All gel electrophoresis experiments were done in a Shandon Disc Electrophoretic Apparatus. In all experiments samples were layered onto the separating gel directly, and no stacking gel was used. R_f values are evaluated with reference to distance migrated by bromphenol blue.

AMINO ACID ANALYSIS

The amino acid content of purified citrate synthase was determined with a Beckman Model 121 Automatic Amino Acid Analyzer. Acid hydrolysis in 6N HCl of approximately 0.72mg of enzyme per hydrolysis sample was carried out at 110 degrees centigrade for 24, 48 and 72 hour periods *in vacuo*. Duplicate samples from each hydrolyzate were taken at the end of each period and analyzed for amino acid content in the automatic amino acid analyzer. A separate protein sample was used to determine total cystine and cysteine by the method of Hirs (1967). The protein was first oxidized with performic acid, hydrolysed in 6N HCl, then analyzed for total cysteic acid on the amino acid analyzer. Tryptophan was determined spectrophotometrically on the Cary 14 Spectrophotometer according to Edelhoch (1967).

N-TERMINAL AMINO ACID DETERMINATION

The N-terminal amino acid in citrate synthase was

analyzed as its dansyl derivative according to the procedures described by Gray (1967).

ULTRACENTRIFUGE EXPERIMENTS

Ultracentrifugations were done in a Beckman Model E Analytical Ultracentrifuge equipped with electronic speed control. All experiments were done at 20 degrees centigrade.

For sedimentation velocity studies on the native enzyme the Schlieren Optical System was used. Samples were run in an An-D rotor at a rotor speed of 60,000 rpm. The buffer system was 0.02M tris-HCl, pH 7.8.

Sedimentation equilibrium experiments were done according to the high speed equilibrium method of Yphantis (1964). The Interference Optical System was used. A six-channel Yphantis centerpiece, sapphire windows and interference counterbalance were used in all experiments. For the runs at pHs 7.0, 7.8 and 9.0, rotor speed was 14,000 rpm, and for the run at pH 7.8 in presence of 0.1M KCl, it was 12,000 rpm. The heavier An-J rotor was used for these runs. The run in guanidine-HCl was done at 36,000 rpm in the An-H rotor. Samples were prepared by dialysing small amounts of protein at about 0.6 mg/ml overnight in the cold against large volumes of the desired solvent. Photographs were taken at least 24 hours after the rotor came to maximum speed. The Raleigh interference fringes were measured on a Nikon microcomparator and the data processed using the computer programme of Roark and Yphantis (1969).

PARTIAL SPECIFIC VOLUME ($\bar{V}$)

$\bar{V}$ of the enzyme in water was taken to be 0.735 ml/gm, the volume obtained from the amino acid composition of the protein. The partial specific volumes of the amino acids are given by Cohen & Edsall (1941). Partial specific volume is given by:

$$\bar{V} = \frac{\Sigma w_i \bar{v}_i}{\Sigma w_i}$$

where w_i = % by weight of the i^{th} residue per mole of protein;
$\bar{v}_i$ = specific volume of the i^{th} residue.

FLUORESCENCE EXPERIMENTS

Fluorescence was measured with an Aminco-Bowman Spectrofluorometer equipped with high pressure Xenon lamp. A one-centimeter light path cuvette was used. NADH and NADH-enzyme complex fluorescence were measured with the exciting wavelength set at 340nm and emission wavelength at 428nm. Tryptophan fluorescence was studied at an excitation wavelength of 288 nm and emission wavelength of 348nm. All reagents were made up fresh just before the experiments, and the concentration of NADH was determined spectrophotometrically using a molar extinction coefficient of 6220 at 340nm. All fluorescence measurements were corrected for absorbance due to NADH at the excitation wavelength.

In the presence of excess NADH, the observed fluorescence from an enzyme solution of citrate synthase is due to the fluorescence of free and bound NADH. If:

F_{ob}=corrected observed fluorescence

$NADH_f$=concentration of free NADH

$NADH_b$=concentration of bound NADH

F_f'=fluorescence of 1 μmole of free NADH

F_b'=fluorescence of 1 μmole of bound NADH, and

$NADH_T$=total concentration of NADH, free and bound;

then we have these relationships:

$$F_{ob} = NADH_f \cdot F_f' + NADH_b \cdot F_b'$$

$$F_{ob} = NADH_f \cdot F_f' + (NADH_T - NADH_f) \cdot F_b'$$

$$F_{ob} = NADH_f \cdot (F_f' - F_b') + NADH_T \cdot F_b'$$

$$NADH_f = \frac{F_{ob} - NADH_T \cdot F_b'}{F_f' - F_b'}$$

or,
$$NADH_f = \frac{NADH_T \cdot F_b - F_{ob}}{F_b' - F_f'} \quad \text{.....1}$$

In equation (1), F_{ob} is the experimental measurable value, $NADH_T$ is known, F_f' can be determined simply by measuring the fluorescence of a solution of free NADH and dividing the corrected fluorescence by the concentration of NADH in μmole and F_b' can be determined from the product (F_f'. enhancement) where enhancement is the ratio of the limiting fluorescence of NADH at very high enzyme concentrations to the fluorescence of the same amount of NADH in the absence of enzyme. In this experiment, the limiting fluorescence of NADH-enzyme complex was obtained by titrating a fixed amount of NADH

with increasing amounts of enzyme and extrapolating to infinite enzyme concentration. Thus, from equation (1) we can find out $NADH_f$, and hence $NADH_b$.

TRYPTOPHAN FLUORESCENCE

The change in protein tryptophan fluorescence was followed during the course of titration in the NADH binding study.

PURIFICATION

The fractions pooled from the Sephadex G-200 column had constant specific activities of 40 to 46, indicating a homogenous preparation. The disc electrophoretic gel pattern showed three bands, the same pattern as obtained by Wright and Sanwal (1971). At pH 7.0 in SDS gels the enzyme showed a single band. Immuno-diffusion technique showed one arc against a monospecific rabbit antiserum (diagram 1). In the analytical ultracentrifuge, the preparation gave a single symmetrical peak with an apparent $S_{20,w}$=10.9s. The UV absorption spectrum showed that the enzyme behaves as a normal protein. The absorption peak was at 278 nm, the ratio of optical densities OD_{280}/OD_{278} obtained for the preparation was 0.98.

KINETIC STUDIES

The effect of NADH inhibition was studied. The same mode of inhibition obtained by Wright & Sanwal (1971) was observed. The double reciprocal plot of 1/v versus 1/acetyl-CoA at saturating and unsaturating oxaloacetate, and increasing [NADH] showed competitive inhibition, the lines converging to the same point on the y-axis. When acetyl-CoA was kept constant and oxaloacetate varied, the double reciprocal plots at varying NADH concentrations again showed the same pattern of inhibition as obtained by Wright & Sanwal (1971). These findings ensures that the citrate synthase being studied is the same enzyme reported by Wright & Sanwal (1971).

RESULTS

Diagram 1. Double diffusion analysis in agar gel of purified citrate synthase against monospecific rabbit antiserum. The upper two wells contained the antiserum; the three lower wells contained purified citrate synthase.

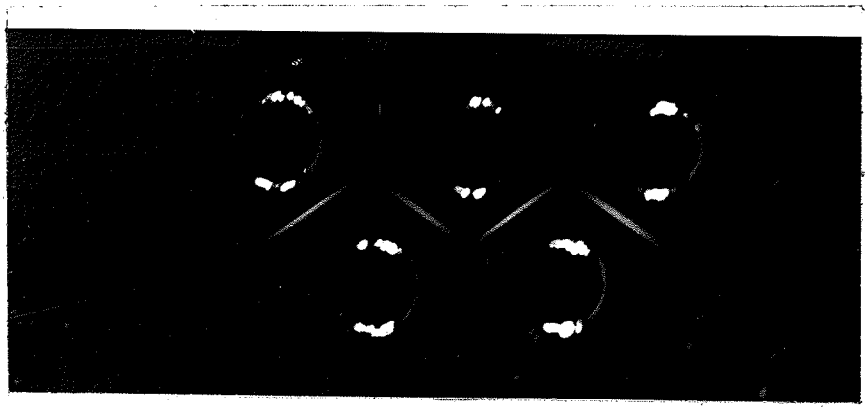


TABLE 1. Amino acid composition of E.coli citrate synthase.

Amino acid	RESULT FROM THIS STUDY		Danson & Weitzman	Wright & Sanwal
	Moles/47,000g.	Nearest integer		
lysine	23.7±0.1	24	24*	17**
histidine	12.2±0.2[a]	12	15	99
ammonia	29.5±0.4[a]	29-30		
arginine	22.0±0.3	22	24	15
aspartate	43.8±0.9[b]	44	45	28
threonine	24.8±0.6[b]	25	27	18
serine	22.7±0.5[b]	23	25	19
glutamate	37.1±0.7	37	36	27
proline	18.8±0.8	19	17	14
glycine	31.1±0.6	31	30	24
alanine	39.1±0.8	39	38	28
valine	22.5±0.1[c]	23	20	10
methionine	16.3±0.9	16	15	11
isoleucine	26.8±0.1[c]	27	26	15
leucine	36.6±0.8	37	34	25
tyrosine	15.1±0.4 (14.7±0.3)[d]	15	15	10-11
phenylalaninep	19.1±0.6	19	22	14
tryptophan	3.88±0.07[d]	4	5	4.6
1/2 cystine	6.53±0.52[e]	6-7	7	6

a. linear extrapolation to zero hydrolysis time.
b. first order extrapolation to zero hydrolysis time.
c. values of 72-hour hydrolysis taken.
d. value determined spectrophotometrically according to Edelhoch (1967).
e. as cysteic acid after performic acid oxidation.

* taken from Danson & Weitzman (1973) corrected to nearest no. of moles/47,000.
** taken from Wright & Sanwal (1971) corrected to nearest no. of moles/47,000.

AMINO ACID ANALYSIS

Acid hydrolysis with 6N HCl for 24, 48 and 72 hours were carried out, and the hydrolysates analysed for amino acid composition. Since steric hindrance by the side chains of Val and Ile sometimes results in a somewhat slower hydrolysis of peptide bonds involving these amino acids, the results for these two amino acids obtained after 72 hours were taken. In the acid hydrolysis, Ser and Thr were destroyed gradually with increasing time, and thus it was necessary to extrapolate back to zero time from the values obtained at the three hydrolysis times. The amount of NH_3 produced increased linearly with time; the extrapolation at zero time revealed the total number of moles of Gln and Asn in the native protein. Except for cystine and tryptophan the rest of the amino acids were determined from the averaged values obtained from the six runs. Cysteine and cystine were oxidized by performic acid to cysteic acid and estimated by the method described by Hirs (1967). Tryptophan was determined spectroscopically as described in Methods. Tyrosine, which can be determined by the same method, was also determined, the value obtained agreed well with the value obtained from acid hydrolysis. The amino acid composition of citrate synthase, based on a molecular weight of 47,000 is shown in Table 1.

END GROUP ANALYSIS

Acid hydrolysis of citrate synthase which had been treated with dansyl chloride yielded a dansylated amino acid

which gave one fluorescent spot on the polyamide thin layer plate. Comparison with dansyl-amino acid standards ran side by side with it showed that the dansylated amino acid was methionine.

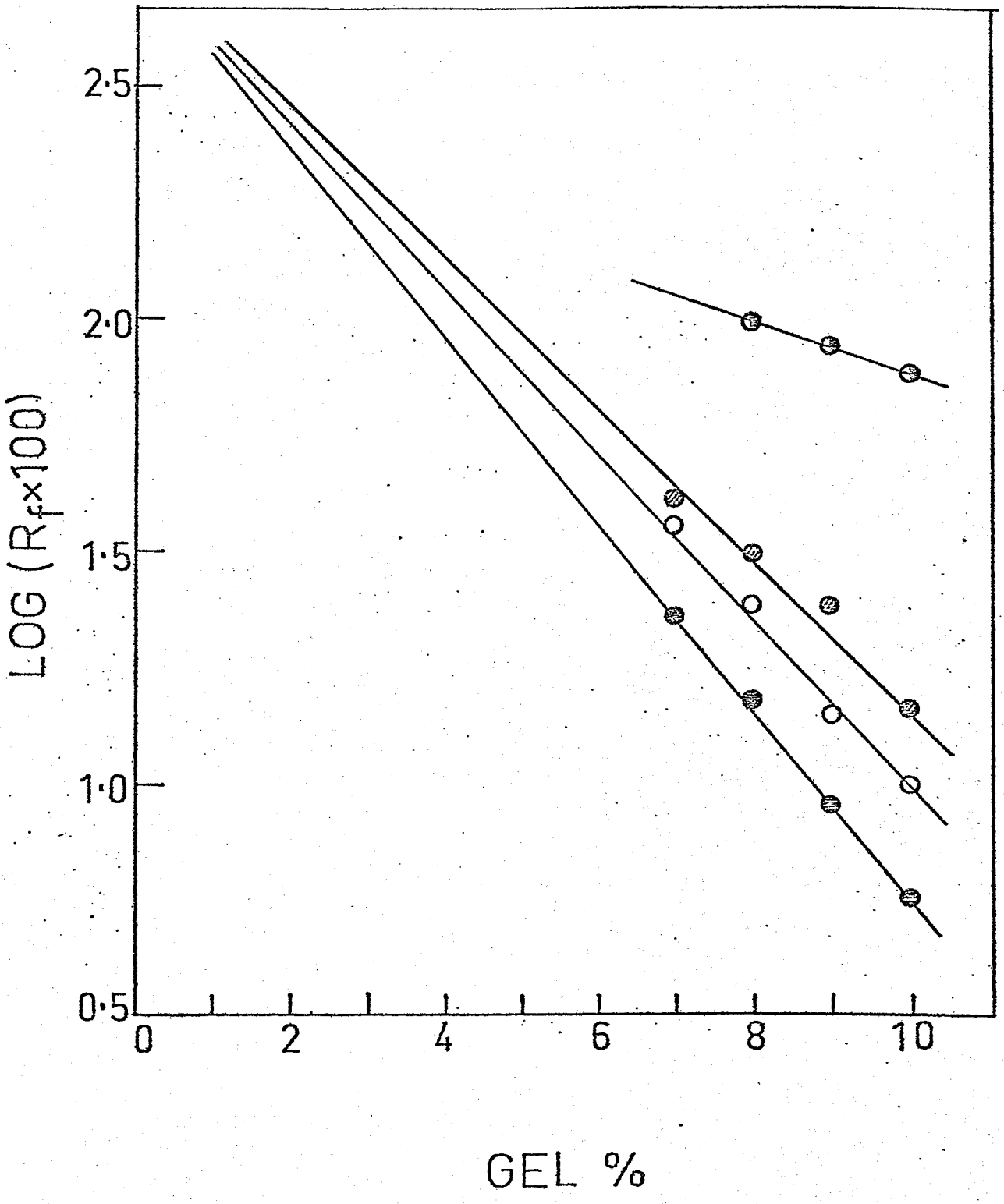
GEL ELECTROPHORESIS

Disc polyacrylamide gel electrophoresis using the Ornstein-Davis system always yielded three distinct, sharp bands, although sometimes a minor band also appeared, which had a higher R_f value than the three normal bands. This multiple band phenomenon was not caused by the presence of the tracking dye bromphenol blue since electrophoretic runs with no tracking dyes added also gave the same band pattern after staining.

Wright & Sanwal (1971) have established that the three band pattern was normal under such conditions for pure citrate synthase. However, it was of interest to study the relationship between these bands. Citrate synthase was subjected to disc electrophoresis on gels with varying degrees of cross-linking. When log R_f of each of these bands was plotted against gel concentration, parallel lines, non-parallel lines intercepting at one point close to the y-axis or non-parallel lines intercepting at several points would be expected, depending on whether the bands represent charge isomers, size isomers or unrelated proteins, respectively. This so-called Ferguson plot is shown in fig.1. The lines cross at a common point at around 2% gel concentration implying that the three bands corresponded to proteins of

Fig.1. The effect of different gel concentration on the the mobility of the 3 distinct bands obtained in disc gel electrophoresis of E.coli citrate synthase. Electrophoreisi performed as described in text, Bis/acrylamide ratio was 1:30, pH 8.0.

(A) fastest moving band of E.coli citrate synthase in disc gel electrophoresis.
(B) middle band.
(C) slowest moving band.
(D) bovine serum albumin monomer.


2·5
2·0
1·5
1·0
0·5
LOG (R_f×100)
0
2
4
6
8
10
GEL %

the same family, with similar charge but different molecular sizes. In preparations where a fourth band was apparent, the log R_f versus % gel plot for this band also crossed the same interception point. The slope of these plots were related to molecular weight. However, we were unable to establish the molecular weights of these different bands. The aggregates covered such a large range of molecular sizes that only a few gel concentrations could be looked at. It is difficult to obtain good, quantitative measurements of slopes based on only a few points. All three citrate synthase bands, however, seem to be larger than the bovine serum albu in monomer (molecular weight 67,000).

Citrate synthase upon treatment with 1% SDS and 1% mercaptoethanol at pH 7.0, 37 degrees centigrade for two hours, gave a single band in SDS gel electrophoresis. The molecular weight corresponds to 44,500±5,000 g/mole as judged from its mobility relative to those of protein standards of known molecular weight. Bromphenol blue was used as tracking dye. The graph for log molecular weight versus mobility of the standards and citrate synthase is shown in fig.2. The error estimated was based on the limit of the extreme upper and lower straight lines that can be drawn through the points. In some SDS gel runs, a second band at 87,000±5,000 also occurs (diagram 2). This minor band may well be citrate synthase dimers undissociated by SDS.

When citrate synthase was incubated with the bifunctional reagent dimethyl suberimidate, which provided inter- and intra-subunit crosslinkages between lysyl residues of the enzyme prior to SDS gel electrophoresis, the final electrophoretic pattern should reveal species with molecular weights representing all possible subunit combinations of the enzyme.

Diagram 2. SDS gel electrophoresis of purified E.coli citrate synthase. 7% gels containing 0.1% SDS, 0.1% mercaptoethanol were used. From left to right:
Citrate synthase, 44,500±5,000;
G_3PDH, 36,000;
Catalase, 60,000;
Bovine serum albumin, 67,000.

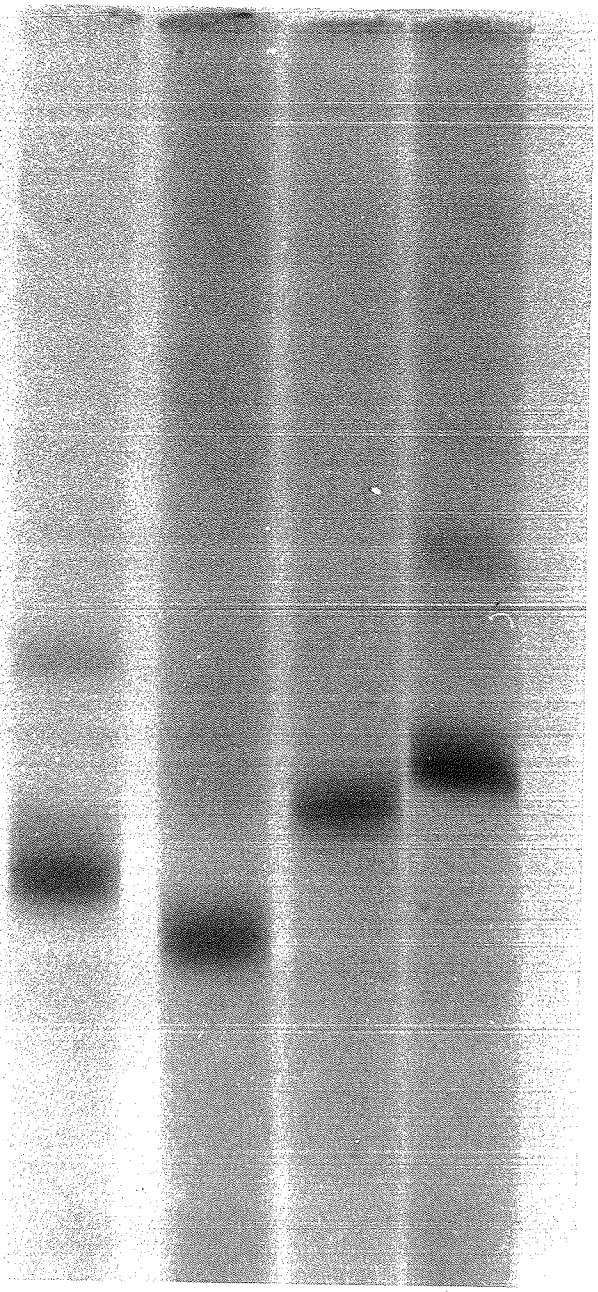


Fig.2. Molecular weight determination of *E.coli* citrate synthase by SDS gel electrophoresis. Position of the citrate synthase band on the calibration line is marked by an arrow. Marker protein molecular weights are:
Bovine serum albumin (BSA) 67,000;
Catalase (CAT) 60,000;
Ovalbumin (OVA) 43,000;
Glyceraldehyde-3-phosphate dehydrogenase (G_3PDH) 36,000;
Lysozyme (LYS) 14,400.

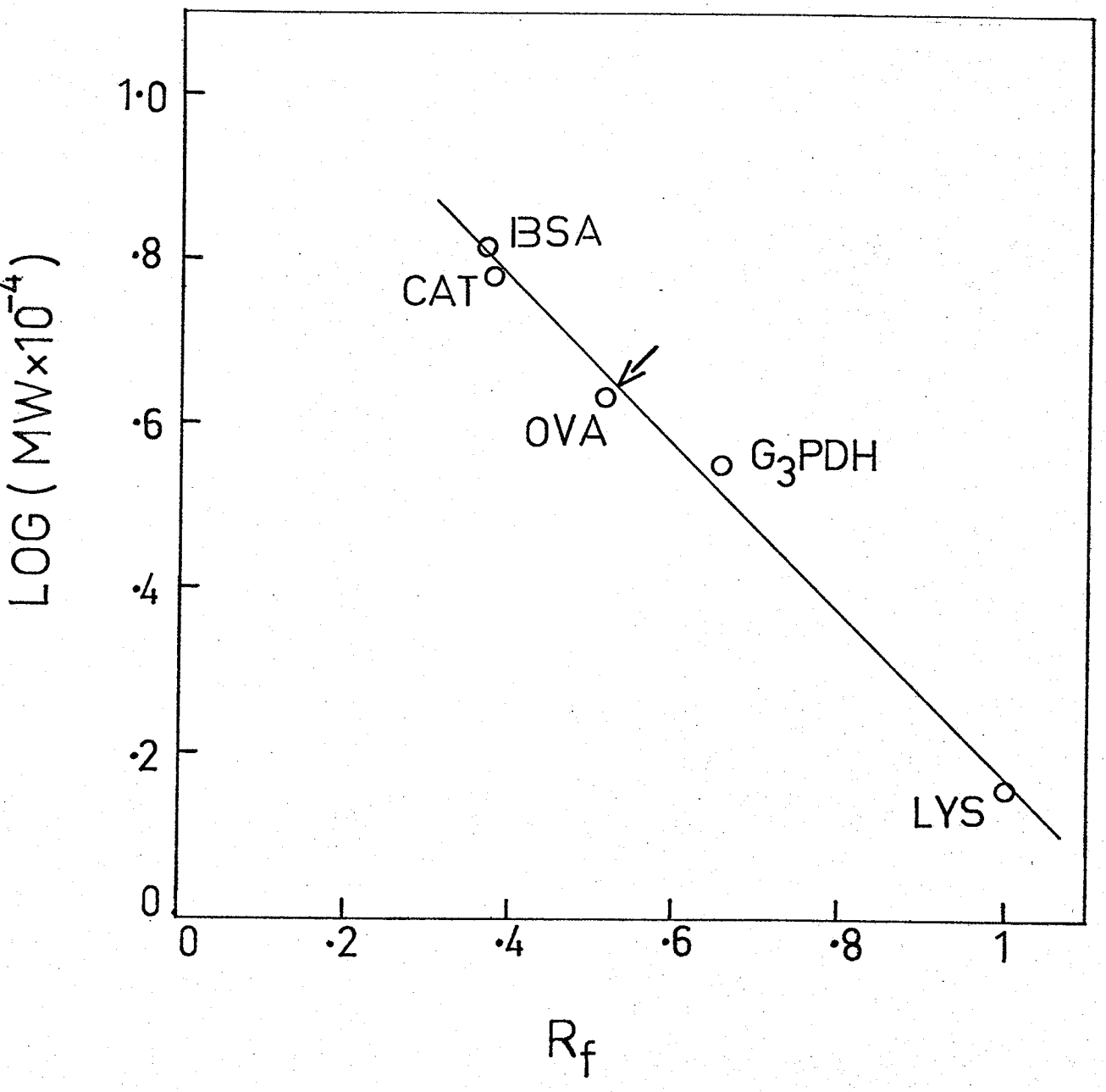

1·0
·8
·6
·4
·2
0
LOG (MW×10⁻⁴)
BSA
CAT
OVA
G3PDH
LYS
0
·2
·4
·6
·8
1
Rf

Diagram 3. SDS gel pattern of dimethyl suberimidate cross-linked proteins. From left to right: G_3PDH, citrate synthase, bovine serum albumin. Note that the dimer (second lowest band) is the major product of the series of citrate synthase bands. Citrate synthase bands corresponding to higher molecular weights are less intense and did not show up well in the photograph.

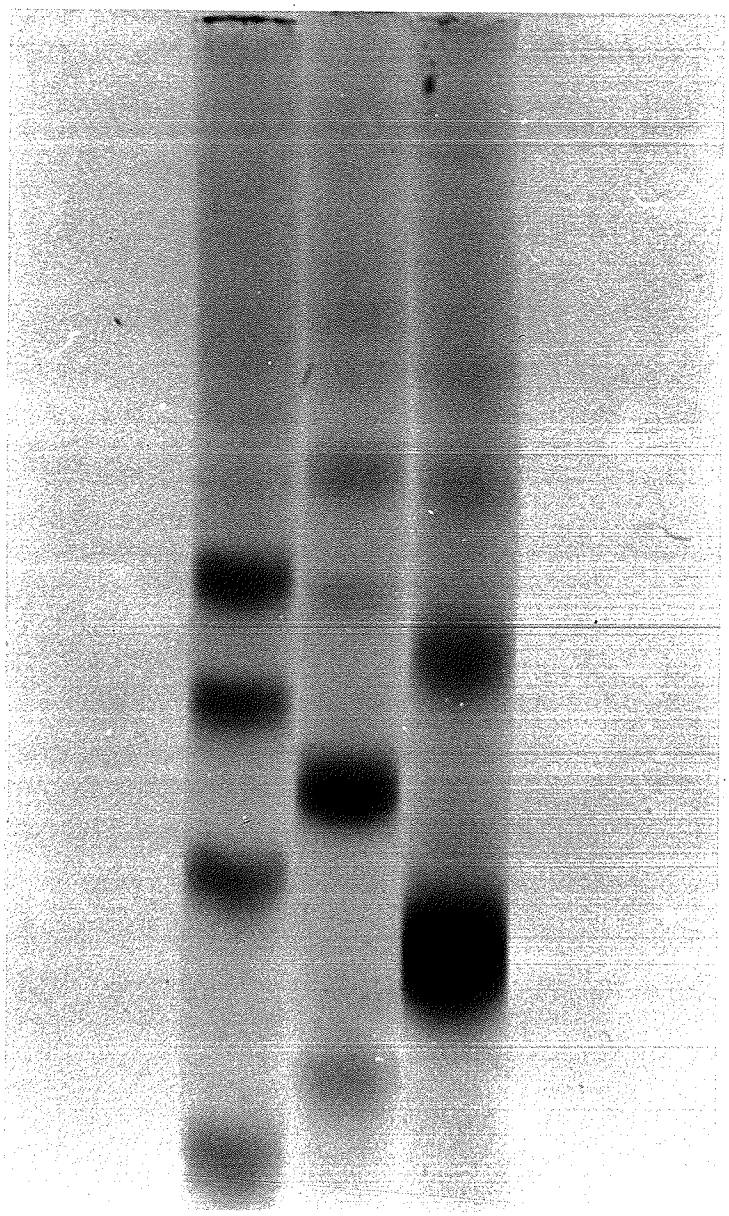


Fig. 3. Estimation of the molecular weights of cross-linked subunits of citrate synthase from E.coli. All cross-linked proteins were dissociated with 1% SDS and subjected to electrophoresis (diagram 3) on 3.5% acrylamide gels, pH 8.5. Approximately 50μg proteins were applied to each gel. The subunit masses of the marker proteins are:
Glyceraldehyde-3-phosphate dehydrogenase (G-1) 36,000;
Catalase monomer (C-1) 60,000;
Bovine serum albumin monomer (B-1) 67,000.
Their oligomers are multiples of their monomeric values.
Positions of the citrate synthase bands are shown by arrows.

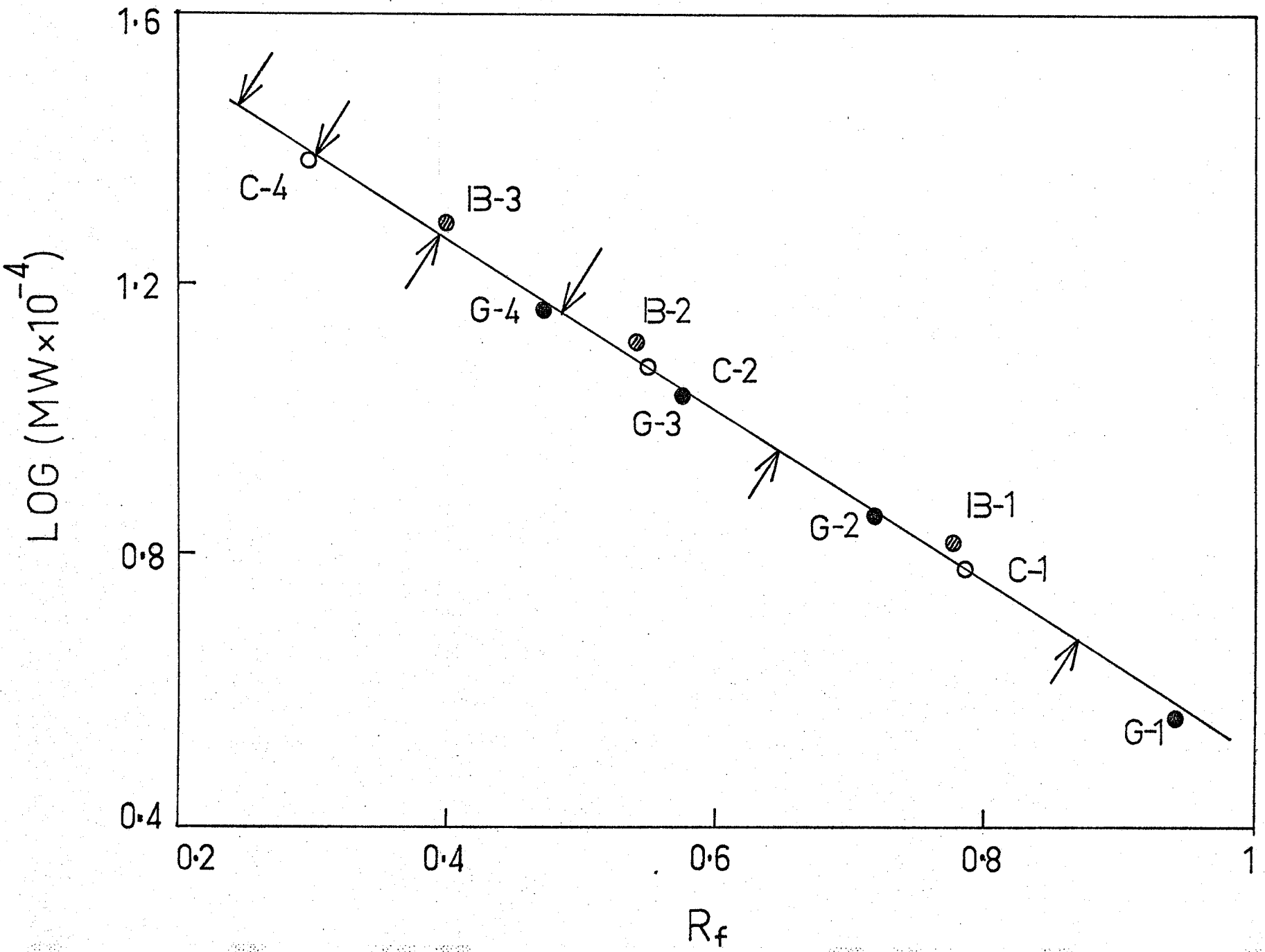

1·6
1·2
0·8
0·4
LOG (MW×10^{-4})
0·2
0·4
0·6
0·8
1
R_f
C-4
IB-3
G-4
IB-2
C-2
G-3
IB-1
G-2
C-1
G-1

Table 2. Molecular weight of dimethyl suberimidate cross-linked species of citrate synthase resolved by SDS-acrylamide gel electrophoresis.

Band	Molecular weight*	Multiple of 47,000
1	49,000±2,100**	1.04±0.04
2	91,000±5,200	1.95±0.11
3	146,000±6,000	3.11±0.13
4	189,000±8,000	4.02±0.17
5	247,000±11,000	5.25±0.23
6	290,000±13,000	6.17±0.28

* data from fig.3
** the error estimated here is based on an uncertainty of ±0.05cm in measuring the distances migrated by citrate synthase bands in the gel.

The gel electrophoretic pattern of such an experiment is shown in diagram 3. Indeed at least 6 such bands could be seen. The molecular weights of the proteins corresponding to each of these bands were estimated from a calibration curve consisting of monomers, dimers, trimers, etc. of glyceraldehyde 3-phosphate dehydrogenase, catalase, and bovine serum albumin. The result is shown in fig.3. The monomer molecular weight obtained here agreed well with the number from SDS gels. It should also be pointed out that in the dimethyl suberimidate cross-linked gel pattern, bands corresponding to an even number of subunits are always heavier than the bands corresponding to an odd number of subunits. This may be taken to mean that the formation of dimers and its aggregates are slightly favored over the formation of monomers, trimers and pentamers.

ULTRACENTRIFUGE EXPERIMENTS

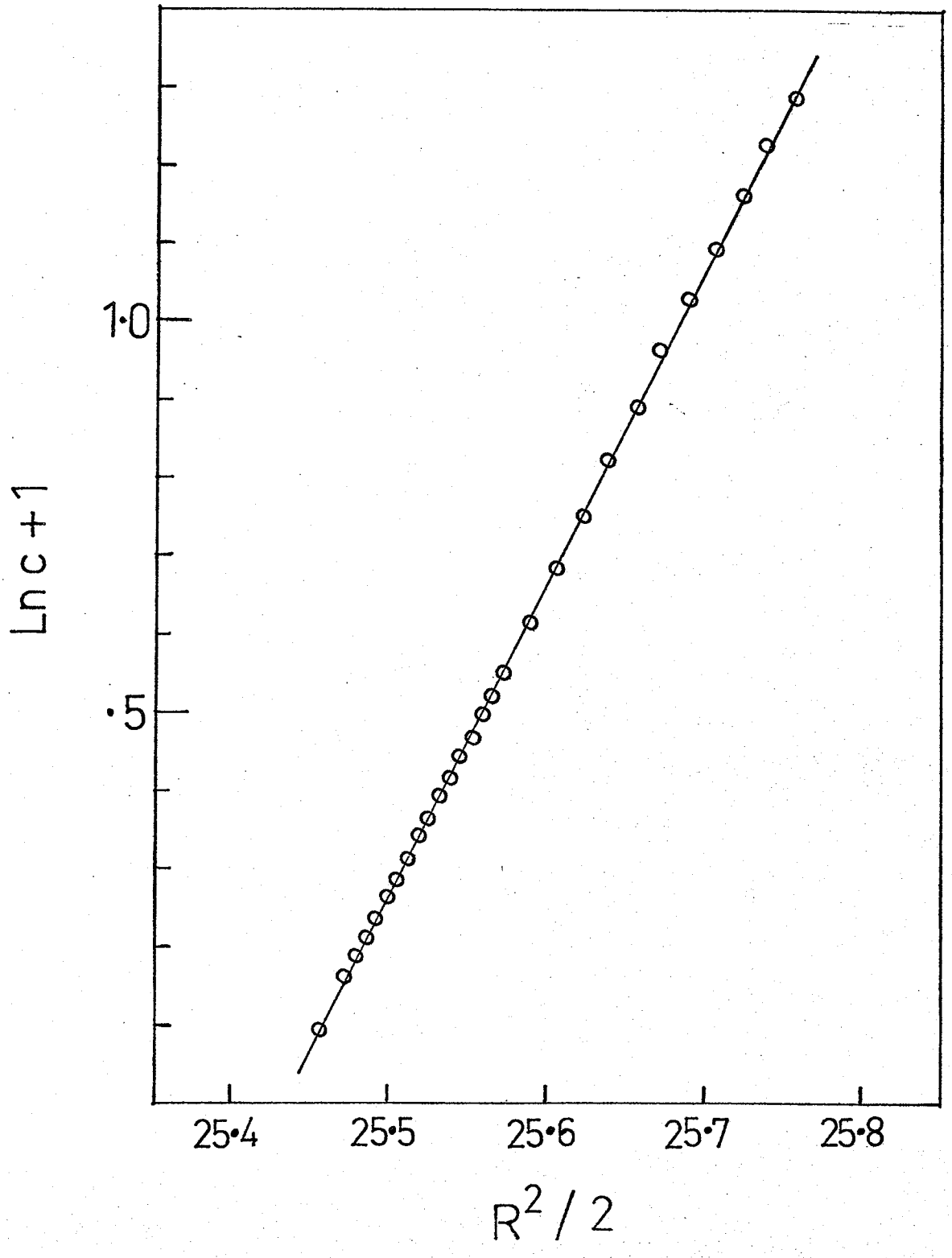
The data from ultracentrifuge experiments are analyzed in several ways. When the system studied is homogeneous, the molecular weight can be obtained from the ln c versus r^2 plot. The 1/molecular weight versus concentration plot can reveal any heterogeneity present in the system under study. This plot gives the weight average molecular weight and the second virial coefficient of homogeneous non-ideal systems. If the heterogeneity of a system is large, the data can be further analyzed by use of the 2-species plot (Roark & Yphantis, 1969). In this plot a given molecular weight average is plotted as a function of the reciprocal of the next lower order average. (Hence $\bar{M}_w$ versus $\bar{M}_n^{-1}$, $\bar{M}_z$ versus $\bar{M}_w^{-1}$, etc.). Associating systems which contain

only two species yield straight lines in such plots. The y-intercept is equal to $(\bar{M}_1 + \bar{M}_2)$ and the slope $(-\bar{M}_1\bar{M}_2)$; where $\bar{M}$ represents molecular weight and subscripts 1 and 2 refer to the two species present. Thus $\bar{M}_1$ and $\bar{M}_2$ can be obtained. Alternatively if the hyperbola $M_k . (1/\bar{M}_{k-1}) = 1$ is drawn, $\bar{M}_1$ and $\bar{M}_2$ are given by the two intercepts of the straight line with this hyperbola. If the plot obtained is concave up, more than 2 species are present, and if charge non-ideality is significant, the initial part of the plot will be concave down. The charge non-ideality effect mentioned above may be reduced by carrying out a non-ideal two species plot.

In this treatment, the different molecular weight averages are expressed as Taylor series expansion; through linear combinations of these molecular weight averages, the coefficients for the second and higher terms can be eliminated; the plot is relatively less influenced by charge non-ideality which is expressed mainly in the second virial coefficient.

The SDS gel experiments showed that the monomer molecular weight of citrate synthase was about 44,500±5,000. This was confirmed by the high speed sedimentation equilibrium experiment of Yphantis (1964) done under denaturing conditions. In 6M guanidine-HCl, 1% mercaptoethanol the enzyme existed as a homogeneous species. When the natural logarithm of concentration, measured in mm of fringe displacement, was plotted against the square of radial distance, a straight line was obtained (fig.4) whose slope corresponded to a molecular weight of 43,500±300 g/mole.

Fig. 4. Sedimentation equilibrium of E.coli citrate synthase in 6M guanidine-HCl. Conditions: initial enzyme concentration 0.6 mg/ml, rotor speed 36,000, Bechman An-D rotor was used.


Ln c + 1
1·0
·5
25·4
25·5
25·6
25·7
25·8
R² / 2

In tris-HCl buffer pH 7.8, the native enzyme showed an association-dissociation equilibrium (fig.5). The species present had molecular weights ranging from less than 90,000 to about 185,000, at which point it levelled off. Examination of the 2-species plot (fig.6) showed some non-ideality and much heterogeneity.

When 0.05M KCl was present, a longer linear region in the 2-species plot (fig.8) was obtained. Similar non-ideality was seen and least square line through the linear region of the plot gave $\bar{M}_1$=92,000±5,800; $\bar{M}_2$=281,000±10,000. A non-ideal 2-species plot was carried out for the same set of data. The least square line through the linear region of the plot gave $\bar{M}_1$=96,960 and $\bar{M}_2$=315,000 (fig.9).

At the same pH when 0.1M KCl was included, a concentration of KCl known to fully activate the enzyme under assay conditions, the enzyme was homogeneous with a molecular weight of 285,000, and showed a slight negative dependence of molecular weight on concentration.

At pH 7.0, the enzyme was very heterogeneous (fig.7); the 2-species plot showed no non-ideality, and indicated the presence of a variety of species. Extrapolation to the hyperbola could not be performed because of the curvature, there could be species from about 50,000 to above 500,000. The lack of non-ideality was consistent with the fact that pH 7.0 was closer to the isoionic point than pH 7.8.

At pH 9.0, the enzyme was homogeneous, showing one species with molecular weight 97,800 (fig.5).

Fig. 5. Sedimentation equilibrium of *E.coli* citrate synthase in non-denaturing media, showing the effect of pH and KCl on the $\bar{M}_w^{-1}$ versus concentration plot. Buffer system used was 0.02M tris-HCl, containing 1mM EDTA. Runs A,B, and C are at 14,000 rpm., E is at 12,000 rpm., all using the Backman An-J rotor. A, pH 9.0; B, pH 7.8; C, pH 7.0; D, pH 7.8 with 0.1M KCl; E, pH 10.0. Error bars are shown for selected points of each plot in order to show the confidence limits of the experiments.

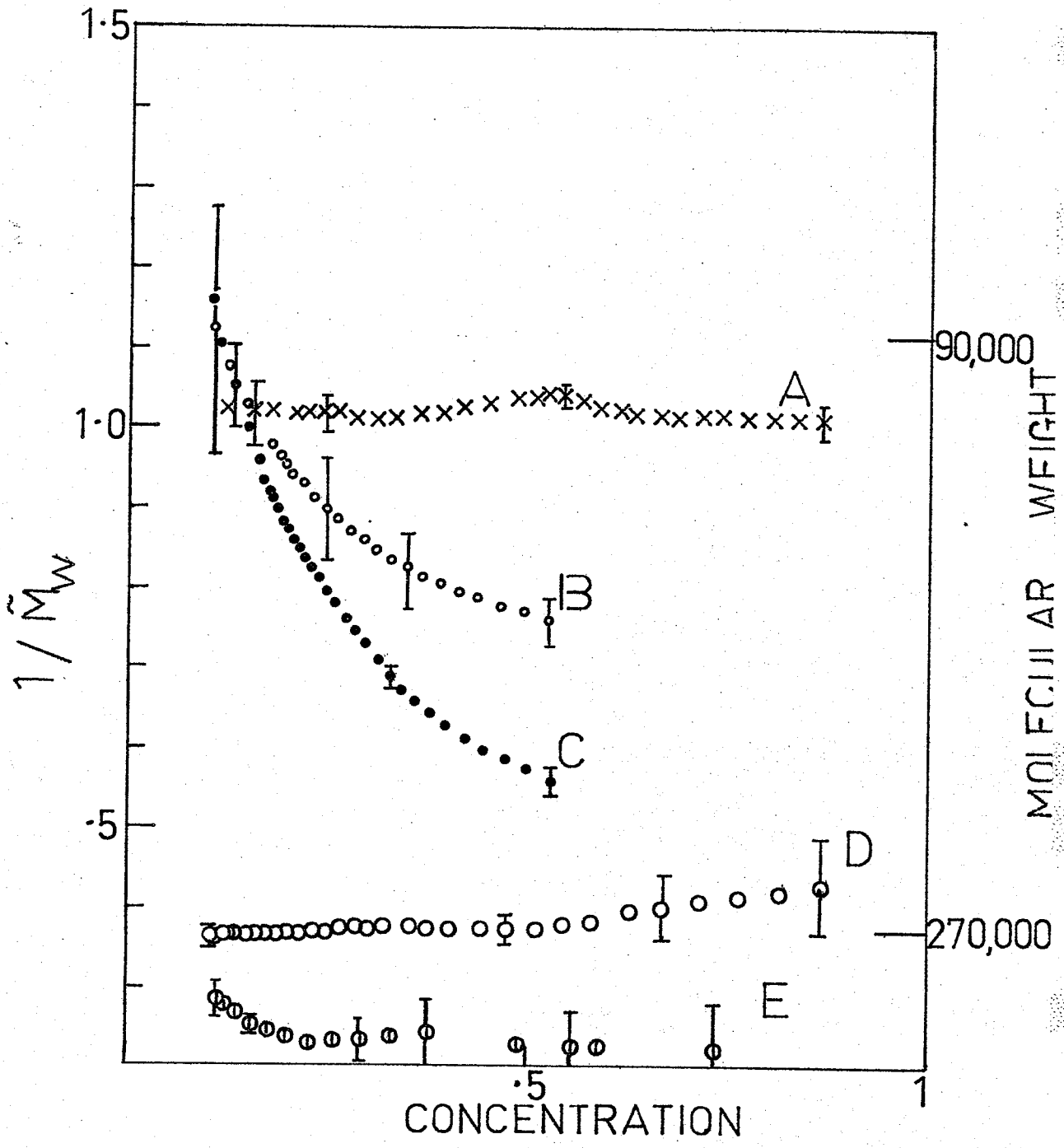

1·5
1·0
·5
1 / M̃w
A
B
C
D
E
90,000
270,000
MOLECULAR WEIGHT
·5
1
CONCENTRATION

Fig. 6. Sedimentation equilibrium of E.coli citrate synthase. 2-species plot of the data taken from Fig.5 curve B (pH7.8). Reduce molecular weight averages are used in the plot. (●) σ_w versus σ_n^{-1}; (o) σ_z versus σ_w^{-1} Corresponding molecular weightx10,000 is shown on the right ordinate. Solid line is the hyperbola calculated for $\sigma_n=\sigma_w=\sigma_z=\sigma_{z+1}$. The expected homogeneous oligomers are marked on the hyperbola. Note that the initial part of both curves are concave down slightly, indicating the presence of non-ideality.

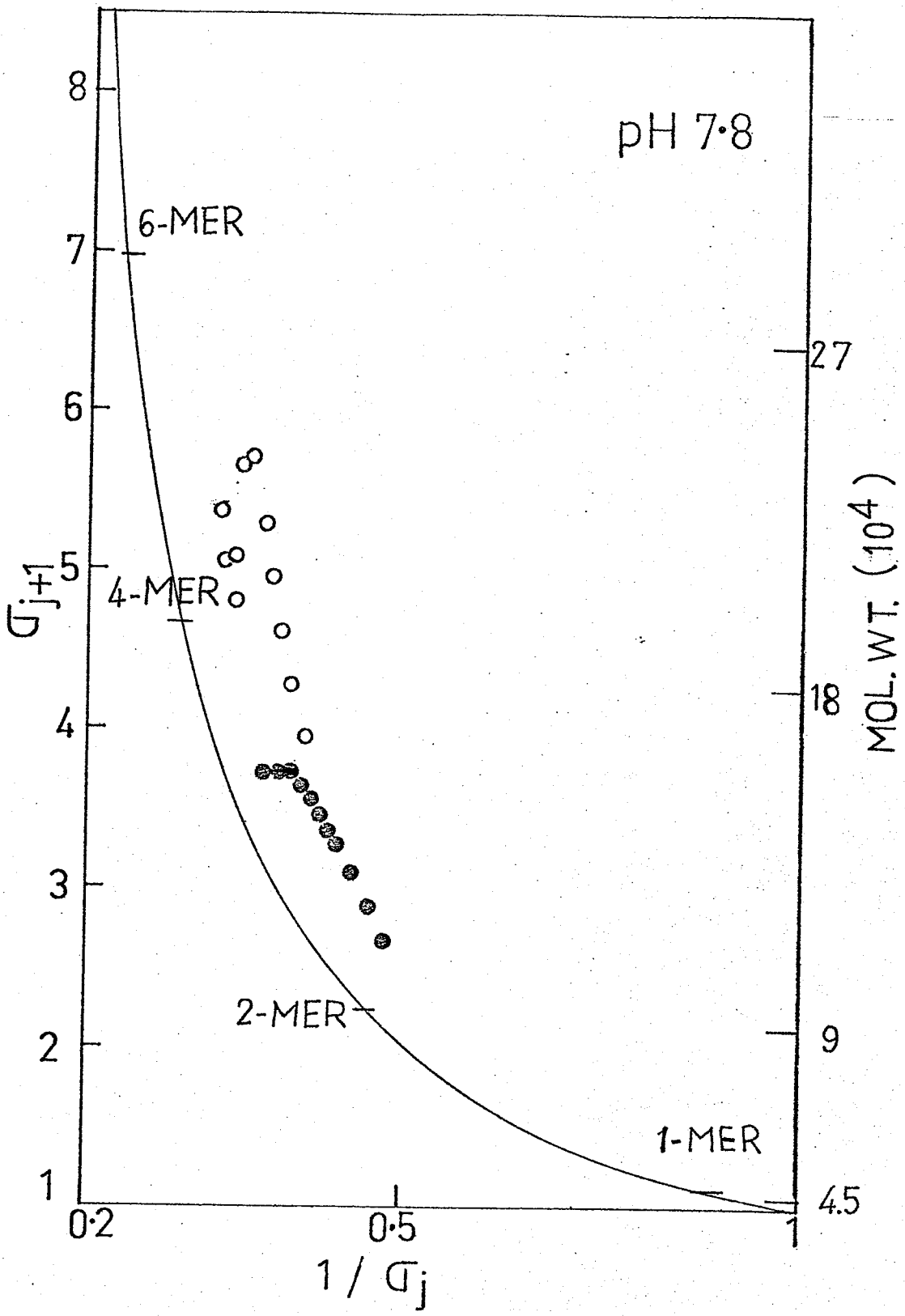

pH 7·8
6-MER
4-MER
2-MER
1-MER
8
7
6
5
4
3
2
1
27
18
9
4.5
MOL. WT. (10^4)
G_{j+1}
0·2
0·5
1
1 / G_j

Fig. 7. Sedimentation equilibrium of E.coli citrate synthase. 2-species plot of the data taken from Fig.5 curve C (pH7.0) ± 1 (see Fig.6 for legend).
(●) σ_w versus σ_n^{-1}; (o) σ_z versus σ_w^{-1}.
Note that the points are concave up, and spread over a wider region, showing that the sample is more heterogeneous at pH 7.0 than at pH 7.8. Non-ideality if present would have been vastly masked by the heterogeneity of the sample.

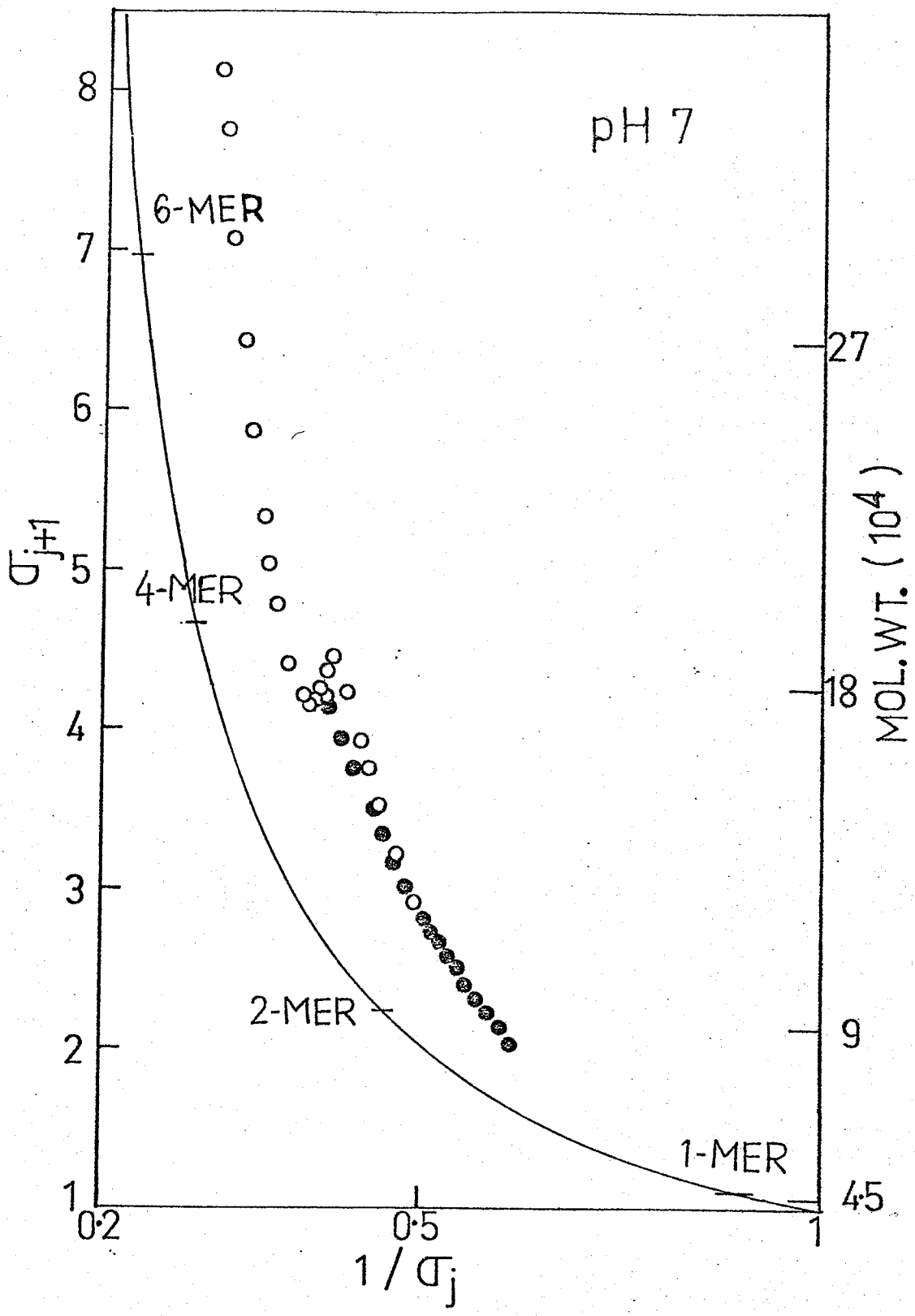

pH 7
6-MER
4-MER
2-MER
1-MER
8
7
6
5
4
3
2
1
σ_{j+1}
27
18
9
45
MOL. WT. (10^4)
0·2
0·5
1
$1/\sigma_j$

Fig. 8. Sedimentation equilibrium of *E.coli* citrate synthase. 2-species plot of a run in 0.02M tris-HCl buffer pH 7.8, 1mM EDTA, containing 0.05M KCl. (◐) σ_w versus σ_n^{-1}; (●) σ_z versus σ_w^{-1}; (o) σ_{z+1} versus σ_z^{-1} The least square line through the "linear region" of the curves is shown. The "linear region" include points up to the inflection point of the curves where they begin to concave down.

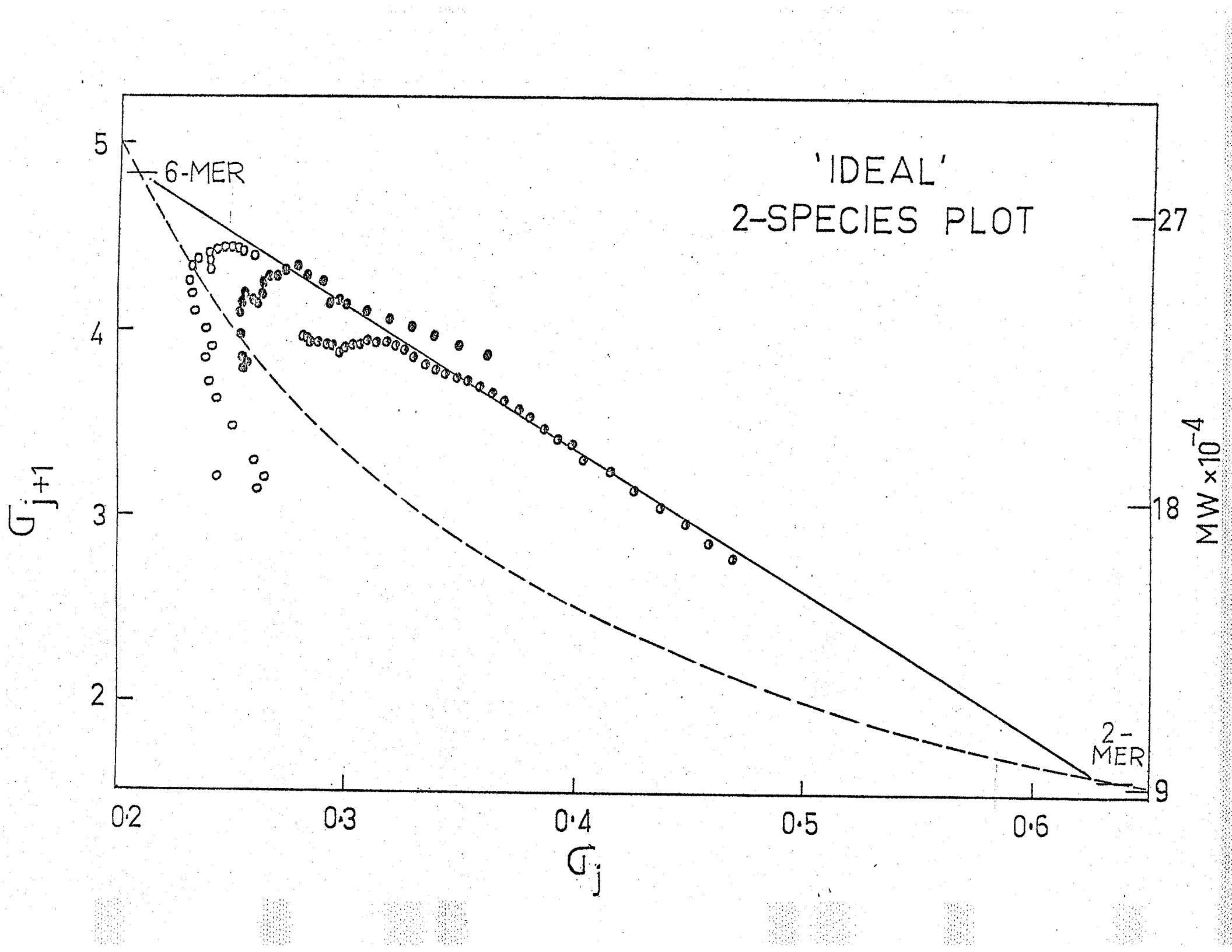

'IDEAL'
2-SPECIES PLOT
6-MER
2-MER
5
4
3
2
27
18
9
$MW \times 10^{-4}$
G_{j+1}
0·2
0·3
0·4
0·5
0·6
G_j

Fig. 9. Sedimentation equilibrium of *E.coli* citrate synthase. "non-ideal" 2-species plot of the same set of data as used in Fig. 8.

(o) $2(\sigma_w - \sigma_z)$ versus $\sigma_{y_1}^{-1}$

(•) $\frac{\sigma_z}{\sigma_w}(\sigma_z + \sigma_w - \sigma_{z+1})$ versus $\sigma_{y_2}^{-1}$

Again, the least square line through the "linear region" of the curves is shown. The last six points that concaved downwards from each curve was omitted.

σ_{y_1} and σ_{y_2} are ideal moments as defined in Roark & Yphantis (1969).

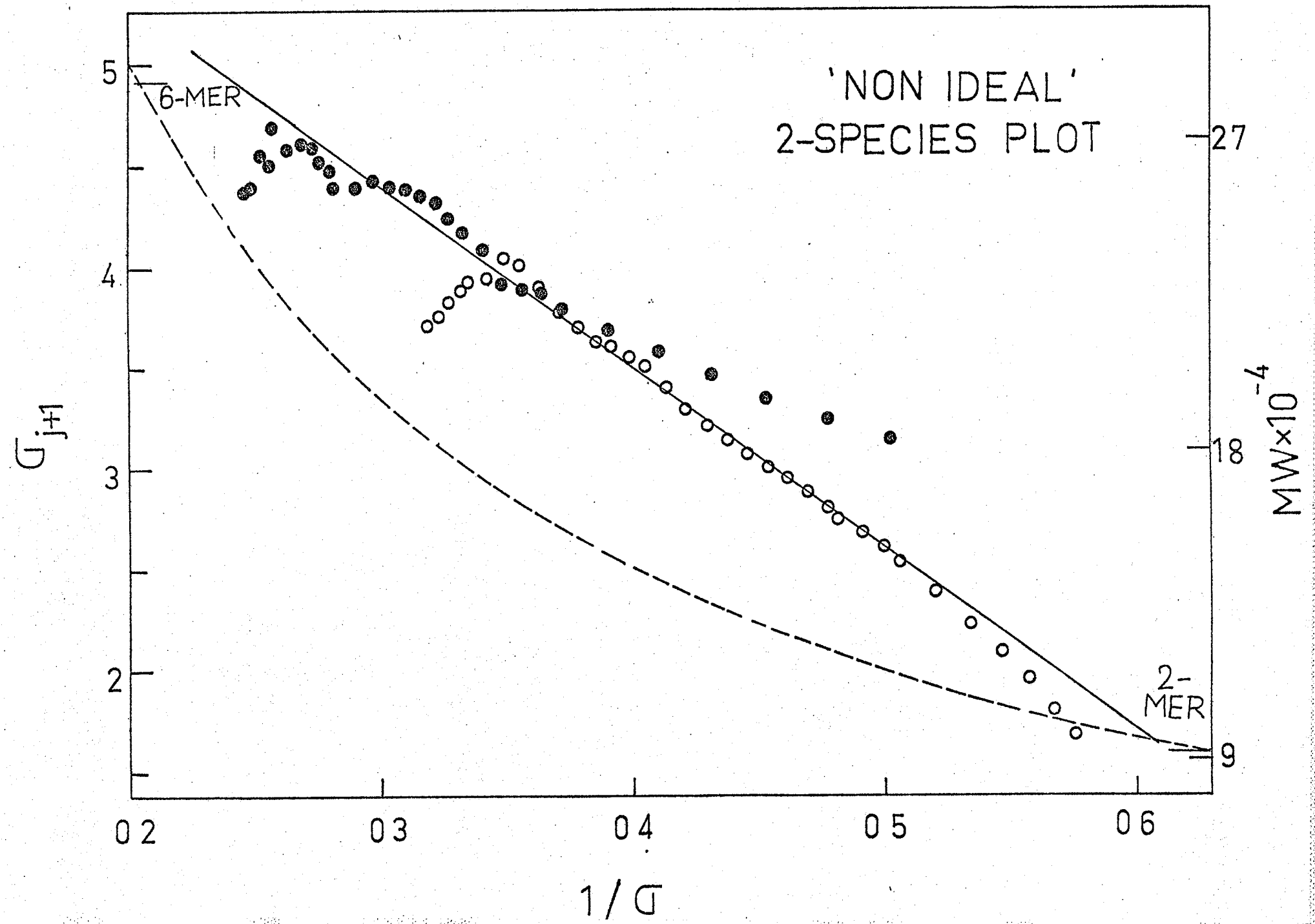
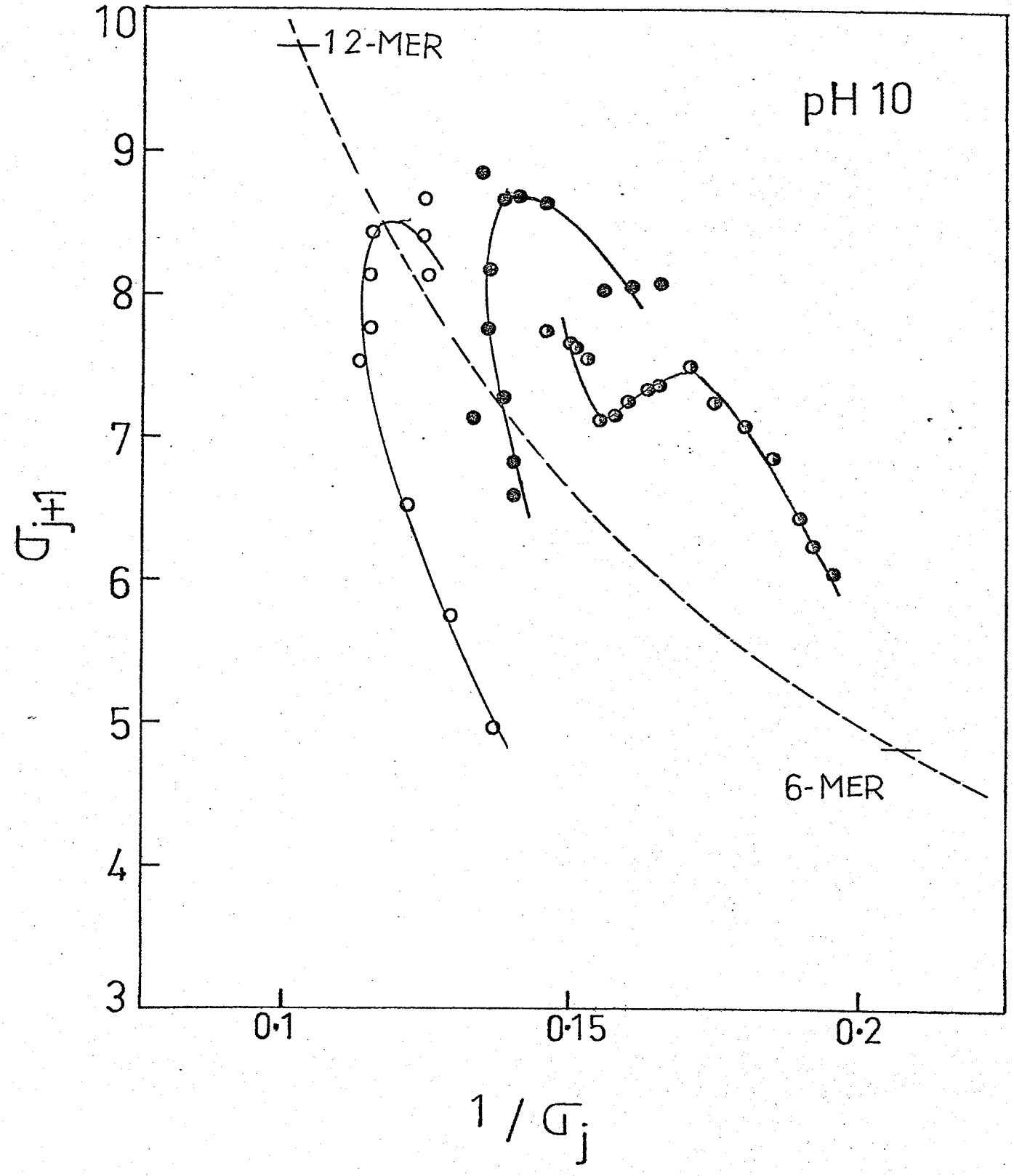

'NON IDEAL'
2-SPECIES PLOT
6-MER
2-
MER
5
4
3
2
27
18
9
G_{j+1}
MW×10^{-4}
0 2
0 3
0 4
0 5
0 6
1 / G

Fig. 10. Sedimentation equilibrium of E.coli citrate synthase. 2-species plot of the data taken from Fig.5 curve E. (pH 10.0). (For legend see Fig.6)
(◐) σ_w versus σ_n^{-1}; (●) σ_z versus σ_w^{-1}; (○) σ_{z+1} versus σ_z^{-1}
Note the downward curvatures of these plots, which showed that large charge non-ideality effect is present in the system.


10
9
8
7
6
5
4
3
12-MER
pH 10
6-MER
0·1
0·15
0·2
σ_{ff}
$1 / \sigma_j$

At pH 10.0, the enzyme associated to species with molecular weight above 350,000 (fig.5). The 2-species plot revealed large charge non-ideality in the system (fig.10).

NADH BINDING EXPERIMENTS

Free NADH absorbs at 340nm and 259nm. When excited at 340nm, NADH yields an emission spectrum which peaks at 458nm. In the presence of citrate synthase, NADH binds in such a way that the NADH fluorescence is greatly enhanced, and at the same time the fluorescence peak shifts from 458nm to 428nm (fig.11b).

The enhancement was determined to be 11.5 in one experiment and 12 in another experiment at a lower NADH concentration, averaging 11.8 ± 0.2. This enhancement factor was assumed to be the same for all the NADH binding sites on the enzyme (fig.11b).

The binding of NADH was examined through a Scatchard plot of the data. In this plot, $\bar{n}/N_f$ was plotted against $\bar{n}$, where $\bar{n}$ is the number of NADH molecules bound per molecule enzyme hexamer when the free NADH concentration is N_f. The intercept on the x-axis gives the number of binding sites, N, and the intercept on the y-axis gives N/K_D. The slope is a measure of the association constant K_a. If there were subunit interaction, it would show as downward or upward curvatures depending on whether binding of the ligand becomes harder or easier as $\bar{n}$ increases.

Fig. 11a. Emission spectrum of NADH-citrate synthase complex and NADH alone. Excitation wavelength is 340nm. The peaks at 340nm and 680nm arise from light scattering effect and its overtone.

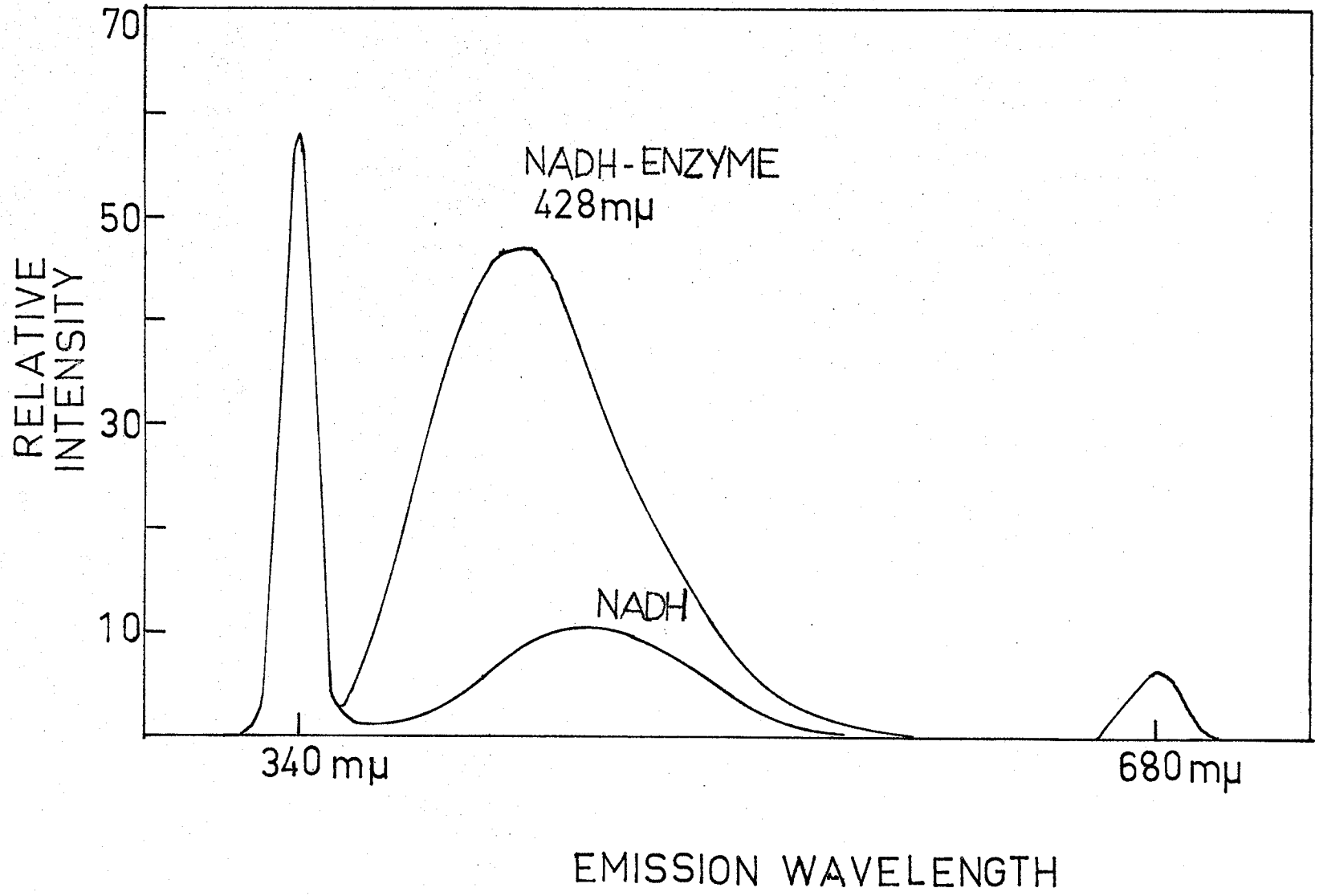
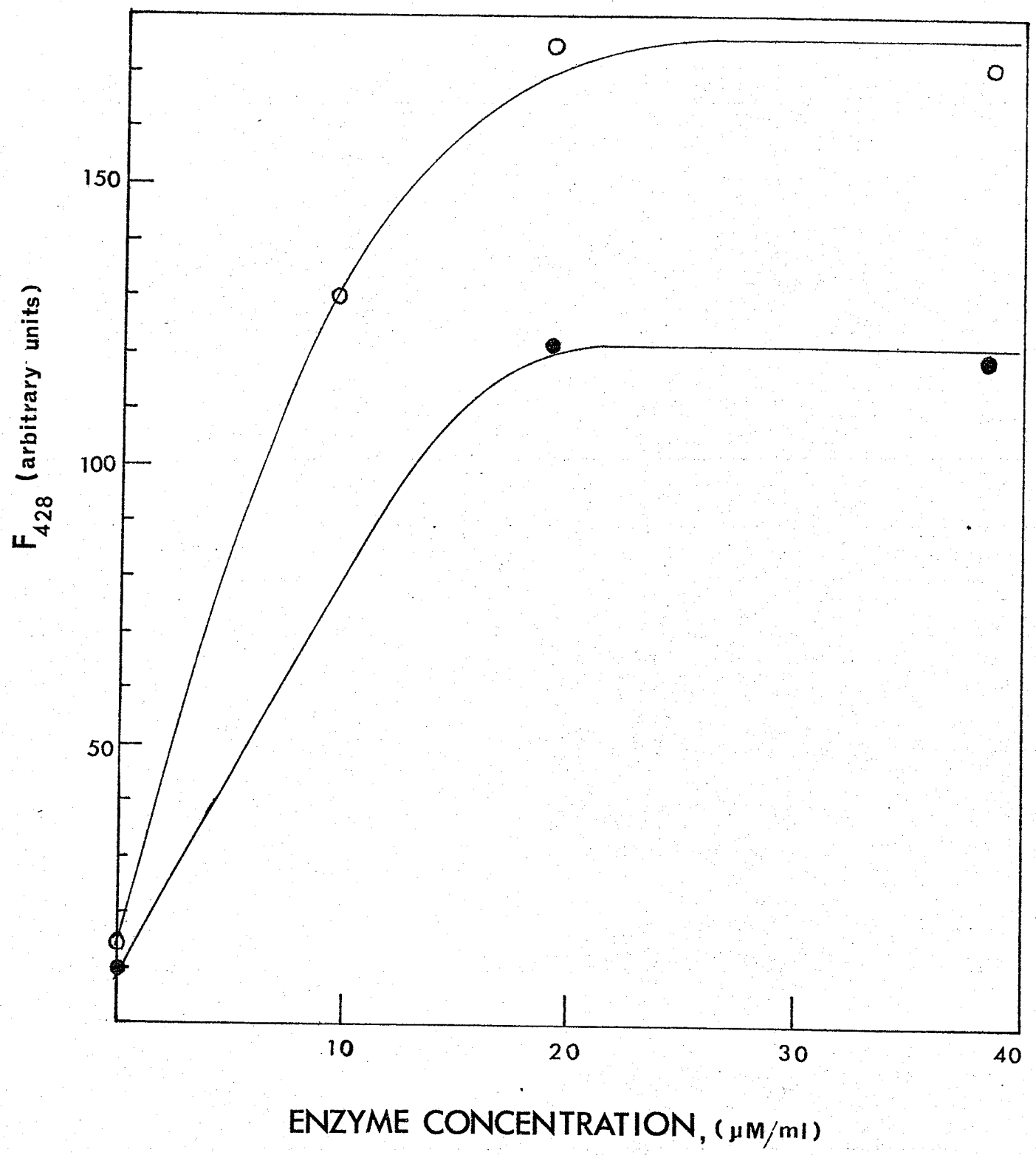

70
50
30
10
RELATIVE INTENSITY
NADH-ENZYME
428mμ
NADH
340 mμ
680 mμ
EMISSION WAVELENGTH

Fig. 11b. Effect of citrate synthase on fluorescence of NADH at 428nm. (○) 4.9μM NADH; (●) 3.2μM NADH. All solutions contained the indicated amounts of NADH and citrate synthase in 0.02M tris-HCl, pH 7.8 in a final volume of 1.00ml.


150
100
50
F_{428} (arbitrary units)
10
20
30
40
ENZYME CONCENTRATION, (μM/ml)

In 0.02M tris-HCl buffer, pH 7.8, and an enzyme concentration of 2.66μmoles per ml, the Scatchard plot for NADH binding (fig.12) showed a long initial region which extrapolated to very close to 3 sites bound per hexamer (assuming monomer molecular weight to be 47,000). The lower end of the plot tailed off to a little over 3 sites. The K_D for the linear part of the plot which extrapolated to 3 sites was 2.3μmole. The fact that 3 sites bound per hexamer were observed pointed to half site reactivity of our enzyme. Half site reactivity was also observed in other systems, e.g. bovine liver glutamate dehydrogenase (Coffee et al 1971).

EFFECT OF ACETYL-COA, OXALOACETATE AND α-KETOGLUTARATE ON NADH BINDING

The Scatchard plots for NADH binding in the presence of these effectors are shown in fig.13. The Scatchard plot for NADH alone is shown as a dotted line in this Figure.

The presence of 1mM acetyl-CoA, a substrate, lowered the affinity of the enzyme for NADH. The data extrapolated to 2.8 sites per hexamer with a K_D of 6.1μmole.

In the presence of 2mM α-ketoglutarate, the other allosteric inhibitor, NADH binding was much tighter than when the latter was present alone. The Scatchard plot indicated 2.9sites bound per hexamer. The K_D was lowered to approximately 0.7μmoles. At an unsaturating concentration of α-ketoglutarate, 0.5mM, the data extrapolated to about the same number of sites, but K_D increased slightly over the value at 2mM α-ketoglutarate.

Table 3. Effect of KCl on dissociation constant for the NADH-citrate synthase complex.

Conditions	K_D in μM, in presence of KCl				
	0	5mM	10mM	0.1M	1M
no additions	2.34±0.13	n.d.	2.05±0.25	4.05±0.35	150±20*
+oxaloacetate 0.2mM	1.50±0.11	2.27±0.20	6.99±2.25	5.32±0.26	51±13*
+acetyl-CoA 1mM	6.12±0.32	n.d.	6.62±0.61	42.90±3.90	190±34*
+α-ketoglutarate 2mM	0.69±0.06	0.77±0.11	0.57±0.06	1.38±0.20	4.84±0.29

* These values were calculated from the intercepts of the vertical axis of Scatchard plots, assuming 1.0 sites/dimer

At a saturating concentration of oxaloacetate, 0.2mM, NADH binding was again tighter than when no effector was present. The data extrapolated to 3 sites. Some binding beyond 3 sites was also observed at the higher NADH concentrations.

EFFECT OF KCl ON NADH BINDING

KCl is known to be an activator of citrate synthase. In the presence of 0.1M KCl, the enzyme is completely desensitized towards NADH inhibition (Faloona & Srere, 1969). It is of interest to find out whether the desensitization toward NADH inhibition is due to a loss of NADH binding ability of the enzyme in 0.1M KCl, or to other changes of the enzyme under such conditions.

Our sedimentation equilibrium experiments have shown that at pH 7.8, in the presence of 0.1M KCl, the enzyme exists as a hexamer. Under the same conditions, the binding data showed there were again 3 NADH bound per enzyme hexamer (fig.13), although the binding was somewhat less tight compared to the binding when KCl was not present. Thus the enzyme could still bind NADH when 0.1M KCl was present, but the NADH bound could no longer inhibit its activity.

The effects of 0.1M KCl plus acetyl-CoA, or oxaloacetate, or α-ketoglutarate were all similar, in that all binding curves showed 3 sites on the x-axis, while the affinity of the enzyme for NADH decreased. The estimated K_D's are listed in Table 3.

Fig. 12. Binding of NADH to E.coli citrate synthase. The buffer system was 0.02M tris-HCl pH 7.8. 2.53μM hexamer citrate synthase was used. $[NADH]_T$ was up to 60μM. The data were plotted by the method of Scatchard. Hexamer molecular weight is assumed to be 282,000.

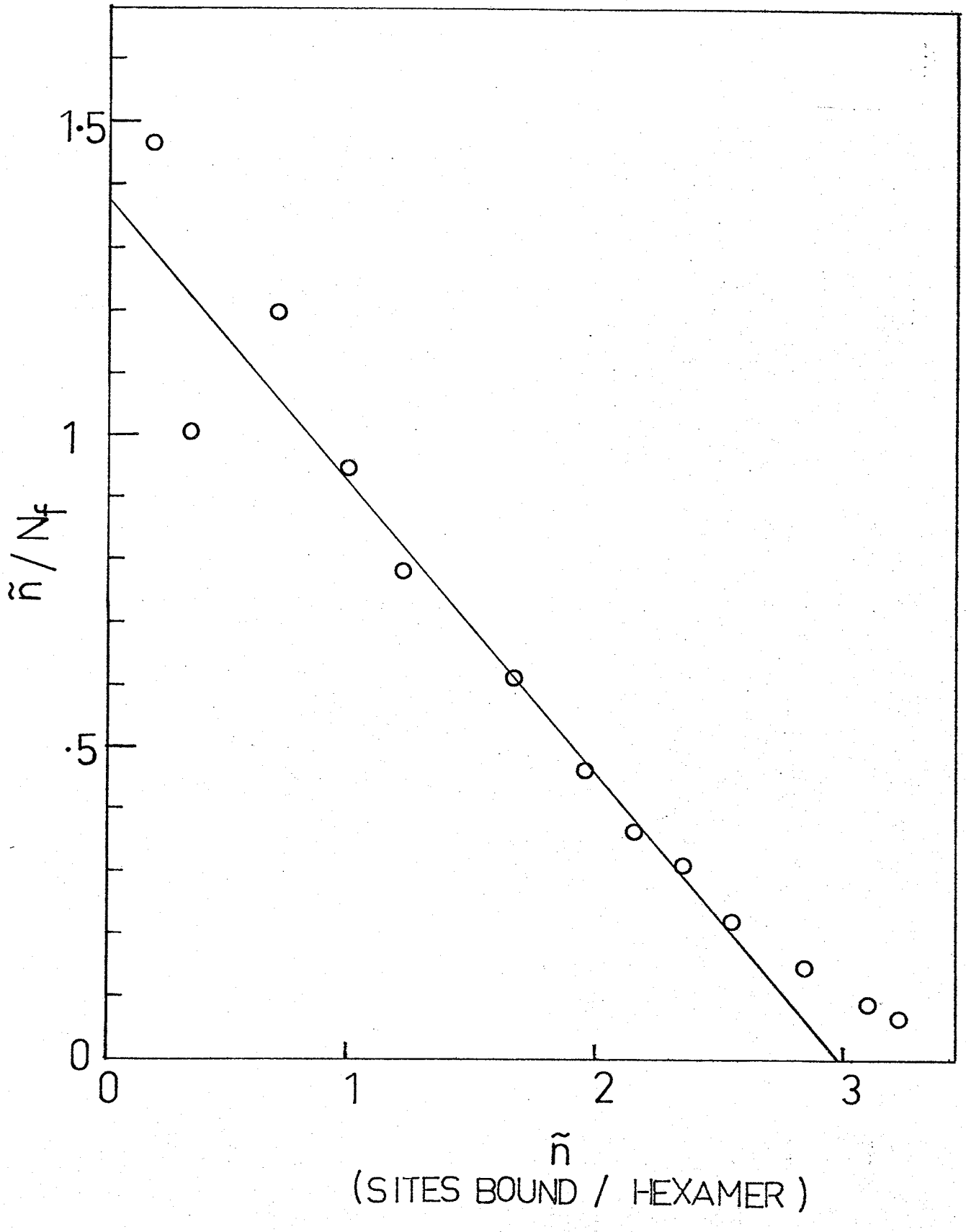
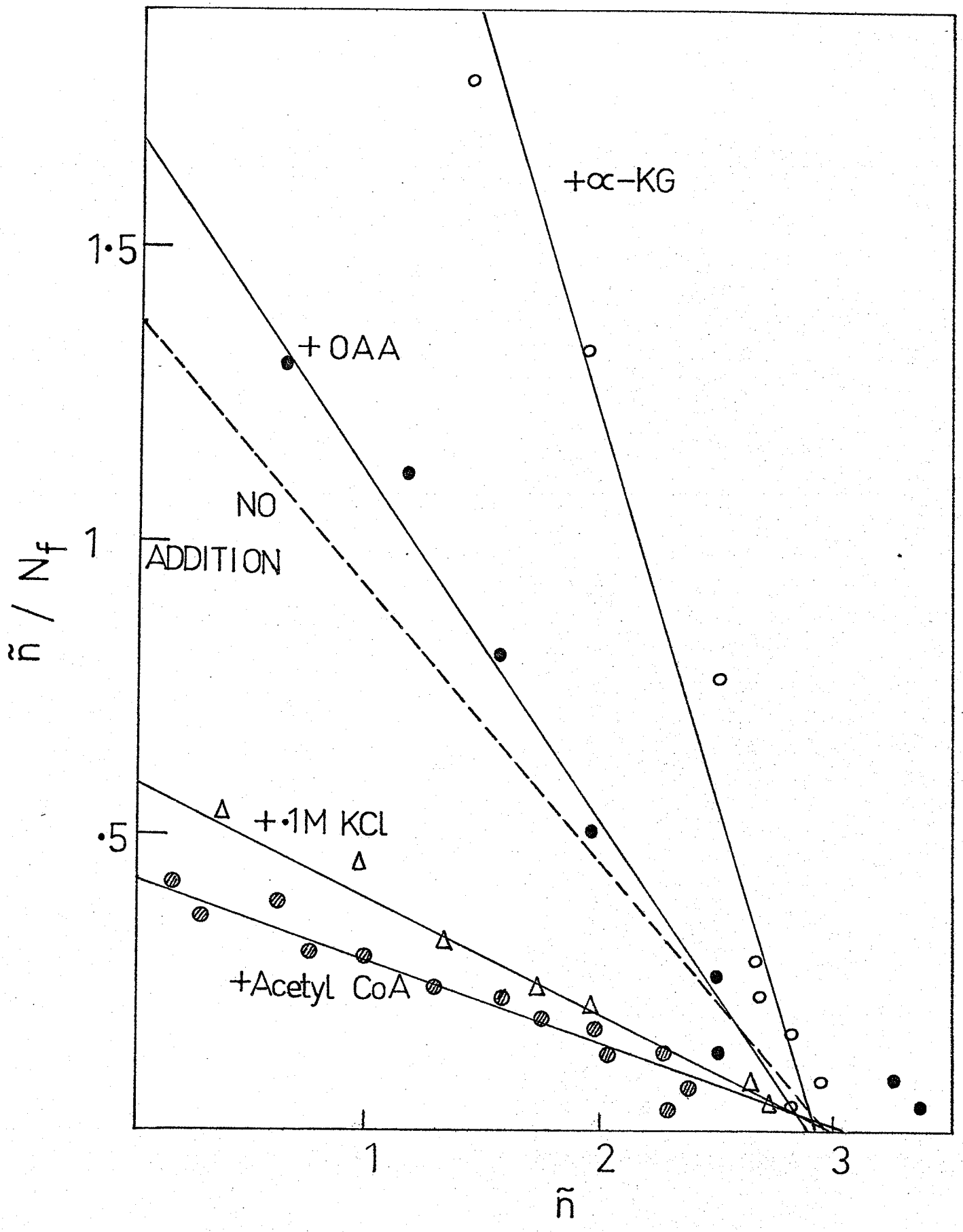

1·5
1
·5
0
0
1
2
3
ñ / Nf
ñ
(SITES BOUND / HEXAMER)

Fig. 13. Scatchard plot of NADH binding to E.coli citrate synthase in the presence of saturating concentrations of various effectors. 1.26μM enzyme used. (○) 2mM α-ketoglutarate; (●) 0.2mM oxaloacetate; (△) 0.1M KCl; (⊘) 1mM acetyl-CoA. The buffer system was the same as described in the legend of Fig.12. The dotted line is the binding curve for NADH alone, taken from Fig. 12.


+α-KG
1·5
+OAA
NO
ADDITION
1
+·1M KCl
·5
+Acetyl CoA
1
2
3
ñ
ñ / N_f

The effect on NADH binding of varying the concentration of KCl in the presence of various effectors was studied. The rate of change of $\log K_D$ (for NADH binding) with log[KCl] was about the same with all the effectors, meaning that the effect of KCl on the enzyme is the same no matter which effector is present. At 1M KCl, there was very little NADH binding under all circumstances. This was presumably due to the high ionic strength introduced by 1M KCl (see Table 3).

EFFECT OF pH ON NADH BINDING

The NADH binding curves at pHs 6.2, 6.6, 7.0, 8.8, 9.15, and 10.0 are shown in fig.14. Binding at pHs lower than 7.8 showed curvature in the Scatchard plots, and the curves extrapolated to a little more than 3 sites per enzyme hexamer. The absolute value of the slopes of the curves decreased as pHs increased from 6.2 to 7.8, which meant binding of NADH became looser as pH went up. Above pH 7.8, the binding became so weak that extrapolation to the x-axis, to give the number of sites, was not feasible. The K_D for these curves can be calculated from the y-intercept by assuming N=3.

These data were further analyzed using a plot of pK_D versus pH. In such a plot the slope of the linear region should reveal the number of protons involved in the interaction between NADH and the enzyme. The slope we obtained for such a plot was -1.33. This means that an average of 1.33 protons were taken up per molecule of NADH associated with the enzyme. If the binding of NADH was an ordinary process, we would expect a slope of -1, i.e. when one NADH is bound, one proton is taken up, and vice versa. Since the slope we obtained is

Fig. 14. Scatchard plot of NADH binding to E.coli citrate synthase at different pH values. 1.20μM enzyme used. (A) pH 6.2; (B) pH 6.6; (C) pH 7.0; (D) pH 8.8; (E) pH 9.15 and (F) pH 10.0. Tris-HCl buffer 0.02M was used in all cases. The dotted line is the binding curve for NADH alone, taken from Fig.12.

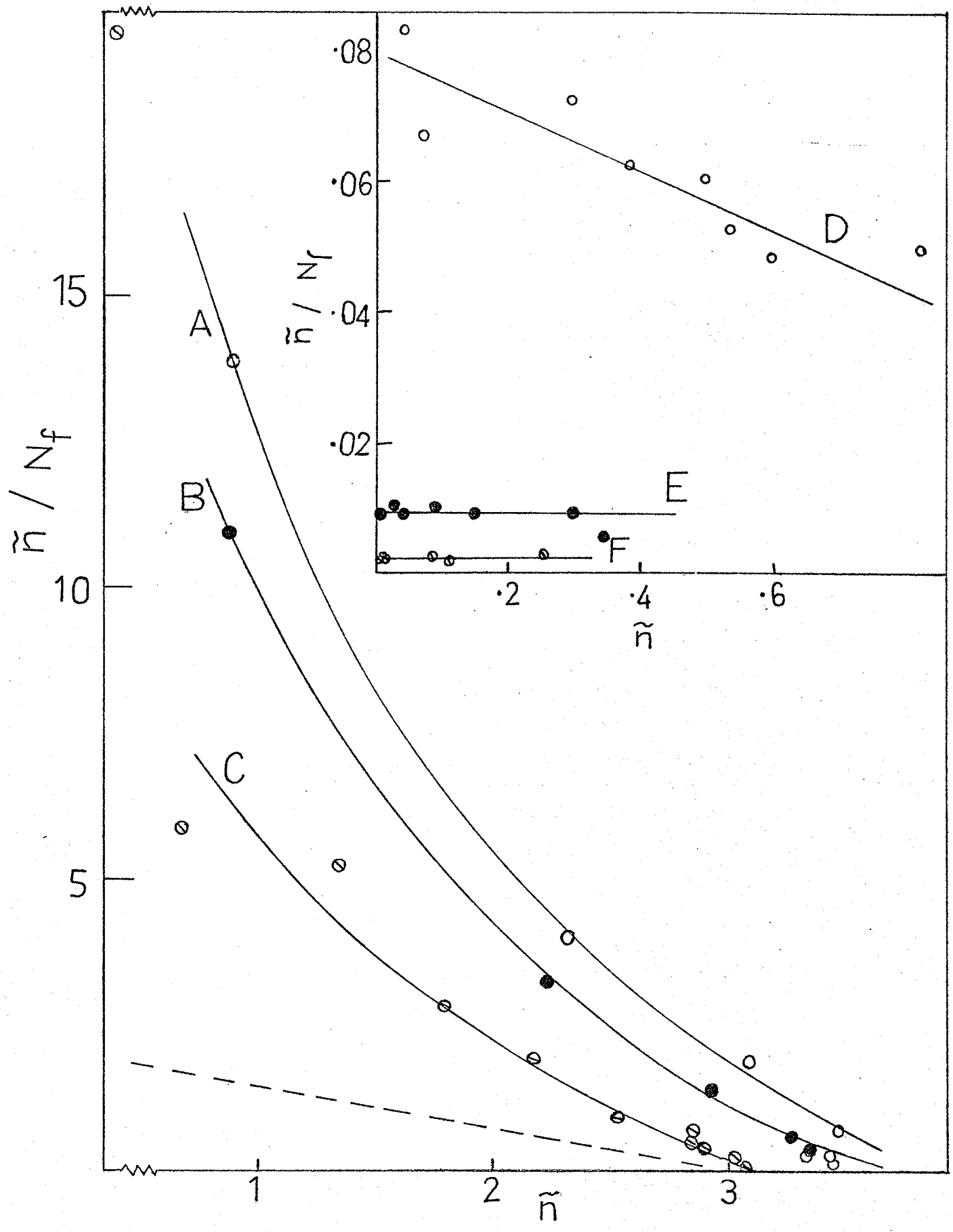
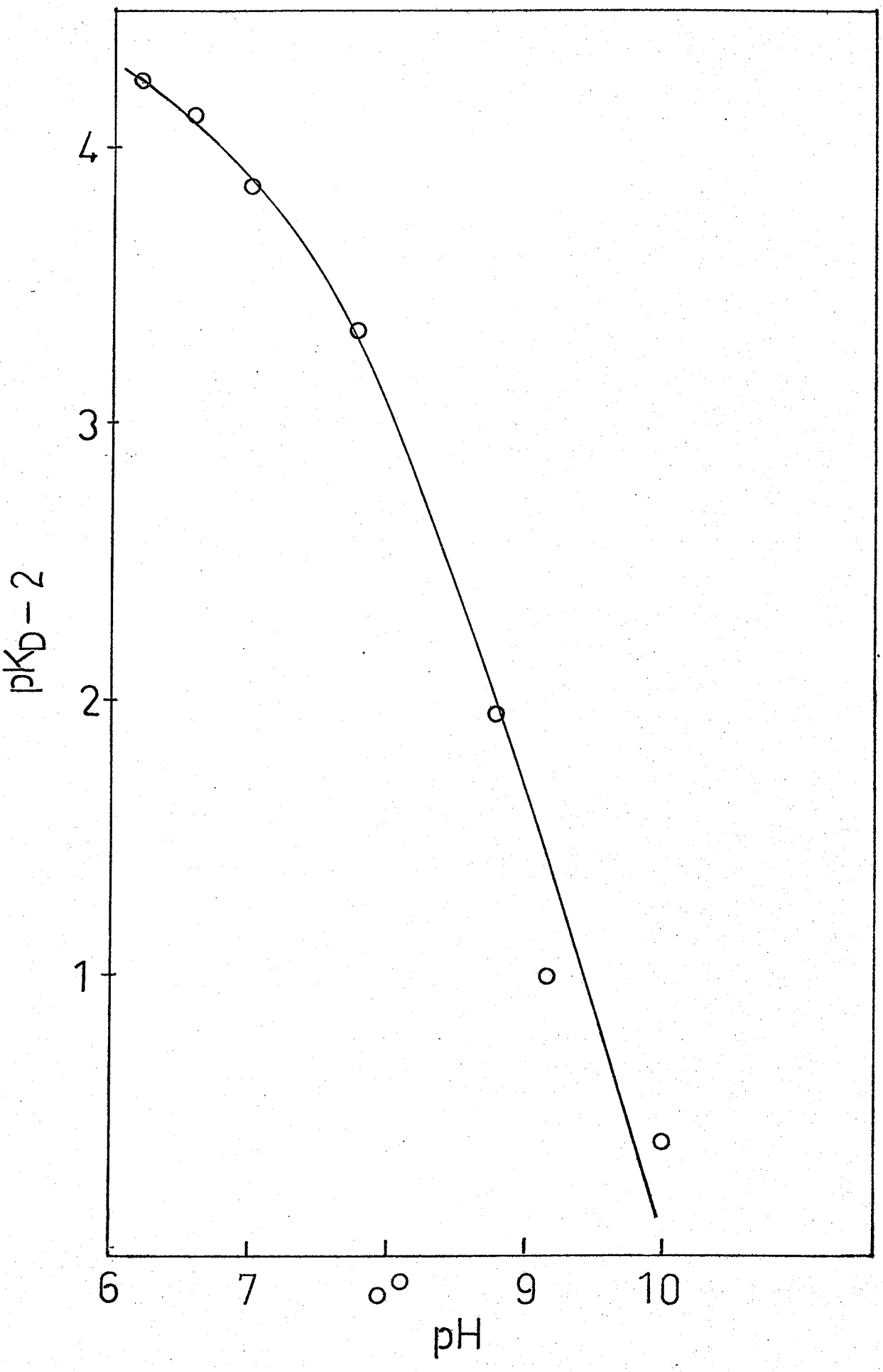

15
10
5
1
2
3
$\tilde{n}$
$\tilde{n} / N_f$
A
B
C
D
E
F
·08
·06
·04
·02
·2
·4
·6
$\tilde{n} / N_f$
$\tilde{n}$

Fig.15. (pK_D-2) is plotted against pH for the binding of NADH to citrate synthase at various pH values. K_D were obtained from Fig.14 by extrapolating to the y-axis, assuming 3 NADH sites bound per enzyme hexamer. The errors on the points are about twice the diameter of the points, i.e. ±0.02 units.


4
3
2
1
pK_D − 2
6
7
8
9
10
pH

-1.33 instead of -1, it is possible that when the enzyme binds one NADH, its conformation is slightly altered such that an average of 0.33 proton will be taken up in addition to the 1 proton taken up by the ligand binding process itself. The fact that we observed more than one proton being taken up for each NADH bound may be a further piece of evidence for a conformational change induced in the enzyme by NADH binding.

QUENCHING OF TRYPTOPHAN FLUORESCENCE

The fluorescence of a substance is generally more affected by the environment than is the absorption. The fluorescence is said to be quenched when the fluorescence yield is lowered by substances which have little influence on the absorption spectrum. One of the ways fluorescence can be quenched is by internal radiationless transfer. Tryptophan has an absorption peak at 288nm. Its emission peak is at 348nm, which is close enough to the NADH absorption peak that when NADH is bound to the enzyme, quenching of tryptophan fluorescence through radiationless transfer is possible.

The tryptophan fluorescence quenching was followed simultaneously with our NADH binding studies. The fraction of fluorescence quenched was obtained by dividing the amount of fluorescence quenched, for a given amount of NADH bound, by the original fluorescence of the protein (or protein + other effectors). Fig.16 is a plot of fraction of tryptophan fluorescence quenched as a function of the number of NADH molecules bound to the enzyme. Straight lines are obtained

Table 4. Tryptophan fluorescence quenching. Percentage quenched when NADH is bound to the enzyme with or without the presence of other effectors or substrates.

Conditions	Weighted average slope Q_{av}	% quenching for 3 NADH bound
No additions	0.175±0.005	52.5±1.5
+ 10mM KCl	0.176±0.016	52.8±4.8
+ 0.2mM oxaloacetate	0.163±0.016	48.9±4.8
+ 0.5mM α-ketoglutarate	0.174±0.012	52.2±3.6
+ 2.0mM α-ketoglutarate	0.178±0.014	53.4±4.2
+ 1.0mM acetyl-CoA	0.214±0.023	64.2±6.9
+ 1.0mM acetyl-CoA + 10mM KCl	0.206±0.032	61.8±9.6

in each case when 10mM KCl, oxaloacetate, α-ketoglutarate, acetyl-CoA or no effector is included. The plots for NADH alone, NADH + oxaloacetate and NADH + acetyl-CoA are shown in the Figure. The equation of each line will be :

$$\frac{F_0 - F}{F_0} = Q\bar{n}$$

where $(F_0-F)/F_0$ is the fraction of tryptophan fluorescence quenched and $\bar{n}$ is the number of NADH molecules bound per molecule of enzyme. The weighted average slope of each line, Q can be calculated by :

$$Q_{av} = \text{weighted average slope} = \frac{\Sigma_1^k w_i q_i}{\Sigma_1^k w_i}$$

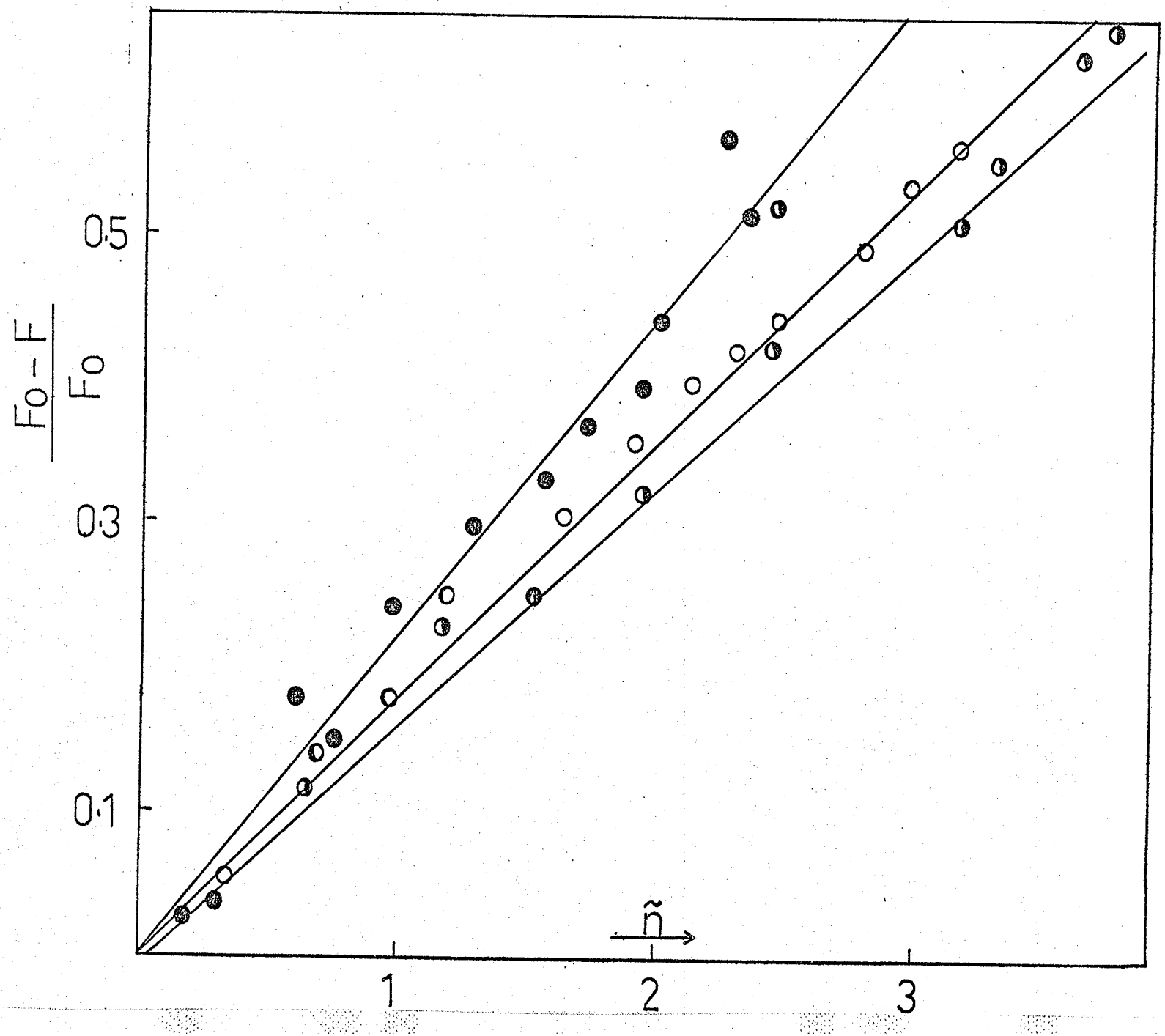
where q_i is the slope calculated from each experimental point.

w_i, the weighing factor, is the observed quenching if the number is less than 0.5 and (1-observed quenching) if the number is greater than 0.5.

k is the number of experimental points taken.

The standard deviation is calculated in the usual way. As 1/3 of the calculated standard deviation is enough to account for 70% of the observed values, we have quoted this number instead of the calculated standard deviation in Table 4. This table lists the weighted average slope of the lines, the standard deviation, and the percent of tryptophan fluorescence quenched when 3 NADH molecules are bound.

Fig. 16. Tryptophan fluorescence quenching of the NADH-enzyme complex. Fraction quenched is plotted against saturation of NADH. Excitation wavelength was 288nm, the fluorescence quenched was followed at 348nm. (●) 1mM acetyl-CoA, citrate synthase and NADH; (○) citrate synthase and NADH; (◐) 0.2mM oxaloacetate, citrate synthase and NADH.


0.5
0.3
0.1
$\frac{F_0 - F}{F_0}$
$\tilde{n}$
1
2
3

The table shows that when 3 NADH sites were filled, the percentage quenching was 52.5. When acetyl-CoA was present, quenching increased to about 64%. Inclusion of other effectors did not seem to change the percentage quenched. A possible explanation is that acetyl-CoA was able to alter the conformation of citrate synthase so that NADH was still bound and tryptophan fluorescence could be quenched more efficiently, while the other effectors were not able to cause this change.

DISCUSSION

SUBUNIT STRUCTURE OF E.coli CITRATE SYNTHASE

The subunit molecular weight of citrate synthase as estimated by SDS gel electrophoresis was 44,500±5,000. The smallest molecular weight species obtained in the SDS gel electrophoresis of the dimethyl suberimidate cross-linked enzyme was 49,000±2,100, which agreed with the SDS gel electrophoresis result within limit of error. The subunit molecular weight obtained by sedimentation equilibrium done at 6N guanidine-HCl was 43,500± 300 , where neither non-ideality nor heterogeneity was observed. These subunit molecular weights agreed reasonably well with each other, but are all lower than the reported 62,000 (Wright & Sanwal, 1971; Danson & Weitzman, 1973) for E.coli citrate synthase. In fact these numbers are closer to the subunit molecular weight of citrate synthase obtained from animal sources, which is reported to be about 50,000. Considering all information obtained from our sedimentation equilibrium runs and gel experiments, we have an averaged subunit molecular weight of 46,720±3,200.

Our amino acid analysis results confirmed those reported by Danson & Weitzman (1973), and are slightly higher than the values reported by Wright & Sanwal (1971). The values for tryptophan and cysteic acid, which required separate determinations, however agreed well between those reported by Wright & Sanwal and those reported by us and Danson & Weitzman.

The subunits of citrate synthase isolated from E.coli may all be of one type. From our SDS gel electrophoresis experiment, we know that the subunits of citrate synthase are similar in size, since they all migrate as one band.

The results of peptide mapping of Wright & Sanwal (1971) and our amino acid analysis further showed that the sequence of the subunits are similar. The former workers have resolved 36-38 soluble peptide spots from a tryptic hydrolyzate of the enzyme by the techniques of paper chromatography and electrophoresis. This total number of peptide spots is dependent on the total number of unique lysine/arginine containing peptides generated by tryptic digest. Our amino acid analysis revealed a total of 42-43 lysine+arginine per monomer. This would lead to a prediction of 43-44 peptide spots resolvable if all the six subunits are the same and there is no overlap of peptide spots. On the other hand, we would expect to find the number of peptide spots considerably bigger than 43-44 if the enzyme had more than one type of subunit sequence. The fact that only 36-38 spots were obtained points to the likelihood of one type of subunit only.

The fact that methionine was found to be the only N-terminal amino acid present is consistent with the idea of one type of subunit. This evidence is however not a strong proof since methionine, alanine and serine are all very common N-terminal amino acid residues of proteins found in E.coli.

The number of half-cysteines we obtained from performic acid oxidized enzyme is in very good agreement with the numbers obtained by Wright & Sanwal (1971) and Faloona & Srere (1969). Wright & Sanwal found 5.6 cysteines per 46,720g while Faloona & Srere got about 5 cysteines per 46,720g of enzyme, both under denaturing conditions. We found 6.3 cysteines for the same amount of enzyme. It is

thus unlikely that there is any disulfide linkage present within the monomer.

It is evident from our SDS gel pattern of the dimethyl suberimidate cross-linked enzyme that the dimer is the main product. Citrate synthases from pig heart, rat heart, rat tissue and moth muscle have shown this dimeric structure(Srere, 1972). The importance of the dimer of citrate synthase from E.coli is further manifested in the 2-species plots shown in figures 8 and 9 where dimer alone is in equilibrium with the hexamer. At pH 9.0 in tris-HCl buffer citrate synthase exists as dimer alone (fig.5)

The presence of KCl is necessary for citrate synthase to exhibit maximum activity. Our sedimentation equilibrium experiments revealed that at pH 7.8, when no KCl was present, the enzyme associated to give a heterogeneous mixture; association went on beyond hexamer. When 0.05M KCl was present at pH 7.8, only 2 species were present, viz. dimer and hexamer. At 0.1M KCl, we had only hexamer (fig.5). It appeared from these results that the effect of KCl was to pull the enzyme to its hexameric form. Faloona & Srere (1971) have reported that the catalytic activity of E.coli citrate synthase will not be at maximum until the KCl concentration reaches 0.1M; it is possible that the hexameric form of the enzyme is necessary for full activity. It is interesting to note that the same authors after purifying the enzyme by a procedure which involved exposure of the enzyme to high concentrations of KCl, obtained for it a molecular weight of 280,000 by gel filtration. Based on a monomer molecular weight of 46,720, the citrate synthase they obtained would be hexamers.

Wright & Sanwal (1971) have studied the effect of pH on the Schlieren sedimentation pattern of citrate synthase. Going from pH 7.0 to pH 11.0, they observed dissociation of an initial peak at $s_{20,w}$=19.8 first to 3 peaks corresponding to $s_{20,w}$ values of 19.8, 13.2 and 5.3; and eventually to one peak with $s_{20,w}$=5.3 at pH 11.0.

We have observed the same trend of dissociation with our citrate synthase in our sedimentation equilibrium experiments. At pH 7.0, species with molecular weights approaching that of a dodecamer were observed; at pH 7.8 heterogeneity as observed in figure 5 was less, but species present included from dimer to hexamer. At pH 9.0, only dimer was present. The fact that at pH 9.0 we have homogeneous dimer while Wright & Sanwal reported presence of 3 species may be explained by the difference in protein concentrations used. They have used a protein concentration of 6 mg/ml in their sedimentation velocity experiments whereas the concentration range we were looking at was below 1 mg/ml.

NADH BINDING TO E.COLI CITRATE SYNTHASE

We were able to observe NADH binding to the enzyme citrate synthase through NADH fluorescence. The binding appeared to be a half site reactivity. Only half as many NADH binding sites were detected as were expected sites from the number of subunits, under all conditions studied, except at pHs 8.8,9.15, and 10.0, where binding was too weak to be extrapolated. The wiping out of the remaining half of expected NADH binding sites might involve a subunit conform-

ational change induced by the NADH bound. In order for the enzyme to do this, it has to be an even numbered polymer, so that when one molecule of NADH is bound to a citrate synthase subunit, it alters the conformation of the next subunit making it so difficult for the second molecule of NADH to bind that essentially no further binding can be observed. In this sense, the dimer would be the basic unit of the enzyme in NADH binding.

Half site reactivity may be explained by negative co-operativity (Levitzki, A. & Koshland, D.E., 1969), which is the decrease in binding constant as successive sites are liganded. Let K represent the interaction constant between subunits, subscripts A and B stand for the conformations of subunits depending on whether it is unliganded or liganded. Then K_{BB} is the interaction constant between two liganded subunits and K_{AB} is the interaction constant between a liganded subunit and an unliganded subunit. If for a system, the interaction constant K_{AB} is bigger than the interaction constant K_{BB}, meaning that the interaction of a liganded subunit with an unliganded subunit is favored over the interaction of two liganded subunits, then we have the phenomenon of negative co-operativity. Negative co-operativity will result by increasing K_{AB} while keeping K_{BB} constant. In the case where K_{AB} is very large and K_{BB} very small, it will become very difficult for the ligand to fill any site beyond the half saturation point.

The possibility of having protein species that are unable to bind NADH may also account for the apparent half site reactivity. At pH 8.8, 9.15, and 10.0, NADH binding was very weak, and extrapolation to the number of sites bound was not pos-

sible. However at lower pH and in the presence of various effectors and substrates, half site reactivity was observed constantly, particularly at 0.1M KCl pH 7.8, a condition we know that will give rise to homogeneous enzyme hexamers. This observation suggests that 'nonbinding' protein may not play a significant role in the half site reactivity observed.

The dependence of negative co-operativity on subunit interaction suggests that dimer or an oligomer is the functional unit of citrate synthase in NADH binding. If subunit interaction is abolished, there will not be any co-operativity. From our sedimentation equilibrium studies on citrate synthase at different pH, we have observed the trend of increasing heterogeneity as pH goes down. Species with average molecular weight smaller than a dimer were observed both at pH 7.8 and pH 7.0. At pH 7.0 the dissociation trend points more to monomer as shown in the 2-species plot (fig.7). The presence of citrate synthase monomer should result in loss of subunit interaction and hence give rise to a lifting of negative co-operativity. Our Scatchard plots (fig.12 and 14) indeed seem to show this lift. At pH 7.8, there were slightly more than 3 sites bound per citrate synthase hexamer (270,000). At pH 7.0 the binding affinity for NADH increased by 10 fold and more than 3 sites per hexamer were seen. Considerably more than 3 sites filled per hexamer were observed when pH was down to 6.6 and 6.2.

It has been pointed out above that citrate synthase can be desensitized towards NADH inhibition and such desensitization is produced in the presence of 0.1M KCl or at pH values above 9.0. Our binding results showed

that at pH above 9.0, NADH bound only very weakly to citrate synthase. It is likely that desensitization at pH above 9.0 is brought about by the inability of NADH to bind citrate synthase.

It was noted that the binding of NADH is affected by a few environmental factors. The binding becomes weaker as the enzyme ages: slight decreases in the amount of NADH bound were observed over a period of several weeks for the same batch of purified enzyme. NADH itself is susceptible to both air oxidation and high citrate synthase concentration. Both tend to reduce the effective concentration of NADH such that the experimental saturation would be low. At high total NADH concentrations, the decrease may be accounted for in part by the large correction factor involved in the calculation of N_f; and in part by the loss of fluorescence quantum yield arises from (1) trivial reabsorption of emitted light (2) formation of ground state NADH dimers or (3) formation of excimers. In our results, we have included points obtained when the free NADH concentration was below 100μM.

The binding of NADH to citrate synthase is affected by the presence of other effectors. In the presence of acetyl-CoA, a substrate of the enzyme, NADH binding is loosened. In the presence of α-ketoglutarate, an allosteric inhibitor of the enzyme like NADH itself, the binding is tightened. Oxaloacetate, the other substrate, which has a similar structure to α-ketoglutarate, also tightens NADH binding. Thus acetyl-CoA tends to remove the effect of NADH on citrate synthase, but α-ketoglutarate (and oxaloacetate) tends to pull the enzyme further to the form favoring tighter NADH binding.

In 0.1M KCl our binding results showed that NADH is able to bind to the enzyme. The K_d estimated was 4.7μM as compared to 2.2μM when there is no KCl present. Thus the effect of KCl on NADH binding of citrate synthase resembled that of acetyl-CoA. Since 0.1M KCl does not eliminate NADH binding but desensitizes the enzyme towards the inhibitory effect, it is possible that K^+ is shifting the enzyme to a conformation which is different from the conformation induced by NADH, hence desensitizing the enzyme.

Our tryptophan fluorescence quenching experiments suggest that acetyl-CoA is capable of altering the conformation of citrate synthase, perhaps by pulling the subunits closer together, since the quenching of tryptophan fluorescence by NADH was more efficient. The presence of oxaloacetate or any other effectors did not cause an appreciable change in the pattern of quenching of tryptophan fluorescence. The fluorescence observed, of course, represented the averaged fluorescence due to 4 tryptophan residues (per 46,000 gram citrate synthase), and so it is possible that conformational changes may lead to increase in quenching of some of the tryptophan residues but decrease for others so that the netchange in fluorescence quenching is very small.

Both our NADH binding and fluorescence quenching experiments suggested that the role of acetyl-CoA as a substrate is more prominent than oxaloacetate. Acetyl-CoA is apparently able to shift citrate synthase to a conformation that weakens NADH binding, something which we expect both substrates to do, but oxaloacetate was proved incapable;

and acetyl-CoA is also capable of producing a more noticeable change in the conformation of citrate synthase as revealed by our quenching experiment. The conformation of citrate synthase that is stabilized by acetyl-CoA is presumably the active form or close to the active form of the enzyme. The native enzyme, being unable to catalyze the citrate condensation reaction maximally, is considered to be in a tight state. In the presence of 0.1M KCl, when the activity of the enzyme is maximal, the enzyme would be in a relaxed state; while in the presence of NADH, catalytic activity of citrate synthase is further reduced, and the enzyme would be in an even tighter state. The conversion of citrate synthase to the NADH stabilized state involved an uptake of 1.3 proton per NADH bound (fig.15). Our study suggests that acetyl-CoA is capable of pulling citrate synthase towards the relaxed state of the enzyme while α-ketoglutarate and oxaloacetate are capable of doing the opposite by pulling the enzyme towards the NADH stabilized tight state. Since KCl can desensitize citrate synthase towards NADH inhibition, presumably KCl can shift the NADH stabilized tight state back to the relaxed state.

The author will not try to set up a model to explain the function of citrate synthase based on the limited information available. Further information on the subunit structure of various enzyme substrate complexes and the relationships between subunit structure and enzyme conformion no doubt will help one step towards setting up a model. Different techniques would probably be involved in obtaining this information since both substrates acetyl-CoA and oxaloacetate are unstable, and will not survive the lengthy

sedimentation equilibrium experiments described in this thesis.

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