BBA 65677

CHARACTERIZATION OF SUCCINATE SEMIALDEHYDE DEHYDROGENASE FROM RAT BRAIN

C. KAMMERAAT AND H. VELDSTRA
Department of Biochemistry, The State University, Leiden (The Netherlands)

(Received July 21st, 1967)

SUMMARY

1. After solubilization from mitochondria, the activity of the enzyme succinate semialdehyde:NAD$^+$ oxidoreductase (EC 1.2.1.16) in brain and other organs of the rat was determined. During development the enzyme activity in brain rises 5-fold. The enzyme from rat brain has been purified 300 times.

2. Inhibition by relatively low succinate semialdehyde concentrations is explained on the basis of a reaction of the aldehyde group of the substrate molecule with the enzyme–substrate complex to give an inactive complex. The following kinetic data were obtained for succinate semialdehyde: $K_1 = 4.7 \cdot 10^{-6}$ M; $K_2 = 2.2 \cdot 10^{-3}$ M; for NAD$^+$: $K_m = 9.9 \cdot 10^{-5}$ M.

3. Purified enzyme preparations are inactive without added 2-mercaptoethanol. *p*-Chloromercuribenzoate and *N*-ethylmaleimide inhibit enzyme activity competitively with respect to NAD$^+$. Sodium arsenite inhibits enzyme activity especially in the presence of 2-mercaptoethanol. In this case parallel lines were obtained in a Lineweaver–Burk plot with respect to succinate semialdehyde as well as NAD$^+$.

4. No isoenzymes could be detected. L-Lactate:NAD$^+$ oxidoreductase (EC 1.1.1.27) activity interfered in the staining procedure after agar-gel electrophoresis, but could be inhibited by 0.01 M oxalate.

INTRODUCTION

In the last few years γ-aminobutyric acid has been considered to be a possible inhibitor of synaptic transmission in the central nervous system[1,2]. Biochemical findings concerning the γ-aminobutyric acid-metabolizing enzymes might help to evaluate this hypothesis. Therefore the γ-aminobutyric acid synthesizing enzyme, L-glutamate 1-carboxy-lyase (EC 4.1.1.15) and 4-aminobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.19), the enzyme responsible for transformation of γ-aminobutyric acid into succinate semialdehyde, were studied earlier in our laboratory[3,4]. Succinate semialdehyde dehydrogenase, catalyzing the NAD$^+$-dependent oxidation of succinate semialdehyde to succinate, now has been included in this series of investigations.

ALBERS AND KOVAL[5] reported some properties of a 20 times purified succinate semialdehyde dehydrogenase preparation from monkey brain. PITTS AND QUICK[6] demonstrated an activation by NaCl of succinate semialdehyde dehydrogenase activity from rat brain and measured succinate semialdehyde dehydrogenase activity in developing rat brain[7] as well as in different regions of human brain[8].

This paper deals with solubilization and purification of succinate semialdehyde dehydrogenase from rat brain. Kinetic data and inhibition studies with sulphydryl reagents are reported. Evidence is presented from chromatographic and electrophoretic experiments to show that there are no isoenzymes of succinate semialdehyde dehydrogenase.

MATERIALS AND METHODS

Preparation of soluble succinate semialdehyde dehydrogenase

White laboratory rats were killed by decapitation. Brains were removed and a 10% (w/v) homogenate in H_2O was made with a teflon pestle at 1200 rev./min. This homogenate was treated with 0.5% Triton X-100, or disintegrated either sonically (MSE Mullard ultrasonic disintegrator) or in a French pressure cell (150 kg/cm^2).

Synthesis of succinate semialdehyde

Succinate semialdehyde was synthesized by a modification of the LANGHELD[9] procedure: a solution of 0.5 M glutamate and 0.5 M NaOCl was adjusted to pH 7.0 and heated at 50° for 10 min. Subsequently the solution was adjusted to pH 3.0 and extracted several times with ether. After evaporation, H_2O was added in order to give a 0.01 M succinate semialdehyde solution. The 2,4-dinitrophenylhydrazone prepared from this solution, had a m.p. of 200–202° (see refs. 10–11). (Found: C, 42.7; H, 3.75; N, 19.3. $C_{10}H_9N_4O_6$ requires C, 42.6; H, 3.6; N, 19.9%).

A solution of pure succinate semialdehyde, prepared according to SCHENCK[12], was obtained from VAN KEMPEN *et al.*[4]. At no time could any difference between these succinate semialdehyde solutions be shown.

Succinate semialdehyde concentration was determined spectrophotometrically[11], spectrofluorimetrically[13] and enzymatically, assuming total conversion of succinate semialdehyde by succinate semialdehyde dehydrogenase[6]. These methods gave identical results.

Assay

Routine incubation conditions: 0.1 M sodium pyrophosphate buffer (pH 8.4); 0.33 mM NAD^+; 4.5 mM 2-mercaptoethanol; 0.1 mM succinate semialdehyde; enzyme preparation; total volume, 3.0 ml.

A calibration line was constructed by taking controls, in which succinate semialdehyde was replaced by either $2 \cdot 10^{-5}$ or $2 \cdot 10^{-6}$ M NADH, through the procedure. Incubation was carried out for 15 min at 37°. The reaction was stopped by heating at 100° for 5 min. NADH was measured fluorimetrically, using an excitation wavelength of 352 nm and measuring fluorescence at 460 nm.

Under these conditions the reaction proceeds linearly with time and enzyme concentration. NADH:(acceptor) oxidoreductase (EC 1.6.99.3) was activated by the ultrasonic treatment and interfered with the enzyme assay in this case, but could be

inhibited by Triton X-100, 3.3 mM amytal and eliminated by centrifuging the enzyme preparation at 100 000 × *g* for 1 h.

Chromatography

Chromatography on DEAE-cellulose was performed essentially according to MOORE and co-workers[14,15]. Soluble brain proteins were eluted at 4° with a linear KCl gradient (0–0.25 M) in 5 mM Tris–phosphate buffer (pH 7.4) with 0.01 M 2-mercaptoethanol. Sephadex G-200 was equilibrated with a solution of 5 mM Tris–phosphate (pH 7.4), 0.01 M 2-mercaptoethanol and 0.1 M KCl. Proteins were eluted with a KCl gradient (0.1–0.2 M) in buffer[16]. Protein was determined according to the method of WADDELL[17] and LOWRY and co-workers[18].

Electrophoresis and staining techniques

For separation of possible isoenzymes, enzyme preparations were subjected to electrophoresis in 0.9% Difco special Noble agar in barbiturate buffer (pH 8.4) on object slides at 150 V during 25 min (ref. 19). After electrophoresis the gels were either cut in 0.4-cm slices or incubated in a staining medium of the following composition: 0.1 M sodium pyrophosphate buffer (pH 8.4), 1 mg nitrobluetetrazolium, 0.1 mg phenazinemethosulphate, 2 mg NAD^+ and 0.3 mM succinate semialdehyde; total volume, 3.5 ml (for lactate dehydrogenase see VAN DER HELM[20]).

The gel was incubated for 2 h at 37° in this medium and fixed in ethanol–water–acetic acid (70:25:5, v/v).

RESULTS

Solubilization

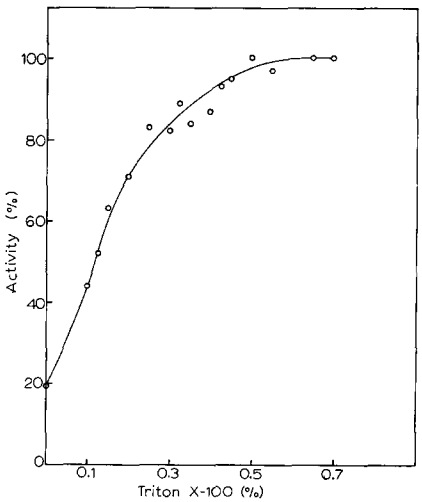
Succinate semialdehyde dehydrogenase is localized within mitochondria[21]. Triton X-100 in a final concentration of 0.5%, stimulates the enzyme activity in 0.32 M sucrose homogenates 5-fold by solubilizing the enzyme (Fig. 1) as does ultrasonic disintegration and treatment in a French pressure cell. Homogenates in H_2O gave 40% of maximal activity and only 20% solubilization.

Activity

In whole brain homogenates from adult rats, total succinate semialdehyde dehydrogenase activity was found to be 200 μmoles/g wet wt. per h. In liver, heart, kidney and spleen, enzyme activity was 171, 79, 21 and 20 μmoles/g wet wt. per h, respectively. The rise of activity in brain during development followed an S-shaped curve (Fig. 2) (*cf.* for succinate semialdehyde dehydrogenase, PITTS AND QUICK[7]).

Purification

After solubilization by treatment in a French pressure cell, the enzyme preparation from brain was centrifuged for 1 h at 100 000 × *g*. A saturated solution of $(NH_4)_2SO_4$ (pH 7.0) was added to the supernatant till 30, 45 and 60% saturation, the precipitate at each stage being isolated by centrifugation. The last pellet was redissolved in 0.01 M sodium pyrophosphate buffer (pH 7.4) containing 5 mM 2-mercaptoethanol. The solution was dialyzed overnight against this buffer and applied to a DEAE-cellulose column. After elution, the fractions with succinate semialdehyde de-



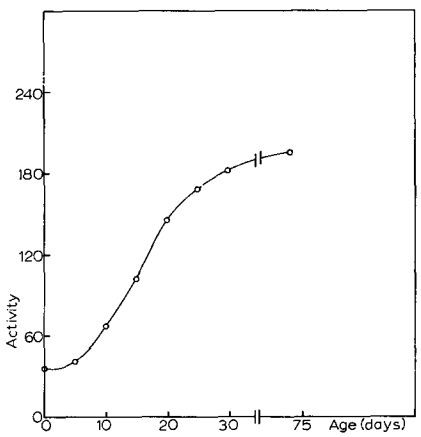


Fig. 1. Stimulation of succinate semialdehyde dehydrogenase activity of a 10% rat brain homogenate in 0.32 M sucrose by Triton X-100. Assay as described in the text.

Fig. 2. Succinate semialdehyde dehydrogenase activity in rat brain during development. Measured with maximal stimulation by Triton X-100, otherwise standard conditions. Activity in μmoles NADH per g wet wt. per h.

hydrogenase activity were pooled and used for further experiments. Additional chromatography on Sephadex G-200 resulted in a great loss of activity with little further purification. The enzyme purification is summarized in Table I.

pH optimum

In sodium pyrophosphate and Tris buffer, maximal activity was found between pH 8.5 and 9.0, when measured in the incubation medium at 37°. In Tris buffer the activity was lower. According to PITTS AND QUICK[6], this can be explained on the basis of enzyme activation by Na^+. 0.5 M NaCl stimulated the enzyme activity in Tris buffer to 85% of the value found for sodium pyrophosphate.

Kinetic data

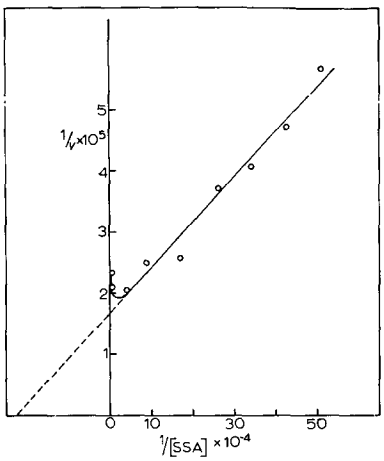
Like other aldehyde dehydrogenases (JAKOBY[22]) succinate semialdehyde de-

TABLE I

PURIFICATION OF SUCCINATE SEMIALDEHYDE DEHYDROGENASE FROM RAT BRAIN

Activity of the homogenate was measured with Triton X-100. Specific activity in mmoles NADH per g protein per h.

Preparation	*Volume (ml)*	*Specific activity*	*% residual activity*	*Purification factor*
Brain Homogenate	67	1.28	100	1.0
Supernatant after disintegration	60	2.23	83	1.7
Pellet 45–60% saturation with $(NH_4)_2SO_4$	3	11.1	33	8.6
DEAE-cellulose	69	317.0	18	250
Sephadex G-200	69	375.0	5	300



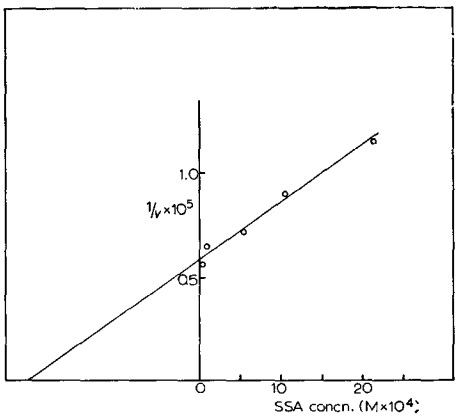


Fig. 3. Dependence of reaction velocity on succinate semialdehyde (SSA) concentration; assay with 0.33 mM NAD^+ at pH 8.5; 4.5 mM 2-mercaptoethanol; purified enzyme preparation. v is expressed in concentration of NADH formed after incubation under otherwise standard conditions.

Fig. 4. Relation between the velocity of the reaction and succinate semialdehyde (SSA) concentrations above 10^{-4} M; assay with 0.33 mM NAD^+ at pH 8.5; 4.5 mM 2-mercaptoethanol; purified enzyme preparation. Units for v as in Fig. 3.

hydrogenase activity is inhibited by its own substrate. Inhibition occurs at succinate semialdehyde concentrations above 10^{-4} M. By measuring the velocity of the reaction at various succinate semialdehyde concentrations under otherwise standard conditions, an apparent K_m for succinate semialdehyde could be determined (Fig. 3): $K_m = 4.7 \cdot 10^{-6}$ M.

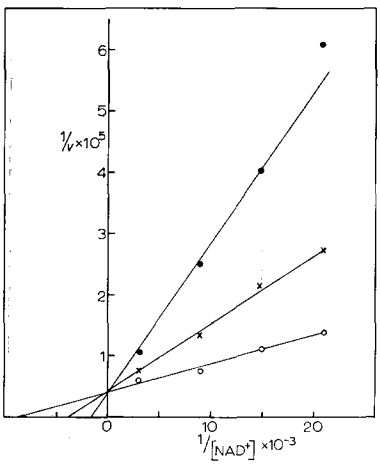
A succinate semialdehyde concentration of $1 \cdot 10^{-4}$ M yields maximal activity.

At high substrate concentrations a linearity was found between the succinate semialdehyde concentrations and the reciprocal value of the reaction velocity (Fig. 4).

K_m for $NAD^+ = 9.9 \cdot 10^{-5}$ M in the presence of $1.0 \cdot 10^{-4}$ M succinate semialdehyde.

–SH groups

While 2-mercaptoethanol stimulates the succinate semialdehyde dehydrogenase activity of freshly prepared brain homogenates by 20% only, purified enzyme preparations were fully dependent on exogenous –SH groups for activity. *p*-Chloromercuribenzoate and *N*-ethylmaleimide inhibited the succinate semialdehyde dehydrogenase activity of freshly prepared brain homogenates without added 2-mercaptoethanol; the degree of inhibition appeared to be dependent on enzyme concentration and duration of preincubation with the inhibitor. The inhibition is progressive with time. After preincubation with *p*-chloromercuribenzoate and *N*-ethylmaleimide the inhibition by *p*-chloromercuribenzoate, but not that by *N*-ethylmaleimide, could be reversed by 2-mercaptoethanol. During preincubation NAD^+ protects the enzyme against inhibition by these sulphydryl reagents. This is consistent with the finding that the inhibition by *p*-chloromercuribenzoate and *N*-ethylmaleimide is competitive with respect to NAD^+ (Fig. 5) but not to succinate semialdehyde.



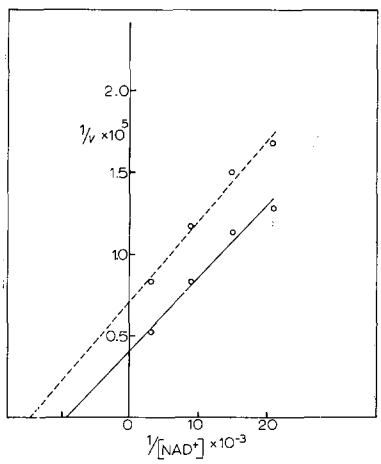


Fig. 5. Reaction velocity as a function of NAD^+ concentration. ○—○, without inhibitor; ×—×, with $7 \cdot 10^{-7}$ M *p*-chloromercuribenzoate; ●—●, with $2 \cdot 10^{-6}$ M *N*-ethylmaleimide. Assay: 0.1 mM succinate semialdehyde, without 2-mercaptoethanol, freshly prepared brain homogenate, treated with 0.5% Triton X-100. Units for v as in Fig. 3.

Fig. 6. Inhibition of succinate semialdehyde dehydrogenase activity by $NaAsO_2$ as a function of NAD^+ concentration. ○—○, without inhibitor; ○— — —○, $5 \cdot 10^{-5}$ M $NaAsO_2$. Assay: 0.1 mM succinate semialdehyde, purified enzyme preparation, 4.5 mM 2-mercaptoethanol. Units for v as in Fig. 3.

5 mM sodium arsenite inhibited enzyme activity 50% without added 2-mercaptoethanol, while in the presence of this compound 50% inhibition was achieved by $7.5 \cdot 10^{-5}$ M arsenite. This effect is quite common for aldehyde dehydrogenases (JAKOBY[22]). Further studies revealed a difference in the type of inhibition with respect to NAD^+ with or without 2-mercaptoethanol in the reaction medium (Figs. 6 and 7). For succinate semialdehyde the pattern of Fig. 6 was found in both cases.

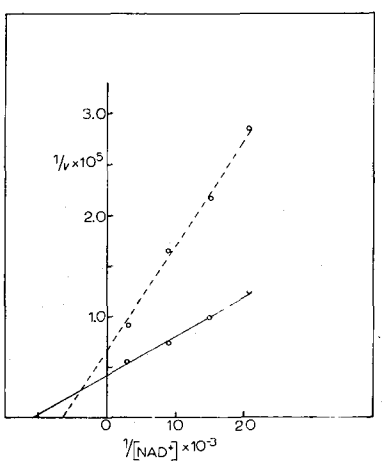


Fig. 7. Inhibition of succinate semialdehyde dehydrogenase activity in freshly prepared rat brain homogenate by 3 mM $NaAsO_2$ at various NAD^+ concentrations (○- - -○). No inhibition: ○—○. No 2-mercaptoethanol in the incubation medium. Units for v as in Fig. 3.

Isoenzymes

After electrophoresis of enzyme preparations in agar gel, the fractions obtained by cutting the gel in 0.4 cm slices, were assayed for succinate semialdehyde dehydrogenase activity. One activity peak was found 1 cm from the starting point in the direction of the anode.

This was confirmed by experiments where succinate semialdehyde dehydrogenase activity in the gel after electrophoresis was made visible by a tetrazolium staining technique. In the staining medium 2-mercaptoethanol had to be omitted because of direct reduction of the tetrazolium salt. The activity of purified enzyme preparations, free of lactate dehydrogenase activity, after preincubation with 2-mercaptoethanol, was found in one band of formazan, 1 cm from the starting point towards the anode.

Preparations with lactate dehydrogenase activity invariably showed four to five bands of formazan. Omitting succinate semialdehyde from the staining medium did not influence this effect. With lactate as substrate, there was a greater formazan production, which yielded the same pattern however. This formazan production without succinate semialdehyde in the medium could be inhibited by 0.01 M sodium oxalate, a lactate dehydrogenase inhibitor[23]. After electrophoresis of preparations with succinate semialdehyde dehydrogenase and lactate dehydrogenase activity, only one band, characteristic for succinate semialdehyde dehydrogenase, appeared when succinate semialdehyde and oxalate were present in the staining medium (Fig. 8). The absence of isoenzymes was also suggested by the finding that during chromatography on DEAE-cellulose as well as Sephadex G-200 the enzyme was eluted in one band of activity.

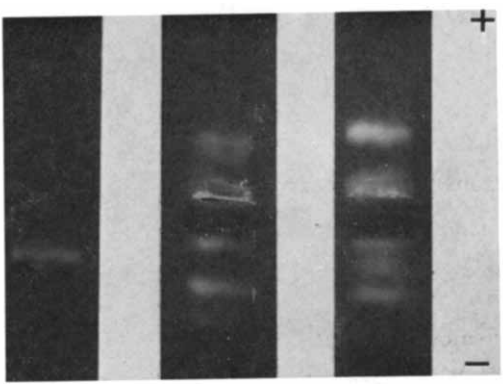


Fig. 8. Patterns, obtained by visualization of enzyme activity by a tetrazolium staining technique after electrophoresis of some enzyme preparations in agar gel. Details described in the text. Left: purified succinate semialdehyde dehydrogenase preparation, preincubated with 5 mM 2-mercaptoethanol. No lactate dehydrogenase activity present. This pattern is also seen after electrophoresis of a freshly prepared rat brain homogenate, treated with Triton X-100, with 0.01 M oxalate in the staining medium. Middle: partly purified succinate semialdehyde dehydrogenase preparation without preincubation with 2-mercaptoethanol. Lactate dehydrogenase activity still present. Right: a freshly prepared rat brain homogenate, treated with Triton X-100.

DISCUSSION

From Fig. 1 it is obvious that total succinate semialdehyde dehydrogenase

activity in homogenates can be measured only after solubilization of the enzyme by disintegration of the mitochondria containing succinate semialdehyde dehydrogenase. The total activity in rat brain homogenates, 200 μmoles/g wet wt. per h, exceeds that of γ-aminobutyric acid:2-oxoglutarate transaminase (140, see ref. 4). As localization studies[21,24] revealed a similarity for these two enzymes, it can be assumed that succinate semialdehyde dehydrogenase is not a limiting factor in γ-aminobutyric acid breakdown in rat brain. In fact, the low K_m for succinate semialdehyde suggests a rapid turnover of the compound, which explains the absence of succinate semialdehyde in brain homogenates[5,25].

The observed inhibition by substrate concentrations above 10^{-4} M can be explained by a reaction between the enzyme–substrate complex and a second succinate semialdehyde molecule:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad \text{(I)}$$

$$ES + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \qquad \text{(II)}$$

$$ES \xrightarrow{k_3} E + \text{product} \qquad \text{(III)}$$

In Reaction II probably the aldehyde group of the succinate semialdehyde molecule is involved, since 0.01 M acetaldehyde inhibits the reaction, but not 0.01 M succinate.

Assuming steady-state kinetics, it follows that

$$v = \frac{k_3 \cdot [E_{total}]}{1 + \frac{K_1}{[S]} + \frac{[S]}{K_2}} \qquad \text{(IV)}$$

where

$$K_1 = \frac{k_{-1} + k_3}{k_{+1}} \quad \text{and} \quad K_2 = \frac{k_{-2}}{k_{+2}}$$

At low succinate semialdehyde concentrations Eqn. IV turns into

$$v = \frac{k_3 \cdot [E_{\text{total}}]}{1 + \frac{K_1}{[S]}} \qquad \text{(V)}$$

From the data presented in Fig. 3, it was calculated that

$$K_1 = 4.7 \cdot 10^{-6} \text{ M}$$

At high substrate concentration, Eqn. IV can be written as

$$v = \frac{k_3 \cdot [E_{\text{total}}]}{1 + \frac{[S]}{K_2}} \qquad \text{(VI)}$$

or

$$\frac{1}{v} = \frac{1}{k_3 \cdot [E_{\text{total}}]} + \frac{[S]}{K_2 \cdot V}$$

Indeed, a linearity between $1/v$ and $[S]$ was found for succinate semialdehyde concen-

trations above $1 \cdot 10^{-4}$ M (Fig. 4). $K_2 = 2.2$ mM. In these experiments corrections for non-enzymatic reduction of NAD^+ by succinate semialdehyde had to be made.

It can be derived from Eqn. IV that maximal velocity is attained when $dv/d[S] = 0$, *i.e.*, when $[S] = \sqrt{K_1 \cdot K_2}$.

In this case this would be $[S] = 1 \cdot 10^{-4}$ M, as is indeed found.

From the inhibition studies with *p*-chloromercuribenzoate and *N*-ethylmaleimide it follows that in the presence of NAD^+ an essential –SH group is inaccessible for these sulphydryl reagents.

Inhibition by arsenite is most probably due to the binding of one arsenite molecule with two –SH groups[26,27]. Without added 2-mercaptoethanol there is some protection by NAD^+, which indicates that in this case the above-mentioned essential –SH group is involved. In the presence of 2-mercaptoethanol the inhibition by arsenite is much stronger and non-competitive with respect to NAD^+ and succinate semialdehyde (Fig. 6). This implies that the –SH groups reacting with *p*-chloromercuribenzoate and *N*-ethylmaleimide, are not involved, but more likely –SH groups, formed from fission of –S–S-bridges by the thiol (*cf.* refs. 5, 28).

As to the type of inhibition: the $1/v$ *vs.* $1/[S]$ plot yields, in this case, parallel lines for the reactions with or without arsenite (Fig. 6). This can be explained on the basis of Eqn. V:

$$v = \frac{k_{+3} \cdot [E_{\text{total}}]}{1 + \frac{k_{-1} + k_{+3}}{k_{+1}} \cdot \frac{1}{[S]}}$$

If $k_{+3} \gg k_{-1}$, which is in accord with the irreversibility found for the enzyme-catalyzed reaction[6,24], then an inhibitor which influences k_{+3}, will change K_1 and v in the same way.

We were unable to prove the existence of succinate semialdehyde dehydrogenase isoenzymes differing in charge or size. While most dehydrogenases exist in multiple forms, a recent report from ROBBINS[29] about non-specific bovine liver aldehyde dehydrogenase also showed the absence of isoenzymes in that case. PFLEIDERER AND AURICCHIO[30] suggested that dehydrogenases are built up from sub-units with a mol.wt. of 30 000–40 000. Further experiments will be required to settle whether succinate semialdehyde dehydrogenase, which has a mol.wt. of about 140 000 according to Sephadex G-200 gel filtration, where it is eluted together with lactate dehydrogenase, is built up from four (identical?) sub-units.

During visualization of succinate semialdehyde dehydrogenase activity after agar-gel electrophoresis of preparations with lactate dehydrogenase activity, we found, apart from one band specific for succinate semialdehyde dehydrogenase, bands of formazan, corresponding to lactate dehydrogenase isoenzymes (Fig. 8). This NAD^+-dependent effect was found even in the absence of any substrate and could be eliminated by 0.01 M oxalate, an inhibitor of lactate dehydrogenase[23].

We conclude therefore that lactate dehydrogenase is responsible for this so-called "nothing" dehydrogenase reaction[31,32].

ACKNOWLEDGEMENTS

The skilful assistance of Miss A. P. M. JONGSMA, MR. C. VAN DER HEIDEN, MR. P. J. VAN DER KOOY and MR. J. T. M. VREEBURG is gratefully acknowledged.

REFERENCES

1 A. TAKEUCHI AND N. TAKEUCHI, *J. Physiol.*, 177 (1965) 225.
2 K. KRNJEVIĆ AND S. SCHWARTZ, *Federation Proc.*, 25 (1966) 627.
3 C. J. VAN DEN BERG, G. M. J. VAN KEMPEN, J. P. SCHADÉ AND H. VELDSTRA, *J. Neurochem.*, 12 (1965) 863.
4 G. M. J. VAN KEMPEN, C. J. VAN DEN BERG, H. J. VAN DER HELM AND H. VELDSTRA, *J. Neurochem.*, 12 (1965) 581.
5 R. W. ALBERS AND G. J. KOVAL, *Biochim. Biophys. Acta*, 52 (1961) 29.
6 F. N. PITTS AND C. QUICK, *J. Neurochem.*, 12 (1965) 893.
7 F. N. PITTS AND C. QUICK, *J. Neurochem.*, 14 (1967) 561.
8 J. J. SHERIDAN, K. L. SIMS AND F. N. PITTS, *J. Neurochem.*, 14 (1967) 579.
9 K. LANGHELD, *Ber.*, 42 (1909) 392, 2360.
10 S. OCHOA, *J. Biol. Chem.*, 155 (1944) 87.
11 B. A. PRESCOTT AND H. WAELSCH, *J. Biol. Chem.*, 164 (1946) 331.
12 G. O. SCHENCK, *Ann.*, 584 (1953) 156.
13 R. A. SALVADOR AND R. W. ALBERS, *J. Biol. Chem.*, 234 (1959) 922.
14 B. W. MOORE AND R. H. LEE, *J. Biol. Chem.*, 235 (1960) 1359.
15 B. W. MOORE AND D. MCGREGOR, *J. Biol. Chem.*, 240 (1965) 1647.
16 F. N. PITTS, C. QUICK AND E. ROBINS, *J. Neurochem.*, 12 (1965) 93.
17 W. J. WADDELL, *J. Lab. Clin. Med.*, 48 (1956) 311.
18 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
19 R. J. WIEME, *Clin. Chim. Acta*, 4 (1959) 317.
20 H. J. VAN DER HELM, *Clin. Chim. Acta*, 7 (1962) 124.
21 L. SALGANICOFF AND E. DE ROBERTIS, *J. Neurochem.*, 12 (1965) 287.
22 W. B. JAKOBY, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 7, Acad. Press, New York, 1963, p. 203.
23 W. B. NOVOA, A. D. WINER, A. J. GLAID AND G. W. SCHWERT, *J. Biol. Chem.*, 234 (1959) 1143.
24 C. KAMMERAAT, *Thesis*, Leiden, 1966.
25 Y. TSUKADA, Y. NAGATA AND G. TAKAGAKI, *Proc. Japan. Acad.*, 33 (1957) 510.
26 L. A. STOCKEN AND R. H. S. THOMPSON, *Physiol. Rev.*, 29 (1949) 168.
27 L. A. STOCKEN AND R. H. S. THOMPSON, *Biochem. J.*, 40 (1946) 529.
28 M. W. NIRENBERG AND W. B. JAKOBY, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 206.
29 J. H. ROBBINS, *Arch. Biochem. Biophys.*, 114 (1966) 585.
30 G. PFLEIDERER AND F. AURICCHIO, *Biochem. Biophys. Res. Commun.*, 16 (1964) 53.
31 H. BARNETT, *Biochem. J.*, 84 (1962) 63P.
32 H. ANDERSEN, *Acta Histochem.*, 21 (1965) 120.