MAGNETIC RESONANCE AND FLUORESCENCE STUDIES ON PYRUVATE DEHYDROGENASE COMPLEXES AND THEIR SMALL MOLECULAR WEIGHT CONSTITUENTS

Dit proefschrift met stellingen van

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doctorandus in de Chemie, geboren op 3 november 1944, is goedgekeurd door de promotor Dr. C. Veeger, hoogleraar in de Biochemie en de co-promotor Dr. F. Müller, wetenschappelijk hoofdmedewerker in de vakgroep Biochemie.

De Rector Magnificus van de Landbouwhogeschool
J.P.H. van der Want

Wageningen, 13 juli 1976

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(with a summary in Dutch)

Proefschrift

ter verkrijging van de graad
van doctor in de landbouwwetenschappen,
op gezag van de Rector Magnificus,
Dr.Ir. J.P.H. van der Want
hoogleraar in de Virologie
in het openbaar te verdedigen
op woensdag 8 september 1976
des namiddags te vier uur in de Aula
van de Landbouwhogeschool te Wageningen

ISN=104093-03

These investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

STELLINGEN

1

Het niet in aanmerking nemen van het Nuclear Overhauser Effect van niet direct gebonden protonen op de intensiteit van quaternaire ^{13}C-resonanties leidt tot verkeerde toekenningen.

E. Breitmaier and W. Voelter (1972) Eur.J.Biochem. 31, 234-238.
Dit proefschrift.

2

Bij de berekening van energie-overdracht leidt het gebruik van levensduren, die uitsluitend bepaald zijn uit de faseverschuiving in de fasefluorimeter, tot dubieuze resultaten.

O.A. Moe, Jr., D.A. Lerner and G.G. Hammes (1974) Biochemistry 13, 2552-2557.
G.B. Sheperd and G.G. Hammes (1976) Biochemistry 15, 311-317.

3

De door Weltman *et al.* vermelde zuiverheidscriteria voor N-(3-pyreen)maleimide sluiten de aanwezigheid van N-(3-pyreen)isomaleimide niet uit; dit kan het meten van twee levensduren verklaren.

J.K. Weltman, R.P. Szaro, A.R. Frackelton, Jr., R.M. Dowben, J.R. Bunting and R.E. Cathou (1973) J.Biol.Chem. 248, 3173-3177.
D.M. Barrat, A.P. Davies and M.T.A. Evans (1971) Eur.J.Biochem. 24, 280-283.

4

Het herhalen door Benga en Strach van de experimenten waarmee Griffith en McConnel de spinlabeltechniek introduceerden en het daarbij aantonen dat een klein deel van de label niet covalent gebonden was, is in vergelijking niet meer dan een korte mededeling waard.

G. Benga and S.J. Strach (1975) Biochim.Biophys.Acta 400, 69-79.
O.H. Griffith and H.M. McConnel (1966) Proc.Nat.Acad.Sci. 55, 8-11.

5

De door Kipnich *et al.* aangegeven redenen voor de verbeterde signaal/ruis verhouding van *hun* 270 MHz Brucker NMR verklaart niet meer dan 10 % van deze verbetering.

H.D. Kipnich, R. Eshe and W. Maurer (1976) J.Magn.Res. 22, 161-164.

6

Het door o.a. Krampitz voorgestelde hydroxyethylthiaminepyrofosfaat als reactie-intermediair bij enzymatische decarboxylatie is op grond van chemische en biochemische argumenten onwaarschijnlijk.

L.O. Krampitz (1969) Ann.Rev.Biochem. 38, 213-240.
H.R. Mahler and E.H. Cordes (1966) Biological Chemistry, Harper and Row Ltd, Londen, 350-352.
A.A. Gallo and H.Z. Sable (1976) J.Biol.Chem. 251, 2564-2570.
J.A. Gutowski and G.E. Lienhard (1976) J.Biol.Chem. 251, 2863-2866.

7

De suggestie van Oldfield *et al.*, dat ^{13}C-NMR bij hogere magneetvelden weinig zinvol is voor eiwitten, gaat maar zeer gedeeltelijk op voor quaternaire C-atomen, gebonden aan een N-atoom.

E. Oldfield, R.S. Norton and A. Allerhand (1975) J.Biol.Chem. 250, 6368-6380.

8

Het dragen van verantwoordelijkheid door een vrijwillig lekenbestuur voor het functioneren van een centrum met beroepskrachten, zoals gebruikelijk in het sociaal-cultureel werk, is in feite onverantwoord en kan aanleiding geven tot frustraties bij zowel staf als bestuur.

9

Indien de Landbouwhogeschool zou worden ondergebracht bij het Ministerie van Onderwijs en Wetenschappen, mag dit geen aanleiding zijn om, bij de gesuggereerde herstructurering van de departementen, het Ministerie van Landbouw en Visserij op te heffen.

Proefschrift van H.J. Grande.

Een blijvend bewijs van mijn diepgaande inzichten voor het nageslacht; dat is wat mij voor ogen zweeft.

Heer Ollie B. Bommel in
"Het losgetrilde inzicht"

Aan Digna
mijn ouders
Marjolein
Sander

Voorwoord

Het werk, in dit proefschrift beschreven, is uitgevoerd op het Laboratorium voor Biochemie te Wageningen en mogelijk gemaakt door financiele steun van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.).

Mijn speciale dank wil ik uitspreken aan allen met wie ik heb gepubliceerd en wier werk ik in dit proefschrift heb mogen gebruiken.

Tevens wil ik allen bedanken die mij zo geduldig hebben ingelicht over de biochemische achtergronden van de eiwitten en de eiwitcomplexen die ik onderzocht. Ook wil ik hen danken voor het verklaren van de talloze afkortingen die de biochemie rijk is, waarbij zij mij tevens leerden mijn fysisch-chemisch taalgebruik aan te passen.

Mijn meer fysisch-chemisch getinte collega's wil ik bedanken voor hun stimulerende discussies, en de studenten die met mij gewerkt hebben voor hun hulp.

Velen hebben óf door hun hulp, óf door het op peil houden van mijn humeur en mijn lichamelijke conditie, bijgedragen aan de totstandkoming van dit proefschrift.

Een speciale vermelding verdienen de secretaressen van de vakgroep Biochemie, die mijn afschuwelijke handschrift hebben moeten omzetten in leesbare tekst, onze onvolprezen tekenaar, alsmede diegenen die op de afdeling tekstverwerking hebben meegewerkt aan het snelle persklaar maken van dit proefschrift.

Contents

List of abbrevations

a	hyperfine coupling constant
A.	*Azotobacter*
ADP	adenosine 5'-diphosphate
ANM	N-(1-anilino naphthyl-4)maleïmide
ATP	adenosine 5'-triphosphate
CoA	co-enzyme A
CoASAc, acetyl CoA	acetyl co-enzyme A
CNDO	complete neglect of differential overlap
E.	*Escherichia*
EDTA	ethylene diamine tetra acetate
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucloetide, oxidized form
FMN	flavin adenine mononucleotide, oxidized form
Hz	Hertz
J	exchange integral
LTA	dihydrolipoyl transacetylase component
M	molarity
MINDO	modified intermediate neglect of differential overlap
MO	molecular orbital
mol. wt.	molecular weight
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NEM	N-ethyl maleïmide
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
PDC	pyruvate dehydrogenase complex
PDH	pyruvate dehydrogenase component
ppm	parts per million
PPP-SCF	Pariser, Parr & Pople-self consistent field method
TMS	tetra methyl silane
TPP	thiamine pyrophosphate
Tricine	N-(tris(hydroxymethyl)methyl glycine)
Tris	tri(hydroxymethyl)amino methane

List of enzymes

In this thesis the non-systematic names of enzymes are used. This list includes the trivial and the systematic names of the investigated enzymes; also is included the enzyme number according to the Report of the Commission for Enzymes of the International Union of Biochemistry.

EC number	Systematic name	Trivial name
1.2.4.1.	pyruvate:lipoate oxidoreductase (acceptor acylating)	pyruvate dehydrogenase complex
4.1.1.1.	pyruvate decarboxylase (2-oxoacid carboxy-lyase)	pyruvate dehydrogenase component
2.3.1.12.	CoASAc:dihydrolipoate S-acetyltransferase	dihydrolipoyl transacetylase
1.6.4.3.	NADH:lipoamide oxido reductase	lipoamide dehydrogenase

Introduction

The pyruvate dehydrogenase complex is a multi-enzyme complex present in many organisms. It is present in animal- and in plant tissue but also in obligate and facultative aerobic bacteria |1| and functions between glycolyis and the citric acid cycle. The complex is therefore very important for the regulation of the metabolism as stated among others by Reed |2|. Most sugars are converted into pyruvate via the glycolyis cycle or an analogue path (e.g. Entner-Doudoroff path for *A. vinelandii*). Pyruvate can then react with the pyruvate dehydrogenase complex, on which pyruvate is decarboxylated. The thus formed acetyl group is attached to coenzyme A (CoA), which is an essential compound for several important pathways in the cell, such as the citric acid cycle which requires acetyl-CoA, and the fatty acid synthesis cycle. This complex thus regulates the amount of acetyl-CoA in the cell.

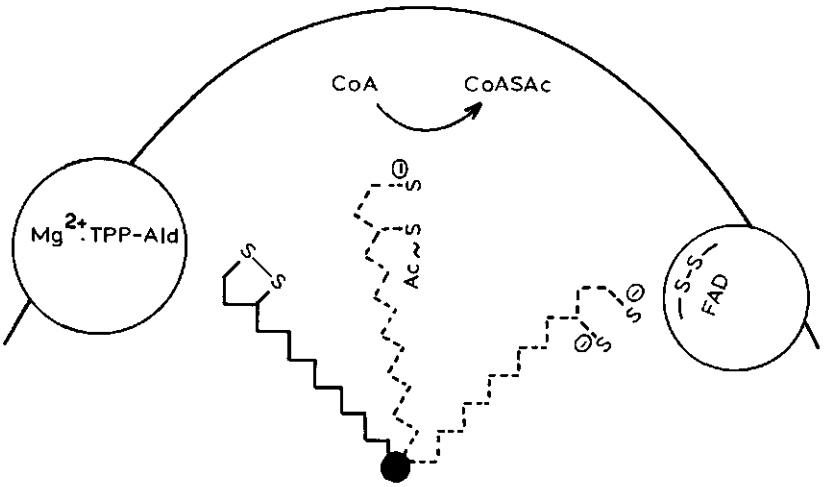
Because of the importance of the pyruvate dehydrogenase complex in regulating the metabolism it is of interest to study its properties. The wide variety of organisms in which the complex is present makes a comparison of the complexes isolated from different sources possible. A comparison in which their similarities are investigated can lead to the recognition of their common and thus most essential features, but also to an understanding of the large differences observed.

The molecular weight of the pyruvate dehydrogenase complexes isolated from several sources varies widely e.g. 1.2×10^6 for *A. vinelandii* |3|, 4×10^6 for *E. coli* |2| and 9×10^6 for pig heart |4|. Also the enzyme-subunit structure differs quite widely. The complexes contain at least three enzymes (*cf.* Fig. pg. 13); pyruvate dehydrogenase (which decarboxylates pyruvate), dihydrolipoyl transacetylase (which is responsible for acetyl transfer, also to CoA) and lipoamide dehydrogenase (which accepts the reducting equivalents and transports them to NAD^+). The mammalian complexes contain sometimes also as regulatory enzymes a kinase and a phosphatase which are normally absent in the complexes of *E. coli* and *A. vinelandii*. These enzymes are of significance in mammalian complexes because the mammalian pyruvate dehydrogenase complexes regulate very differently as compared with bacterial complexes such as those from *E. coli* and *A. vinelandii*.

In mammalian complexes the pyruvate decarboxylation-activity is inhibited by phosphorylation of one of the pyruvate dehydrogenase peptide chains; reactivation of the pyruvate dehydrogenase is then obtained after dephosphorylation |2|. In bacterial complexes the regulation must be differently accomplished because no phosphorylation-dephosphorylation system is operating. The mechanism which is of importance in bacterial complexes might involve metabolite modulators, including nucleotides and acetyl CoA (cf. ref |2| p.245). Of importance is then whether these regulations are accompanied by structural reorganization of the complex or are due to regulation of the enzyme components itself. It is therefore of interest to find ways in which the complex and especially the active site(s) can be studied in detail without disturbing the aggregation state of the complex.

Physical techniques such as EPR, NMR and fluorescence are well suited for this purpose. Detailed information can be obtained by these techniques, even on these large complexes, if small molecules are available which can function as "probes". By studying these probe molecules bound to the protein, differences in local environment can be detected. It is possible to attach such probes to all enzyme components of the pyruvate dehydrogenase complex.

Pyruvate dehydrogenase needs as cofactors Mg^{2+} and TPP. Mg^{2+} can be replaced by Mn^{2+}, maintaining both the partial pyruvate dehydrogenase activity and the overall activity of the complex. Replacement of Mg^{2+} by Mn^{2+} opens the possibility to use magnetic resonance techniques, such as EPR and relaxation methods in NMR. EPR and solvent relaxation, induced by paramagnetic species, are well suited for quantitation and determination of the kind of ternary complexes (enzyme-metal-TPP) and quaternary complexes (enzyme-metal-TPP-pyruvate) formed |5|. Specific line broadening effects of TPP in the presence of Mn^{2+} give information about the conformation. On the other hand TPP can be replaced by its fluorescent analogue thiochrome-pyrophosphate for fluorescence studies. Other TPP-analogues can be used for this purpose too. Apart from information about binding to the pyruvate dehydrogenase component itself, also information about distances in the complex can be obtained by measuring the energy transfer to other absorbing species as e.g. used by Moe e.a. |6| to estimate the distance to FAD of the lipoamide dehydrogenase in the *E. coli* complex. In most flavoproteins the flavine, either FMN, FAD or another isoalloxazine analogue, is quenched to a large extent by its environment. However, lipoamide dehydrogenase isolated from all sources is highly fluorescent, even to a larger extent than FAD in water |7|. Therefore it is possible to study its fluorescent properties, without modification of the enzyme. Analysis of the amount of fluorescence, the fluorescence life time and the polarisation will yield valuable information about the microenvironment of this prosthetic group.



In the complex it can also be used to estimate distances from the FAD to another group, by measuring the energytransfer. Fluorescence studies on lipoamide dehydrogenase from pig heart |8, 9| illustrate these possibilities. From the energytransfer of tryptophane to FAD the distance is estimated in this case.

Much less applicable for physical methods is dihydrolipoyl transacetylase which plays a central role in the complex as transport enzyme of the acetyl group. It functions also as structural unit as can be concluded from recombination experiments of the complex from its isolated enzyme components |2|. Pyruvate dehydrogenase and lipoamide dehydrogenase mixed together do not combine, while also separately they do combine with the dihydrolipoyl transacetylase |2|. The transacetylase consists of identical polypeptide chaines, but these form together a structural unit of high symmetry as found from electron microscopy. Its structure is dependent on the pyruvate dehydrogenase complex where it is isolated from |2|. The peptide chains itself contain a covalently bound lipoic acid, which can be reduced in the complex either via the pyruvate dehydrogenase (in the presence of Mg^{2+}, TPP and pyruvate) or via the lipoamide dehydrogenase (in the presence of NADH).

In the first case the disulphide bridge of lipoic acid is reduced and the acetyl-group is attached to one of the sulphur atoms, in the other case two sulphydryls are formed. It is shown in the thesis of T.W. Bresters |10| that for the *A. vinelandii* complex it was possible to react the so-called specifically reduced sulphydryl group(s) of the dihydrolipoyl transacetylase with maleïmide--analogues. This observation made it possible to attach both fluorescence labels and spinlabels (stable paramagnetic nitroxideradicals) to the complex. Because the lipoylmoiety of the transacetylase has a transport function and rotates between the prosthetic groups (Fig.) of the other two enzymes |2|, introduction of a label on this group will probably not disturb the conformation of the complex to

a large extent. The central role of just this lipoyl moiety makes it a very interesting group to study. If a fluorescent label or spinlabel is attached to this group, information about distances in the complex can be obtained. The use of thiochrome pyrophosphate instead of thiamine pyrophosphate in combination with such a label will give information about the distance between the pyruvate dehydrogenase and the lipoyl moiety. Similar information can be obtained relative to FAD. Also conformational changes of the complex can be detected via this labelled lipoyl moiety. Summarizing it can be stated that all the enzyme components of the complex contain or can be made to contain probes (FAD, Mn^{2+}, thiochrome pyrophosphate, fluorescent and spinlabel maleïmide analogues) which can be used to study the complex in more detail.

The aim of the reported investigations is to illustrate that the above described methods indeed lead to a more fundamental insight in the conformation and conformational changes of large enzyme complexes such as the pyruvate dehydrogenase complex.

This thesis exists of five articles of which the first two have already been published and the others are submitted for publication. The first article illustrates the power of relaxation studies in the presence of paramagnetic species (Mn^{2+}) to elucidate the conformation of the coenzyme TPP. The second article introduces specific labelling and Mn^{2+} binding-techniques to the smallest pyruvate dehydrogenase complex yet known, that isolated from *A. vinelandii*. The third article compares fluorescence and spinlabel properties of the *A. vinelandii* complex and the *E. coli* complex. The last two articles are centered around the properties of the FAD-group of lipoamide dehydrogenase. In these articles ^{1}H-NMR and ^{13}C-NMR are used to obtain more information about the electronic structure of the isoalloxazine, the part of FAD being involved in redox reactions. In these articles not only derivatives of isoalloxazine are described, but also its isomeres, alloxazines, and the cationic species of these compounds. The cationic species can also be of particular importance as model of reaction intermediates which may occur in reactions catalyzed by flavoproteins.

These five articles are followed by a general discussion of some of the observations made. Additional results, which are not published yet, will be discussed there.

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Eur. J. Biochem. 37, 563–569 (1973)

I A Nuclear-Magnetic-Resonance Study of the Manganese · Thiamine-Pyrophosphate Complex in solution

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(Received March 16/June 18, 1973)

Manganese causes a broadening of the resonance lines in the nuclear magnetic resonance (NMR) of thiamine pyrophosphate (TPP). This broadening was used to calculate values for the transverse relaxation times T_2. The longitudinal relaxation time T_1 was determined using progressive power saturation and also by Fourier-Transform NMR. Since the paramagnetic contribution to T_2^{-1} is always larger than to T_1^{-1}, T_1 was free of scalar quantities and therefore it was used to evaluate internuclear distances according to the Solomon-Bloembergen relationships. From these calculated distances a folded structure was derived. The effect of temperature in the proton absorptions in NMR indicate a τ_M of 10^{-6} s similar to that of ADP and ATP. Combinations of ^1H and ^{31}P NMR showed that coupling occurred between the 5′-β-CH_2 and the adjacent phosphorus and that at higher temperatures the pyrophosphate moiety was rotating more freely as indicated by a narrowing of the phosphate resonances. EPR measurements showed that a 1:1 complex of TPP and Mn^{2+} was formed. The association constant and free energy (ΔG^{298}) of the complex are given.

In recent years considerable work has been done on the binding of metal ions to ADP and ATP in order to obtain some insight into the conformational structure of these and similar biologically relevant complexes in solution. Generally it is found that these molecules possess a folded structure [1–3].

Perahia *et al.* [4] have carried out molecular orbital calculations on different possible conformations of free ATP and metal · ATP complexes to determine whether ATP might have an intrinsic tendency to fold in the free state. They concluded that the folded form, as found by X-ray studies [5], had a considerably higher stability than the extended form of the metal complex, the energy difference being as much as 48.6 kcal · mol^{-1}. For the free ATP in solution they calculated an even more stabilized form (6 kcal), than in the crystalline metal complex. In this free ATP the terminal phosphate group of ATP in solution is even closer to the adenine ring than in the crystalline form, possibly due to an H-bond between the phosphate-OH and the nitrogen in position 7.

The complex of magnesium and thiamine pyrophosphate (TPP) plays an important role as a cofactor in several enzymatic reactions. It is known that in many cases Mn^{2+} can replace Mg^{2+} without loss of enzymatic activity. The position of Mn^{2+} with respect to the TPP molecule and the three-dimensional structure of the molecule in solution are still unknown. For this reason we have undertaken a detailed study of this complex by electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) techniques. Some results were published in a preliminary form [6]. During the preparation of this paper a study on the transverse relaxation time T_2 of the Ni · TPP complex was published [7].

Abbreviations. TPP, thiamine pyrophosphate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Thiamine pyrophosphate hydrochloride (TPP · HCl) and Tris base were purchased from Sigma Chemical Co., crystalline $MnCl_2 \cdot 4\ H_2O$ was obtained from Merck and ^2H_2O from Fluka.

In NMR experiments 0.3 M solutions of TPP · HCl were brought to pH 6.60 ± 0.02 (approximately p^{2}H 7) with a 3 M NaO^{2}H solution. A 10 mM $MnCl_2 \cdot 4\ H_2O$ solution in ^2H_2O was used as a metal ion source. All solutions were freshly prepared before each experiment. Samples of 0.5 ml were placed in 5-mm tubes and small volumes of metal ion were delivered by means of a micro-syringe.

Transverse relaxation times, T_2, were estimated from the line widths of the proton resonances using a Jeol-C 60H spectrometer [8]. Longitudinal relaxation times, T_1, were measured by means of the

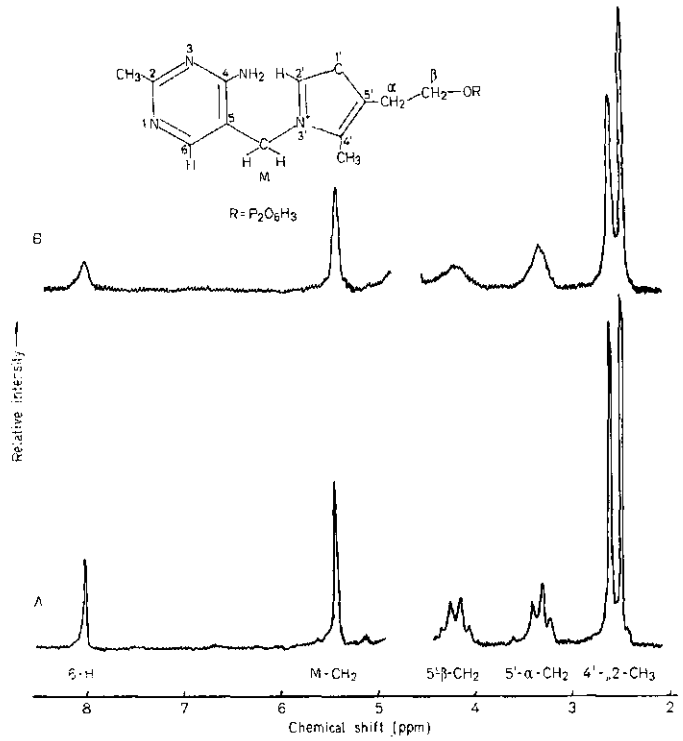

Fig. 1. *60-MHz ^{1}H-NMR spectrum of TPP in the absence and presence of Mn^{2+}.* (A) 0.3 M TPP, pH 6.6, 25 °C. (B) as (A) but in the presence of 0.1 mM Mn^{2+}

progressive saturation method [8,9] and the Carr-Purcell method [10]. The radio frequency of the Varian XL-100 spectrometer used for the saturation experiments, was calibrated by measuring the "ringing" frequency [11]. A Jeol JNM-PS-100 equipped with Fourier Transform was used to measure T_1 by means of the Carr-Purcell method. ^{31}P spectra were also recorded on the Varian XL-100 spectrometer.

X-band (9.45 GHz) EPR spectra were recorded with a Varian E-3 spectrometer; 0.5 mM $MnCl_2 \cdot 4\,H_2O$ in 0.1 M Tris-HCl pH 7.4 was titrated with 8 mM TPP in the same buffer. The spectral amplitude was taken as a measure of uncomplexed $Mn(H_2O)_6^{2+}$ [12].

RESULTS AND DISCUSSION

It is well known that the relaxation times of nuclei such as protons are influenced by paramagnetic substances. This is reflected in a broadening of their magnetic resonance lines (T_2) and different saturation behaviour (T_1). Theoretical considerations of these processes have been discussed in detail by Solomon and Bloembergen [13,14].

In our studies T_2, the spin-spin relaxation time, was obtained from the line widths [8] of the magnetic resonances at half peak height ($\Delta\nu$)

$$T_2^{-1} = \pi\Delta\nu \qquad (1)$$

The paramagnetic contribution to the line width is given as pT_{2p} where p is defined as:

$$p = [Mn^{2+}]_{Total}/[TPP]_{Total} \qquad (2)$$

and

$$\frac{1}{T_{2p}} = \frac{1}{T_2} - \frac{1}{T_2^0} \qquad (3)$$

in which T_2^0 is the transverse relaxation time of the different protons of thiamine pyrophosphate and T_2 is the transverse relaxation time of the same protons when Mn^{2+} is added to the solution. The various NMR absorptions have been assigned [15,16] as given in Fig. 1 A.

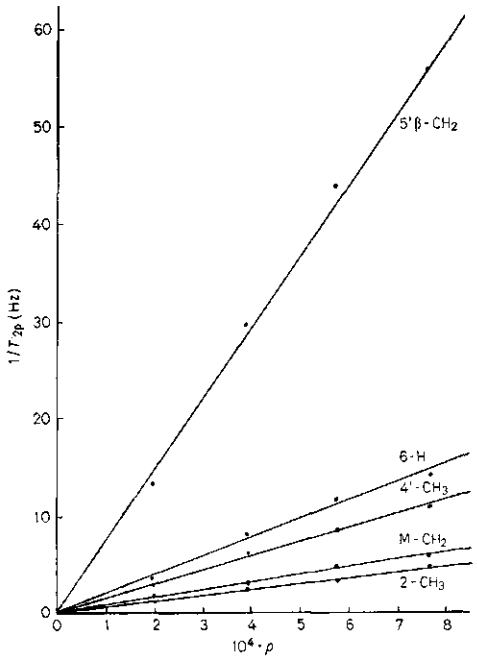

Fig. 2. *Correlation between the paramagnetic contribution to the transverse relaxation rate and the relative Mn^{2+} concentration.* Conditions as mentioned in Materials and Methods. Temperature 25 °C. $p = [Mn^{2+}]/[TPP]$

The exchangeable protons of the 4-amino group and the 2'-carbon are not observed, since measurements were carried out in ^2H_2O. In Fig. 1 the ^2HOH peak has been omitted. The quartet of the 5'-β-CH_2 is located at the low-field side of this ^2HOH peak. This quartet is due to the coupling of these protons with the phosphate group. The J-value (the nuclear spin-spin coupling), measured in phosphor resonance, is 6 Hz. The same value is found for the 5'-α-CH_2 group. The effect of adding Mn^{2+} to the solution is illustrated in Fig. 1 B. Clearly all lines are broadened, but not to the same extent. To obtain accurate values for pT_{2p}, T_{2p}^{-1} was measured as a function of p as given in Fig. 2. pT_{2p}^{-1} was obtained from the slope of these lines. The T_2 values of the 5'-α-CH_2 were not taken into account because of the triplet splitting of the absorption line. Values of pT_{2p} are listed in Table 1.

The resonance of the $-CH_2-$ adjacent to the pyrophosphate moiety is the one most affected by Mn^{2+}. This resonance is almost completely lost at concentrations of 0.2 mM Mn^{2+}. This shows that Mn^{2+} binds to the pyrophosphate group. It can also

Table 1. *Relaxation times of the various protons of TPP in the presence of Mn^{2+} at 27 °C and 60 °C*
The experimental conditions are as described in Materials and Methods. The values are corrected for diamagnetic contributions and calculated from the Fourier-Transform data. The values obtained from the progressive saturation method are about 20% higher. The errors in pT_{1p} are about 5% (least-square-fit data)

Protons in position	pT_{1p}		pT_{2p}
	60 °C	27 °C	27 °C
	μs	μs	μs
6(H)	292	152	52
2(CH_3)	1210	470	192
4'(CH_3)	237	142	61
5'-α(CH_2)	128	90	—
5'-β(CH_2)	32	21	15
M(CH_2)	423	254	88

be seen that the protons of the thiazolium CH_3(4'CH_3) are considerably more affected than the protons of the 2-CH_3, indicating that the former are closer to the Mn^{2+}. In fact of all the resonances, that due to the 2-CH_3 group was the least affected by Mn^{2+} (Fig. 3).

These experimental data were correlated with theoretical parameters according to the following equation derived by Swift and Connick [17] and Luz and Meiboom [18]:

$$\frac{1}{pT_{2p}} = \frac{q}{\tau_M} \frac{(1/T_{2M})^2 + 1/T_{2M}\tau_M + \Delta\omega_M^2}{(1/T_{2M} + 1/\tau_M)^2 + \Delta\omega_M^2} + \frac{1}{T_{OS}} \quad (4)$$

In this equation q is the number of TPP molecules bound to Mn^{2+}, T_{2M} the relaxation time of a coordinated TPP molecule. $\Delta\omega_M$ the chemical shift difference between free and coordinated TPP molecules, τ_M the residence time of a TPP molecule in the coordination sphere of the metal ion and $1/T_{OS}$ the outer sphere contribution to the relaxation rate due to TPP molecules beyond the inner coordination sphere. From Fig. 1 it is clear that the shift difference $\Delta\omega_M$ between free and coordinated TPP is much less than the line broadening. In this case Eqn (4) reduces to:

$$\frac{1}{pT_{2p}} = \frac{q}{T_{2M} + \tau_M} + \frac{1}{T_{OS}} \quad (5)$$

To distinguish between outer and inner sphere relaxation it is necessary also to measure the paramagnetic contribution to the longitudinal relaxation time T_1. If the relaxation is determined by outer sphere relaxation pT_{1p} and pT_{2p} should be identical because pT_{1p} is governed by a similar relation:

$$\frac{1}{pT_{1p}} = \frac{q}{T_{1M} + \tau_M} + \frac{1}{T_{OS}} \quad (5')$$

The values of pT_{1p} at two temperatures (27 °C and 60 °C) are given in Table 1. The values given were measured with the Carr-Purcell method [10] using Fourier-Transform because of its greater accuracy

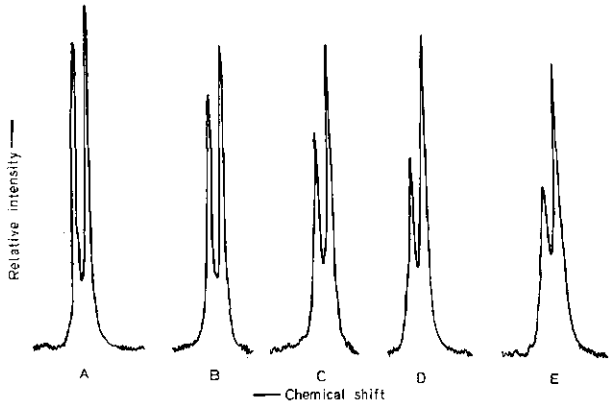

Fig. 3. *Effect of various Mn^{2+} concentrations on the methyl resonances of TPP at 27 °C.* [Mn^{2+}]: (A) 0; (B) 50 μM; (C) 0.1 mM; (D) 0.2 mM; (E) 0.5 mM

by comparison with the progressive saturation method (the T_{2p} values from Fig. 2 were determined in a set of separate experiments). A least-squares-fit program on two constants was used to fit the experimental amplitudes to the theoretical curve, at different time-intervals between the 180° and 90° pulses:

$$A = A_0\,(1-2e^{-t/T_1}) \qquad (6)$$

In this way both A_0, the amplitude for t, is infinite, and T_1 could be calculated from the measured amplitude A and the time interval t. In all cases pT_{1p} is larger than pT_{2p} and from this it can be concluded that the relaxation cannot be dominated by outer sphere relaxation. If τ_M is responsible for the relaxation, pT_{1p} becomes equal to pT_{2p}. However, for the same reasons described above, this can be excluded for the temperatures given in Table 1. Therefore, we obtain:

$$\frac{1}{pT_{2p}} = \frac{q}{T_{2M}} \qquad (7)$$

and

$$\frac{1}{pT_{1p}} = \frac{q}{T_{1M}} \qquad (7')$$

In order to determine the number of molecules (q) of TPP coordinated to Mn^{2+}, TPP was added in small quantities to an aqueous solution of Mn^{2+}. The intensity of the EPR lines, which decrease during the titration, was taken as a measure of uncomplexed $Mn(H_2O)_6^{2+}$ [12]. The results at 25 °C, shown in the form of a Scatchard [19] plot (Fig. 4) indicated that a 1:1 complex ($q = 1$) had formed. The association constant and the free energy ΔG^{298}, calculated from this plot for the binding process were 5.0 (± 0.2) mM^{-1} and −2.9 kcal·mol^{-1}, respectively. At different temperatures, up to 60 °C, the q-value did not change and the apparent association constant was only slightly dependent on temperature. The association constant ($\mu = 0.08$, 25 °C) is lower [20] than those found for ADP · Mn^{2+} and ATP · Mn^{2+} complexes (16 mM^{-1} and 56 mM^{-1}, respectively).

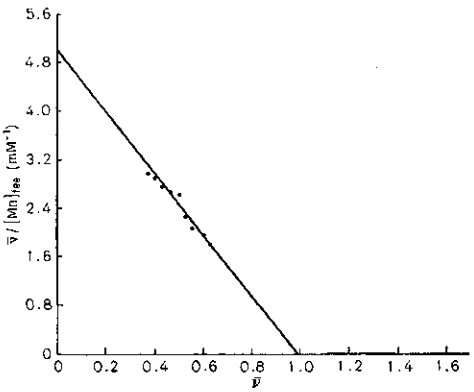

Fig. 4. *Titration of Mn^{2+} with TPP at 25 °C.* The results were obtained by EPR and are given in the form of a Scatchard plot ($\bar{\nu} = [Mn]_{bound}/[TPP]_{total}$). For experimental conditions see Materials and Methods

It is now possible to correlate the experimentally determined values of $1/pT_{1p}$ and $1/pT_{2p}$ with the theoretically derived expressions [13, 14] for T_{1M} and T_{2M}:

$$\frac{1}{pT_{1p}} = \frac{1}{T_{1M}} = \frac{2}{15}\,\frac{S(S+1)\,\gamma_I^2\,g^2\,\beta^2}{r^6} \times \left(\frac{3\,\tau_c}{1+\omega_I^2\,\tau_c^2} + \frac{7\,\tau_c}{1+\omega_S^2\,\tau_c^2}\right) + \frac{2}{3}\,\frac{S(S+1)\,A^2}{\hbar^2}\left(\frac{\tau_e}{1+\omega_S^2\,\tau_e^2}\right) \qquad (8)$$

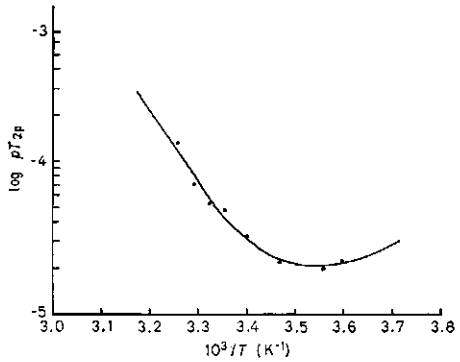

Fig. 5. *Effect of temperature on the transverse relaxation time of 4'-CH_3 protons in the presence of Mn^{2+}.* For details see text

$$\frac{1}{pT_{2p}} = \frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6} \times \left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right) + \frac{1}{3}\frac{S(S+1)A^2}{\hbar^2}\left(\tau_e + \frac{\tau_e}{1+\omega_S^2\tau_e^2}\right) \quad (9)$$

These equations consist of a dipolar and a scalar term. The former being represented by the first and the latter by the second product in Eqns (3) and (9).

In these formulas S is the electron spin quantum number 5/2 for Mn^{2+}, γ_I the nuclear magnetogyric ratio for a proton, r the ion-proton internucleus distance, β the Bohr Magneton, ω_I and ω_S the Larmor angular precession frequencies for the proton and electron spin, respectively. A the hyperfine coupling constant and τ_c and τ_e correlation times.

The correlation time for hyperfine interaction τ_e is defined as:

$$\frac{1}{\tau_e} = \frac{1}{\tau_S} + \frac{1}{\tau_M} \quad (10)$$

τ_S is the electron spin relaxation time and τ_M the exchange time as already mentioned in Eqn (4).

From temperature-dependent studies (Fig. 5) it is clear that at temperatures below 20 °C, τ_M becomes dominant [*cf.* Eqn (5)]. By extrapolation of the low temperature region towards 27 °C a τ_M value in the order of 10^{-5} to 10^{-6} can be estimated. Furthermore, τ_S is 10^{-9} s for $Mn(H_2O)_6^{2+}$ [21] and therefore τ_e is determined by τ_S. Since ω_S is 4.13×10^{11} at 23,487 gauss $\omega_S^2\,\tau_e^2 \gg 1$, the scalar term in Eqn (9) for $1/T_{2M}$ reduces to:

$$\frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\tau_e \quad (11)$$

and becomes negligible in Eqn (8) for $1/T_{1M}$.

Table 2. *Distances, hyperfine coupling constants and activation energy of the protons of the TPP complex*
The values were calculated as mentioned in the text

Proton	r		A	$\Delta H^{\ddagger}$
	27 °C	60 °C	27 °C	
	nm	nm	kHz	kcal
6-H	0.58	0.65	100	9.1
2-CH_3	0.71	0.83	50	6.3
4'-CH_3	0.58	0.63	84	6.2
5'-α-CH_2	0.54	0.57	—	6.8
5'-β-CH_2	0.42	0.45	102	7.5
M-CH_2	0.64	0.69	78	6.8

From our data in Table 1 it appears that in all cases $1/T_{1M}$ is significantly smaller than $1/T_{2M}$. From this it follows that only T_{1M} values can be used for distance calculation, since T_{2M} is determined by a scalar and a dipolar term.

The only unknown variable is the correlation time τ_c. This correlation time consists of a τ_e part and a τ_r part, in which τ_r is the rotation correlation time of the tumbling of the complex. For a small complex such as Mn · TPP, τ_r will be in the order of 10^{-10} and determines therefore τ_c. We were working with a system comparable to ATP. Therefore we used a τ_c value of 3×10^{-10} s as was derived for ATP complexes by Sternlicht *et al.* [1]. With this τ_c value the equations for T_{1M}^{-1} and T_{2M}^{-1} become:

$$\frac{1}{T_{1M}} = \frac{6}{15}\left(\frac{S(S+1)g^2\beta^2\gamma_I^2}{r^6}\right)\tau_c \quad (12)$$

$$\frac{1}{T_{2M}} = \frac{7}{15}\left(\frac{S(S+1)g^2\beta^2\gamma_I^2}{r^6}\right)\tau_c + \frac{1}{3}S(S+1)\tau_e\left(\frac{A}{\hbar}\right)^2 \quad (13)$$

With these equations estimates of the internuclear distances for all observed protons were made. The values obtained are only estimates because of uncertainty in the value for τ_c. However, since the distance r appears to the sixth power in Eqn (12) errors in the assumed value for τ_c will cause only small changes in the distances calculated. Further, estimates for the hyperfine coupling constants (A/h) were made using the relationship derived from Eqns (12) and (13):

$$\frac{1}{T_{2M}} - \frac{7}{6}\frac{1}{T_{1M}} = 114\,\tau_e\left(\frac{A}{h}\right). \quad (14)$$

The results of our calculations are given for two different temperatures in Table 2. The distances calculated indicate the following structure: the Mn^{2+} is bound to the pyrophosphate moiety which is folded over the thiazolium ring towards the quaternary nitrogen. Thus, the Mn^{2+} is situated above the aromatic proton. The pyrimidine ring is positioned

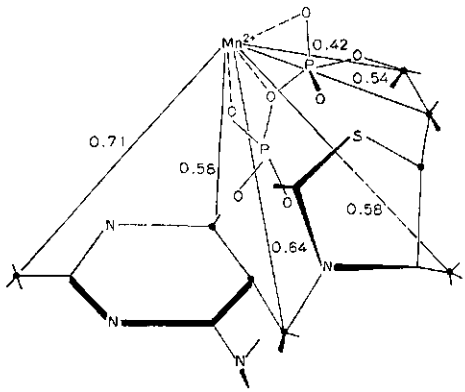

Fig. 6. *Schematic drawing of the Mn · TPP complex.* Distances are measured in nanometres

almost perpendicular with respect to the thiazolium ring. This arrangement is shown schematically in Fig. 6. The pyrimidine ring lies perpendicular to the plane of the paper, the thiazolium ring, with the CH_3 group pointing backwards, almost in the plane of the paper and the 2'-proton is situated above the 4—5 bond of the pyrimidine ring. The pyrophosphate moiety is bent behind the thiazolium ring. In this way the Mn^{2+}, the 6-H and the 4'-CH_3 group are in the same plane, as required by the calculated relative short distances between Mn^{2+} and these protons. From this it follows that the M-CH_2 is positioned in front of this plane in agreement with our experiment.

It has been suggested that a close proximity of the 2'- and amino protons is important for the reactivity of TPP [22]. This idea is supported by our model. The structure as derived from our studies, which is in accordance with the model proposed by Gallo *et al.* [7], differs from the crystal structure of TPP·HCl [23]. The two rings of TPP have the same orientation in the crystalline state and in solution, but in the crystalline state the pyrophosphate group is not located in the vicinity of the thiazolium nitrogen atom. In contrast to our results and those of Gallo *et al.* [7] the pyrophosphate in the crystalline state is removed from the thiazolium nitrogen atom and is located at the opposite site of the thiazolium ring. The difference could be due to the difference in protonation of the pyrophosphate groups in solution and in the crystalline state. In the crystalline state the molecule will be neutral, while in solution at pH 7 the phosphate groups are partially ionized and will therefore be attracted to the positive nitrogen of the thiazolium ring. Also crystal effects and H-bridges could be of importance.

It is obvious from the calculated values for the hyperfine term A, that it is also dependent on the relative distance between Mn^{2+} and the different protons (Table 2). This hyperfine interaction is mostly determined by the contact interaction of the electrons of the Mn^{2+} with its nuclear spin. The proton nuclear spin is polarised through the electrons of the Mn^{2+}. The valency electrons of Mn^{2+} are located in 3d-orbitals, which must be mixed with some s-character [24]. Only electrons in the s-orbitals have a finite density at the Mn^{2+}-nucleus and can interact with the nuclear spin *via* this contact interaction. This mixing of s-character is caused by distortion of the spherical symmetry of the free ion, upon complex formation. The electron density at the protons is therefore also dependent on the symmetry of the environment of the Mn^{2+}. This is illustrated by the A-value of the protons at 5'-β, which should be much higher if it was dependent only on the distance to Mn^{2+}. For this reason evaluation of T_2, which also depends on A, results in a qualitative picture of the molecular structure [7].

Upon elevation of the temperature the pyrimidine ring is moving away from the thiazolium moiety. This is indicated by the much larger calculated distances at 60 °C of the 6-proton, and particularly the protons of the pyrimidine CH_3, from the Mn^{2+}. The pyrophosphate moiety is also interacting more weakly with the positive nitrogen of the thiazolium ring as is evident from the distances calculated. The higher mobility of the pyrophosphate moiety is also supported by the ^{31}P-spectra, where the absorptions are much sharper at 54 °C as compared to the phosphor resonance observed at lower temperature. It was ascertained from both phosphate resonance studies and the reversibility of the temperature effect on the spectrum that no hydrolysis of the pyrophosphate group occurred. In order to check whether temperature had any influence on the conformation of the complex, we studied the temperature dependence of T_1 in proton resonance. In these experiments Mn^{2+} was omitted because the metal is not of importance for the structure of the complex, as follows from the low ratios of Mn^{2+} · TPP used in earlier experiments. All protons showed a good linear correlation between the logarithm of $1/T_1$ and the reciprocal temperature. This indicates that there is no distinct conformational change in the temperature range 9—60 °C, but rather that there is a continuous opening of the complex as the temperature is increased. The free enthalpy of activation calculated from these plots are tabulated in Table 2. All the free enthalpies are of the same order of magnitude except that of the pyrimidine ring proton at position 6. The value for the pyrimidine ring proton is in accordance with the proposed model, because the two rings are not able to rotate freely with respect to each other. This is evident from a space filling model, where the

6-H proton and the amino group are blocking the internal movement of the thiazolium ring sterically hindered by the 2'-proton.

R. L. Houghton thanks the Science Research Council for the award of a N.A.T.O. Research Studentship. We wish to thank Dr P. Smit (Department of Organic Chemistry, Wageningen) for the use of the Jeol-60 NMR apparatus and the Department of Organic Chemistry of the University of Utrecht for the use of the XL-100 spectrometer. We thank the Jeol Company for the use of the fourier transform instrument and in particular we thank Keiji Eguchi for help in recording the NMR spectra. The present investigation was supported by the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.)

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Eur. J. Biochem. 59, 355–363 (1975)

II The Pyruvate Dehydrogenase Complex from *Azotebacter vinelandii*

3. Stoichiometry and Function of the Individual Components

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(Received June 25, August 27, 1975)

Labelling studies with *N*-ethylmaleimide show that either in the presence of Mg^{2+}, thiamine pyrophosphate (TPP) and pyruvate or in the presence of NADH the overall activity of the pyruvate dehydrogenase complex from *Azotobacter vinelandii* is inhibited without much inhibition of the partial reactions. The complex undergoes a conformational change upon incubation with NADH. The inhibition by bromopyruvate is less specific.

Specific incorporation of a fluorescent maleimide derivative was observed on the two transacetylase isoenzymes. Binding studies with a similar spin label analogue show that 3 molecules/FAD are incorporated by incubation of pyruvate, Mg^{2+} and TPP, whereas 2 molecules/FAD are incorporated *via* incubation with NADH. The spin label spectra support the idea that in the complex the active centres of the component enzymes are connected by rapid rotation of the lipoyl moiety.

Three acetyl groups are incorporated in the complex by incubation with [2-^{14}C]pyruvate. Time-dependent incorporation supports the view that the two transacetylase isoenzymes react in non-identical ways with the pyruvate dehydrogenase components of the complex. The results show that the complex contains 2 low-molecular-weight transacetylase molecules and 4 molecules of the high-molecular-weight isoenzyme.

Mn^{2+}-binding studies show that the complex binds 10 ions, with different affinities. 2 Mn^{2+} ions are bound with a 20-fold higher affinity than the remaining 8 Mn^{2+} ions. The latter 8 ions bind with equal affinities and are thought to reflect binding to the pyruvate dehydrogenase components of the complex.

It is concluded that the complex contains 8 pyruvate dehydrogenase molecules, 4 high-molecular-weight transacetylase molecules, 2 low-molecular-weight transacetylase molecules and 1 dimeric (2-FAD-containing) symmetric molecule of lipoamide dehydrogenase. Evidence comes from pyruvate-dependent inactivation and labelling studies that the pyruvate dehydrogenase components contain either an –SH group or an S-S bridge which participates in the hydroxyethyl transfer to the transacetylase components.

In preceding papers [1,2] it was shown that the purified pyruvate dehydrogenase complex from *Azotobacter vinelandii* catalyzes the CoA-linked and NAD^+-linked oxidative decarboxylation of pyruvate and the partial reactions characteristic for the components. The involvement of protein-bound lipoic acid in the reaction sequence is therefore highly probable. The question remains to be solved, however, how this lipoyl moiety participates in the overall reaction of a complex in which movement of the individual enzymes is restricted. An additional related problem in the case of the *A. vinelandii* complex is the role of the two transacetylase isoenzymes.

Possible mechanisms, operating either individually or in combination, have been suggested by Reed and coworkers [3], as follows. One or more of the component enzymes change in conformation during the reaction by which the bound prosthetic groups are brought into juxtaposition. The attached lipoyl moiety

Abbreviations. TPP, thiamine pyrophosphate; EPR, electron paramagnetic resonance.

Enzymes. Pyruvate dehydrogenase complex is pyruvate-NAD^+ reductase, decarboxylating, CoA acetylating (EC 1.2.4.1); pyruvate dehydrogenase is pyruvate-$K_3Fe(CN)_6$ reductase, decarboxylating (EC 1.2.2.2); transacetylase or lipoate acetyltransferase (EC 2.3.1.12); lipoamide dehydrogenase (EC 1.6.4.3); pyruvate oxidase is pyruvate-O_2 reductase, decarboxylating (EC 1.2.3.3).

provides a flexible arm permitting rotation between the prosthetic groups of two other enzymes. Interaction between several lipoyl moieties is necessary for acetyl and electron transfer, a proposal which could account for the apparent excess bound lipoic acid generally found in 2-oxo acid dehydrogenase complexes [4, 5].

Active-site-directed inactivation and labelling experiments may be helpful tools in the approach of these problems. From data in the literature it is known that the pyruvate dehydrogenase and lipoamide dehydrogenase components of the *Escherichia coli* complex contain a large number of reactive –SH groups [4, 6, 7], in contrast to the transacetylase components [4, 8]. Thus it can be expected that the action of sulfhydryl reagents may be very complex, as actually shown by Maldonado *et al.* [9] in their studies on the *E. coli* complex with bromopyruvate.

It is the aim of this article to report on some data concerning the lipoyl-site-directed inactivation of the overall activity by sulfhydryl reagents and the selective incorporation of paramagnetic and fluorescent labels derived from maleimide. Together with Mn^{2+} titrations and the incorporation of radioactive pyruvate, conclusions on the composition of the complex could be derived.

MATERIALS AND METHODS

The preparation of the complex as well as the methods for activity determination have been described [1]. Throughout the studies complex without phosphotransacetylase activity was used. The specific activity of the complex was 6–10 μmol NADH produced $min^{-1} \cdot mg\ protein^{-1}$.

Inactivation Experiments

The complex (0.5–1.0 mg/ml) was incubated at 25 °C aerobically with slow stirring or anaerobically (flushing with argon) in 20-ml flasks in the presence of 50 mM Tris-HCl pH 7.1, 2 mM $MgCl_2$, 0.1 mM TPP, 2 mM pyruvate and either *N*-ethylmaleimide, iodoacetate or bromopyruvate in 1 mM concentration. The incubations were started by the addition of enzyme; at the times indicated aliquots were withdrawn and assayed for overall or partial activity. The overall assay was performed in 50 mM Tris-HCl, pH 7.1, without dithiotreitol or reduced glutathione. Oxygen uptake was followed with a Clark-type electrode.

Spin Labelling and Fluorescence Labelling

The complex (5–10 mg/ml) was incubated at either 25 °C (during 6 min) or 4 °C (during 45 min) aerobically in the presence of 50 mM potassium phosphate, pH 7.2, and 5 mM Mg^{2+}, 0.5 mM TPP, 5 mM pyruvate, in the presence or absence of 0.5 mM NADH, and a 10-fold excess based on the flavin content of the label. In case of specific incorporation *via* NADH alone (0.5 mM), the spin label must be added before the reduced pyridine nucleotide (see Table 2); incubation times 10 min (25 °C) and 45 min (4 °C). Anaerobiosis does not affect the amount of label incorporated (*cf.* Fig. 2). The labelled protein was separated from the free label on a Sephadex G-10 column (0.8 × 25 cm). To prevent nonspecific labelling, the complex was prelabelled by reaction at room temperature with a 20-fold excess (based on FAD) of *N*-ethylmaleimide in the absence of pyruvate, Mg^{2+} and TPP during 10 min. The sulfhydryl reagent was removed by Sephadex G-10 filtration. Under these conditions the enzyme was at least 80% active in the overall reaction. Concentrations of bound spin label were calculated either directly by comparing the signal amplitude of the complex with that of free spin label in glycerol/H_2O with a somewhat higher mobility, but with a coinciding line width, or indirectly from the difference between the signal amplitude of the amount of label added (identically treated without protein) and the signal amplitude of the amount of unbound label. Since the direct method gives too high values, in some case a double integration procedure was also carried out; as a standard the same solution of spin label in glycerol/H_2O was used. The electron paramagnetic resonance (EPR) spectra were recorded on either a Varian E-3 or E-6 spectrometer. The measurements were carried out in an aqueous solution sample cell (Varian V-4548) or in capillary (3 mm outer and 0.65–0.75 mm inner diameter). Fluorescence of the sodium dodecylsulphate gels was visually observed by means of an ultraviolet lamp.

Mn^{2+} Titrations

Just prior to the binding studies, the enzyme solution (10 mg/ml containing 10 mM EDTA in 0.05 M Tris-HCl, pH 7.0) was dialysed against two changes of 1 l 0.05 M Tris-HCl, pH 7.0. Binding was studied by EPR measurements carried out at 25 °C in an aqueous solution sample cell (Varian V-4548). The cell was positioned and kept in this position during the titration. The actual titration was carried out by adding Mn^{2+} to the protein in a storage vessel and filling the cell by suction. Free Mn^{2+} was calibrated in the last dialysis buffer under exactly the same conditions.

Label Experiments with [2-^{14}C]Pyruvate

The complex was incubated aerobically in the presence of 50 mM potassium phosphate buffer, pH 7.0, 5 mM $MgCl_2$ and 0.5 mM TPP. The reaction

was started by the addition of an excess of [2-^{14}C]-pyruvate with a specific activity of 0.494 mCi/mmol. After 10 min at 25 °C or 30 min at 4 °C the mixture was separated on a Sephadex G-10 column (0.8 × 25 cm). The enzyme was eluted with 50 mM phosphate buffer pH 7.0 in 0.2-ml fractions. Free pyruvate was eluted with 1 M KCl. Alternatively the labelled protein was precipitated with cold 10% trichloroacetic acid, collected on Whatman filter paper, then washed with 10% trichloroacetic acid and alcohol, respectively. The samples were counted with a Packard model 3375 Tri-Carb liquid scintillation spectrometer. Aquasol was used as scintillation liquid.

Sodium dodecylsulphate gel electrophoresis of the labelled protein was done as described earlier [1, 11] in the absence of 2-mercaptoethanol. After staining with Coomassie brilliant blue the bands were cut out and transferred to counting vials containing soluene (Packard). After swelling, the samples were counted as described above. Corrections were made for background.

FAD determinations were done as described previously [1].

The spin label, 3-maleimido-2,2,5,5-tetramethylpyrrolidinyloxyl was obtained from Synvar (Palo Alto). The fluorescence label *N*(1-anilino-naphthyl-4)-maleimide was prepared according to Kanacka *et al.* [12]. N-[^{14}C]Ethylmaleimide and [2-^{14}C]pyruvate were obtained from the Radiochemical Centre (Amersham, England). Aquasol was obtained from New England. FMN, purified according to Mayhew *et al.* [13] was a gift from Dr S. G. Mayhew. Sepharose-bound lipoate was a gift from Dr J. Visser. *N*-Ethylmaleimide and 3-bromopyruvic acid were obtained from Sigma. Iodoacetic acid was from Merck. All other chemicals have been described previously [1].

RESULTS AND DISCUSSION

Inactivation of the Overall Reaction by Sulfhydryl Reagents and Oxygen

Accepting the proposed sequence for the overall reaction, one sulfhydryl group per lipoyl moiety will be generated during the reaction of the complex with pyruvate plus Mg^{2+} · TPP in the absence of CoA (*cf.* [14]). It thus can be expected that the subsequent addition of alkylating agents like *N*-ethylmaleimide or bromopyruvate will result in an irreversible block and complete loss of the overall activity. In Fig. 1 this is illustrated. Anaerobically in the presence of 0.1 mM pyruvate and 1 mM alkylating agent, the activity is completely destroyed in a process dependent on Mg^{2+} · TPP and pyruvate within a few minutes.

No reactivation can be achieved by adding dithiothreitol in 2 mM or higher concentration. A slow reaction of *N*-ethylmaleimide with other groups than the ones specifically generated in the lipoyl moieties takes place as judged from the control experiments. Although somewhat slower, iodoacetic acid reacts in similar way as *N*-ethylmaleimide in the presence and in the absence of pyruvate.

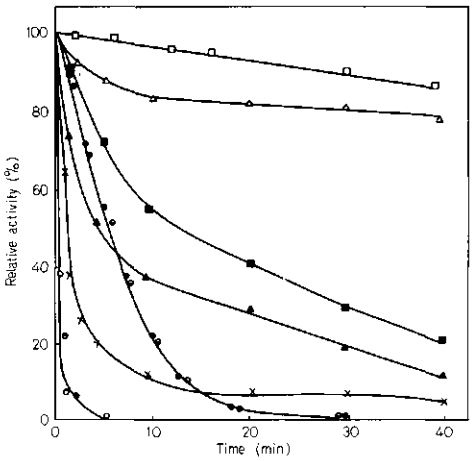

Fig. 1. *Pyruvate-dependent inactivation of the overall reaction by sulfhydryl reagents.* The incubations were anaerobically performed as described in Methods. At the times indicated aliquots were withdrawn and assayed for overall activity. (○——○) Complete mixture with *N*-ethylmaleimide; (◐——◐) complete mixture with bromopyruvate; (●——●) without Mg^{2+} · TPP plus *N*-ethylmaleimide. (◒——◒) without Mg^{2+} · TPP plus bromopyruvate; (×——×) bromopyruvate without pyruvate; (■——■) bromopyruvate without both pyruvate and Mg^{2+} · TPP; (▲——▲) bromopyruvate without pyruvate, while prior to the assay the removed aliquots were incubated for 10 min with 2 mM dithiothreitol; (△——△) *N*-ethylmaleimide without pyruvate; (□——□) without both sulfhydryl reagents and pyruvate

The reaction with bromopyruvate is much more complex although this compound also inactivates in a Mg^{2+} · TPP-dependent process. During the early stages of this inactivation process, partial reactivation of the enzyme can be obtained upon incubating an aliquot with dithiothreitol prior to the assay. These results suggest the initial formation of some reversibly inactivated adduct between bromopyruvate and the Mg^{2+} · TPP-containing complex before it irreversibly inactivates in a secondary process.

In the absence of a sulfhydryl reagent, but in the presence of 0.1 mM pyruvate plus Mg^{2+} · TPP, aerobically within 5 min 30% of the activity is lost (Fig. 2). At higher pyruvate concentrations the extent of inactivation becomes more complete. Exclusion of air or omission of Mg^{2+} · TTP largely prevents this inactivation process. No reactivation can be achieved by using dithiothreitol, but the addition of 2 mM of this compound at any stage during the inactivation prevents further inactivation.

The inactivation of the overall reaction by the combined action of *N*-ethylmaleimide and pyruvate is not accompanied by a comparable drop in the activity of one of the component enzymes (Table 1). This suggests mainly an attack of the inhibitor at the protein-bound lipoyl moiety. The lipoyl moiety is not essential or required for the partial reactions of the complex from *E. coli* [15].

Bromopyruvate decreases the overall activity in a different way. The presence of pyruvate rather seems to protect the activities of the first enzyme of the complex while it appears that bromopyruvate also reacts *via* the normal sequence of steps of the substrate.

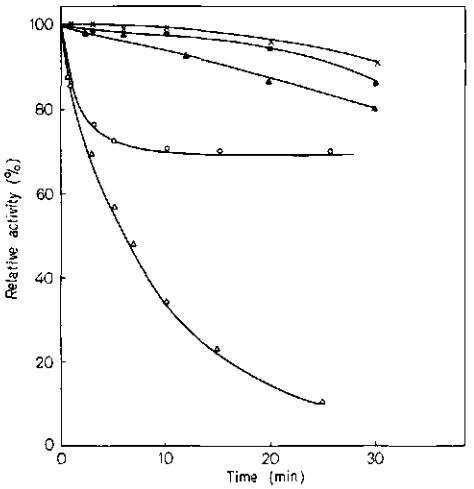

Fig. 2. *Pyruvate-dependent inactivation of the overall reaction by oxygen.* The incubations were performed as described in Methods in the absence of alkylating reagents, with the following pyruvate concentrations: (○——○) 0.1 mM aerobically; (△——△) 2 mM aerobically; (●——●) 0.1 mM anaerobically; (▲——▲) 2 mM anaerobically; (×——×) no pyruvate aerobically

Partially inactivated 'oxidase' (by bromopyruvate) reactivates for about 50% upon addition of 2 mM dithiothreitol. By action of bromopyruvate the lipoamide dehydrogenase activity declines independent of the presence of pyruvate by about 25–30%. Furthermore, the complex is inactivated by oxygen without affecting the activities of the individual components. This observation rules out the involvement of the lipoyl moiety in the 'oxidase' reaction. In addition these data plus our observation that catalase does not influence the pyruvate-dependent inactivation of the overall activity by oxygen, indicate that the 'oxidase' activity itself is not responsible for this phenomenon. In view of the observed pyruvate accumulation when *Azotobacter* grows in the presence of excess oxygen [16], the complex could be sensitive to oxygen in the intact cell.

Reduced, protein-bound lipoic acid generated after transacetylation is reoxidized by transfer of the reducing equivalents to the disulfide and FAD of lipoamide dehydrogenase. The mechanism of reduction of pure oxidized lipoamide dehydrogenase by reduced lipoamide and NADH differs slightly from the pattern observed with the pig heart enzyme. With NADH mainly the 2-equivalent reduced enzyme · NAD^+ complex is formed, with reduced lipoamide the 2-equivalent-reduced red intermediate is formed [17]. Attempts were made to reduce the protein-bound lipoyl moiety *via* the reduced flavoprotein and to block the overall reaction irreversibly by subsequent reaction with *N*-ethylmaleimide (Table 2). The overall activity is zero when the lipoamide dehydrogenase activity is still high. Addition of NAD^+ (0.1 mM) does not influence the rate of the inactivation process. The sequence of the addition is important, since addition of *N*-ethylmaleimide after addition of NADH leads to a lower degree of inactivation than in the reverse order. Clearly a conformational change within the complex occurs upon addition of NADH, protecting the protein-bound lipoate. Similar effects are observed in the presence of spin label.

Table 1. *Effects of* N*-ethylmaleimide, bromopyruvate and oxygen on different activities of the pyruvate dehdyrogenase complex*
The enzyme was aerobically incubated under the conditions as described in Methods without stirring. The pyruvate concentration was 2 mM. The activities of the partial reactions were assayed as described in Methods

	Percentage of residual activity									
	+ *N*-ethyl-maleimide + pyruvate		+ bromo-pyruvate + pyruvate		− alkylating reagents + pyruvate		+ *N*-ethyl-maleimide − pyruvate		+ bromo-pyruvate − pyruvate	
	1 min	10 min	1 min	10 min	1 min	10 min	1 min	10 min	1 min	10 min
Pyruvatedehydrogenase complex	7	0	10	0	85	35	95	85	56	15
Pyruvate dehydrogenase	95	90	71	35	97	80	98	85	50	20
Pyruvate oxidase	98	97	75	32	98	82	98	88	52	15
Transacetylase	93	96	95	93	98	96	95	95	93	95
Lipoamide dehydrogenase	99	93	92	75	100	99	99	94	91	72

Table 2. *Effect of N-ethylmaleimide in the presence of NADH on both the overall and the lipoamide dehydrogenase activity*
The enzyme was anaerobically incubated under the conditions as described in Methods with the additions in the order listed. Between the first and second addition 10 s passed. [NADH] = 0.1 mM, [*N*-ethylmaleimide] = 1 mM. The pyruvate-$K_3Fe(CN)_6$, pyruvate-O_2 and transacetylase activities were not affected

Additions	Percentage of residual activity			
	overall reaction		lipoamide dehydrogenase	
	1 min	10 min	1 min	10 min
N-Ethylmaleimide + NADH	10	0	88	60
NADH + *N*-ethylmaleimide	88	50	98	92
N-Ethylmaleimide	93	85	99	94
NADH	100	98	100	98

Selective Introduction of Maleimide Spin and Fluorescence Label

The experiments described above indicate that the pyruvate-dependent and NADH-dependent inactivation of the overall reaction by *N*-ethylmaleimide is caused by reaction with the sulfhydryl groups of the protein-bound lipoyl moiety. To obtain more evidence about the involvement of the lipoyl moiety and its content, the reaction was carried out with maleimide derivatives carrying either a fluorophore or a radical.

Incubation of the complex, prelabelled with *N*-ethylmaleimide as described in Methods, with *N*-(1-anilinonaphthyl-4)-maleimide yields only in the presence of pyruvate, Mg^{2+} and TPP and subsequent separation on a Sephadex G-10 column a highly fluorescent protein conjugate. After subjecting the fluorescent conjugate to dodecylsulphate gel electrophoresis, avoiding reductive cleavage by the omission of 2-mercaptoethanol, a similar four-banded pattern as described previously [1] is obtained. The fluorescence is located specifically on the second and third band. Since these bands are both ascribed to the transacetylase components of the complex [1] it must be concluded that the probe binds specifically to the generated $-SH$ group of the lipoyl moiety. Identical results are obtained when the complex is incubated with the fluorescent label and NADH.

Further information was obtained by using the paramagnetic label, 3-maleimide-2,2,5,5-tetramethyl-pyrrolidinyloxyl. Fig. 3a shows the EPR spectrum of the spin label, bound to the protein, which was prelabelled with *N*-ethylmaleimide in the absence of pyruvate, Mg^{2+} and TPP. As compared with Fig. 3c, the protein-bound label is of the rather mobile type and shows some anisotropy. The rotational correlation time τ_c calculated according to the semi-empirical method of Mensch and Meier [18], which is essentially based on comparison with spectra of the free label in a series of glycerol/water mixtures, is 0.18 ns. This very high mobility for a label bound to a protein

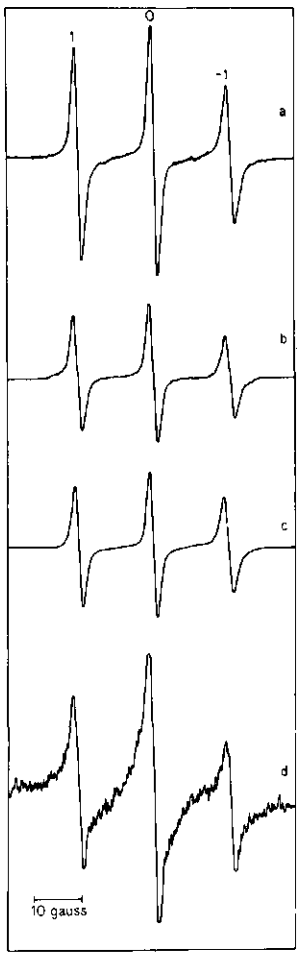

Fig. 3. *EPR spectra of spin label bound to pyruvate dehydrogenase complex.* For conditions see Methods. (a) Complex (3.2 μM), prelabelled, with *N*-ethylmaleimide, was labelled in the presence of pyruvate, Mg^{2+} and TPP, amplification 2×10^5. (b) Complex (3.1 μM), pretreated with *N*-ethylmaleimide, was labelled in the presence of NADH, amplification 2×10^5. (c) Free spin label in 70% glycerol/H_2O, amplification 2×10^4, (this solution was used for calibration, see Methods). (d) Spin label attached to Sepharose-bound reduced lipoate with approximately 2 μM of spin label bound, amplification 10^6. Modulation amplitude 2 G, power 80 mW for all spectra

complex of a million molecular weight supports the idea that the label is bound to the lipoic acid group having a flexible arm of 1.4 nm which connects the enzymes within the complex by rotating rapidly (*cf.* [3]), a view recently opposed by Moe *et al.* [19]. The anisotropy found can be caused by the preferential modes of rotation or by the inhomogeneity of the lipoyl residues bound to different transacetylase iso-

Table 3. *Amount of spin label bound under different conditions to pyruvate dehydrogenase complex of* A. vinelandii
The enzyme was either untreated or pretreated with *N*-ethylmaleimide in the absence of pyruvate, Mg^{2+} and TPP as described in Methods. The enzyme was incubated as described in Methods under the conditions given below. n.d. = not determined

Expt	Incubation	Enzyme prepn	Spin label/FAD from		
			bound label	unbound label	signal integration
1	Pyruvate, Mg^{2+}, TPP	pretreated	3.6	3.0	3.0 ± 0.2
2	No additions	pretreated	< 0.1	n.d.	
3	No additions	untreated	3–4	n.d.	
4	Pyruvate, Mg^{2+}, TPP, NADH	pretreated	3.3	n.d.	
5	NADH	from Expt 1 after Sephadex G-10	3.6	n.d.	
6	Pyruvate, Mg^{2+}, TPP	untreated	3.6[a]	2.9[a]	
7	Pyruvate, Mg^{2+}, TPP + NADH, 4 °C	untreated	3.6[a]	n.d.	
8	NADH, 4 °C	pretreated	2.0	n.d.	1.8 ± 0.2

[a] Corrected for non-specific labelling in parallel experiments.

enzymes. Such a situation can be simulated by reducing Sepharose-bound lipoate [20] with NADH and lipoamide dehydrogenase followed by reaction with spin label. The resulting EPR spectrum (Fig. 3d) shows similar characteristics, *i.e.* a high mobility and an even more pronounced anisotropy. Furthermore it was found from other experiments that despite two –SH groups available in free reduced lipoamide the spin label binds approximately in a 1 : 1 ratio. The amount of protein-bound spin label was calculated by comparison with a standard solution of spin label in a glycerol/water mixture of a somewhat higher mobility, but with a line-width about the same as that of the observed signal (Fig. 3c). Due to the anisotropy found in the protein-bound signal some uncertainty is introduced in the quantitisation of the amount bound from the signal amplitude by comparing it with that of the free spin label. This is prevented by measuring the amount of unreacted spin label and subtracting this from the total amount added. The values obtained under different conditions are given in Table 3 and are expressed as moles of label bound per mole of FAD. It was impossible to determine by dodecylsulphate gel electrophoresis to which proteins the spin label is bound. This can be due to the presence of a small radical contamination in the gel. From this table it is clear that 3 active –SH groups per FAD react with the spin label. Assuming that the spin label reacts with the same proteins as the fluorescence label and that one lipoyl group is present per transacetylase protomer as is the case with the complexes from *E. coli* and pig heart, it can be concluded that the transacetylase to lipoic acid dehydrogenase (dimer) ratio is 6 : 1, which is very high compared with this ratio in other complexes [4, 5].

Some discrepancy is observed when the data obtained from bound and unbound label are compared. This is in our opinion due to the anisotropy observed in the bound label. Double integration of the signals gives a lower number. The determination of free label gives erroneous results in incubations in the presence of reducing agents like NADH. In contrast to the bound label, the free label is reduced slowly by NADH, which makes determination from the amplitude necessary.

Incubation experiments with NADH alone indicate clearly that less spin label is bound. The mobility of the reporter group is slightly higher than that found for the label introduced by incubation with pyruvate (Fig. 3b). The results in the presence of pyruvate + NADH indicate that the maximum number of groups which can be incorporated is 3 per FAD molecule. It must be mentioned in this context that upon incubation with NADH 2 molecules of *N*-[^{14}C]-ethylmaleimide per FAD are incorporated at both 4 °C and 25 °C.

Incorporation of [2-^{14}C]Pyruvate

As shown for the *E. coli* enzyme by Barrera *et al.* [5], [2-^{14}C]pyruvate can be used to determine the amount of lipoyl residues bound to the transacetylase component. Because the complex from *A. vinelandii* contains two transacetylase isoenzymes, it was of interest to determine the distribution of the label over the two components. This was done by dodecylsulphate gel electrophoresis. The complex (12 µM) was incubated with 0.6 mM [2-^{14}C]pyruvate, 5 mM Mg^{2+} and 0.5 mM TPP. The reaction was stopped by addition of sodium dodecylsulphate to a final concentration of 2%. This mixture was heated to 90 °C for 5 min and subjected to dodecylsulphate gel electrophoresis. After staining the four bands were cut out, together with an equal amount of gel in which no bands were visible and which was used for background (less than 10%) correction. After 1 min of incubation, 26% of the radioactivity is present on the pyruvate dehydrogenase component, 69% is associated with

Table 4. *Incorporation of radioactivity from [2-^{14}C]pyruvate in pyruvate dehydrogenase complex from* A. vinelandii
The enzyme was incubated aerobically with [2-^{14}C]pyruvate in the concentrations given below with Mg^{2+}, and TPP under the conditions described in Methods. After incubation, the enzyme was separated from unreacted pyruvate either by passage over a Sephadex G-10 column or by washing with 10% trichloroacetic acid on Whatman paper, as described in Methods

Expt	Conditions	Temperature	FAD concn	[^{14}C]Acetyl/FAD
		°C	μM	mol/mol
1	Pyruvate 20 μM	25	1	3.1
2	No Mg^{2+}, TPP; 20 μM pyruvate	25	1	< 0.1
3	Pyruvate 20 μM, enzyme pretreated with *N*-ethylmaleimide	25	3.5	2.7
4	Pyruvate 220 μM	4	11	3.0
5	Pyruvate 40 μM	4	4.0	2.8
6	Pyruvate 310 μM, washed with acid (1-min incubation)	25	6.3	2.6

the second band, while the 3rd and 4th band contain 2.6% and 1.8% of the total radioactivity. After 6 min of incubation the total radioactivity increases only slightly (7%) but the distribution changes considerably. 11% is now associated with the pyruvate dehydrogenase band, 62% with the second band and 17% and 10% with the third and fourth band respectively. It is clear that with time a redistribution of label occurs, together with a rather small increase of the total amount of radioactivity incorporated. This is also illustrated comparing the results of Expt 6 (Table 4) with those of the other experiments.

The total amount of label incorporated is in good agreement with the results obtained from the spin label experiments. The results point to the presence of 3 lipoyl groups per flavin molecule. No significant difference was found between the results from 1 min incubation and 10 min. In general, the incorporation of the label used is very fast compared with the rate of inactivation of the overall reaction by oxygen in the presence of pyruvate, Mg^{2+} and TPP, as described above. Therefore the experiments could be performed aerobically.

Mn^{2+} Binding Experiments

While the experiments described above suggest a lipoic acid transacetylase to lipoamide dehydrogenase monomer ratio of 3 : 1, a determination of the number of pyruvate dehydrogenase monomers with respect to the flavin content turned out to be more difficult. No specific covalent labelling method exists at this moment.

It has been shown that Mn^{2+} can replace Mg^{2+} in the overall reaction with a K_m that is 10 times lower than found for Mg^{2+} [2]. Because free Mn^{2+} is paramagnetic, the binding of Mn^{2+} to the complex can be followed by EPR spectrometry. A Scatchard plot, resulting from three titrations with different preparations of the complex is shown in Fig. 4. This plot indicates either two types binding sites or negative

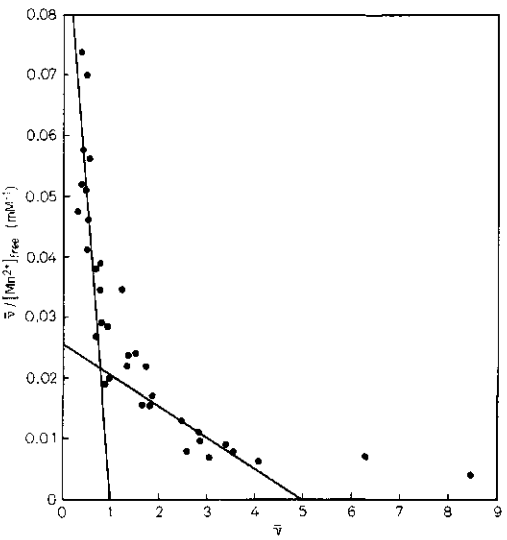

Fig. 4. *Scatchard plot of Mn^{2+} binding to the pyruvate dehydrogenase complex.* Determination of free Mn^{2+} as described in Methods. The data are from three different experiments in which the flavin concentration varied from 10–45 μM. Temperature 25 °C
$\bar{\nu}$ = ratio of bound Mn^{2+} to free Mn^{2+}

cooperative binding to one type of binding site. The latter possibility is rather unlikely because such phenomena are not observed in kinetic experiments [2]. Moreover, our preliminary unpublished results of water relaxation studies in nuclear magnetic resonance spin echo point to the existence of two distinct sites with different relaxation enhancement. Due to the fact that the first binding site has a rather high affinity for Mn^{2+}, *i.e.* $K = (1.1 \pm 0.2) \times 10^6$ l · mol^{-1}, $n = 1$ per FAD, and the concentration of the complex cannot exceed 100 mg · ml^{-1}, no accurate EPR measurements can be done below $\bar{\nu} = 0.5$. The effects of low Mn^{2+} concentrations will be studied further by water relaxation measurements.

The nature of the strong binding site is not clear. There is no kinetic evidence for such a site [2]. It is of interest to mention that Bisswanger [22] has shown that in the *E. coli* complex a small amount of TPP is very tightly bound to the complex. For reasons outlined below the low affinity binding site, *i.e.* $K = (5.1 \pm 0.4) \times 10^6\ l \cdot mol^{-1}$, $n = 4$ per FAD, reflects in our opinion binding to the active site of the pyruvate dehydrogenase and indicates a pyruvate dehydrogenase to lipoamide dehydrogenase monomer ratio of 4 : 1.

From the results given above a picture for the composition of the pyruvate dehydrogenase complex from *A. vinelandii* can be derived. As shown in the preceding paper [1], the complex contains one dimeric molecule of lipoamide dehydrogenase. This leads in the present interpretation to a composition pyruvate dehydrogenase : lipoyl transacetylase (high plus low M_r) : lipoamide dehydrogenase FAD = 8 : 6 : 2. This stoichiometry is based on the number of reactive lipoyl moietys present on the transacetylase components. In this complex apparently two transacetylases are present and it is assumed that both isoenzymes contain one lipoyl moiety.

Although a certain overlap of the closely located bands of the dodecylsulphate gels can be expected, the redistribution of radioactivity indicates that a secondary process proceeds. The low-molecular-weight transacetylase and the pyruvate dehydrogenase components of the complex as well as the high-molecular-weight transacetylase and the lipoamide dehydrogenase components seem to be closely associated [1]. Converting this observation into a physiological speculation it can be visualized that the low-molecular-weight transacetylase is the acetyl acceptor of the pyruvate dehydrogenase and transfers this acetyl group (plus 2 electrons and a H^+) to the high-molecular weight transacetylase, under conditions that no CoA is present. The latter enzyme mainly reduces the lipoamide dehydrogenase. The low radioactivity on the low-molecular-weight transacetylase after 1-min incubation thus could be due to a rapid acetyl transfer and a rate-limiting acetyl donation by the pyruvate dehydrogenase component. Such a kinetic effect is not unlikely in view of the non-saturating [^{14}C]-pyruvate concentration which had to be used for technical reasons (*cf.* [2]). It is clear from the data of Table 4 that radioactivity is transferred from the pyruvate dehydrogenase moiety to the low-molecular-weight transacetylase moiety. This idea is slightly preferred, for the reasons mentioned, over the alternative possibility, *e.g.* both transacetylase isoenzymes are hydroxyethyl acceptors from the pyruvate dehydrogenase moieties, the high-molecular-weight isoenzyme accepting at a higher rate. Consistent with the first possibility are the data of Table 3: two spin label molecules per FAD molecule are incorporated by treatment with NADH, as compared with three by treatment with a saturating concentration of pyruvate or pyruvate plus NADH. A fast reaction with the fluorescence label of both transacetylase isoenzymes is also observed at a saturating pyruvate concentration, while two *N*-[^{14}C]ethylmaleimide groups are incorporated *via* NADH.

From the distribution of radioactivity after prolonged incubation with [2-^{14}C]pyruvate a ratio high-M_r : low-M_r transacetylase of 2 : 1 can be estimated, assuming an equal distribution of lipoyl moieties. However, the densitometer tracings of the gels indicate rather a ratio 1 : 3 in the amount of protein. It is known that different proteins can stain very differently [23], *E. coli* transacetylase stains much more weakly than (equimolar) pyruvate dehydrogenase.

A ratio 8 : 4 : 2 : 2 agrees rather well with the molecular weight determinations [1]. A value of 1.3×10^6 is calculated, based on the molecular weights of the individual chains as determined from dodecylsulphate gel electrophoresis [1], which is slightly higher than the range $1.0 - 1.2 \times 10^6$ found for the intact complex. As it can be expected that the transacetylases behave abnormally in these gels, as concluded by Eley *et al.* [4], a similar correction applied here gives $1.1 - 1.2 \times 10^6$.

The ratio 8 : 6 : 2 and the presence of a fourth component deviates rather strongly from the composition of other pyruvate dehydrogenase complexes. The presence of a single lipoamide dehydrogenase molecule with two similarly bound FAD-moieties, in contrast with the mammalian complex [24], to which the reducing equivalents are transferred is quite interesting from the point of regulation of the overall activity. Because sigmoidal kinetics are found in the overall activity but not in the partial reactions of the pyruvate dehydrogenase component, it was concluded (*cf.* [2]) that subunit interactions play a major role in the regulation of the overall activity. It is tempting to speculate on a regulatory role of the two transacetylase isoenzymes in channelling the reducing equivalents to the lipoamide dehydrogenase component. A tentative model, which is in agreement with the tetrad structures, observed in the electron microscope [17] is given in Fig. 5. The interesting feature of this model is the inequivalent positions which the pyruvate dehydrogenase and transacetylase components occupy with respect to the lipoamide dehydrogenase monomer. In this model the high-molecular-weight transacetylase molecules are positioned in the centres of the planes. However they can be easily shifted towards positions closer to the lipoamide dehydrogenase monomers without affecting the relative symmetry.

Critisism may arise concerning the interpretation of the number of pyruvate dehydrogenase molecules of the complex. Especially since the kinetic experi-

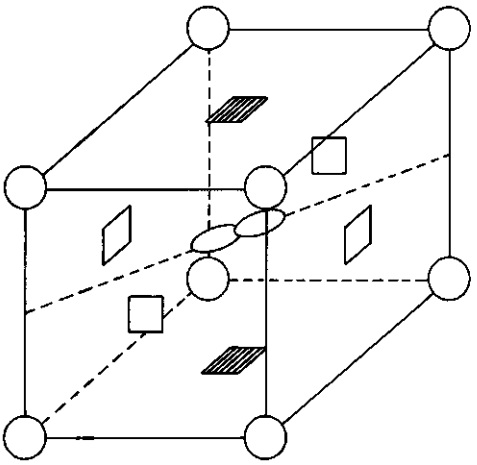

Fig. 5. *A tentative model for the pyruvate dehydrogenase complex from* A. vinelandii. In this model the transacetylase components are considered to be non-identical. The pyruvate dehydrogenase molecules (circles) are thought to be located on the edges of a cube. The two low-molecular-weight transacetylase molecules (shaded squares) are thought to be located in the center of two planes. The four high-molecular-weight transacetylase molecules (open squares) are thought to be distributed in a symmetric way around the two symmetric (*cf.* [22,24]) lipoamide monomers (ellipses)

ments [2], which reflect the binding of $Mg^{2+} \cdot TPP$, were performed at a relatively high metal-ion concentration and thus are not completely comparable with the titration data. The data of Fig. 4 indicate that at high Mn^{2+} concentrations nonspecific binding occurs; however the high-affinity binding site is much more specific. The interpretation that this high-affinity site reflects the presence of two extra pyruvate dehydrogenase molecules of the complex, leads to a too high value for the molecular weight of the complex (M_r $1.3-1.4 \times 10^6$). If the present interpretation of 8 pyruvate dehydrogenase molecules present in the complex is correct, the question at which site the high-affinity Me^{2+} is bound remains. The low-molecular-weight transacetylase molecules and two FAD molecules present in the complex could be meaningful in this context.

The presence of radioactive label on the pyruvate dehydrogenase component after 1 min of incubation with [^{14}C]pyruvate is of great interest with respect to the reversible inactivation at the early stage of incubation with bromopyruvate. It is unlikely that this is due to the presence of hydroxyethyl derivative of TPP, still bound under these conditions. It seems likely from the present data that either an $-SH$ group or an S-S bridge of the pyruvate dehydrogenase component participates in the hydroxyethyl transfer and can lead to alkylation by bromopyruvate or inactivation by O_2. Further studies will concern more detailed insights into the actual functioning of the different components of the complex.

The authors wish to thank Mr B. J. Sachteleben for drawing the figures. This investigation was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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III Symmetry and asymmetry of the Pyruvate Dehydrogenase Complexes from *A. vinelandii* and *E. coli* as detected by fluorescence and spinlabel studies

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SUMMARY

Fluorescence lifetimes measurements of FAD bound to lipoamide dehydrogenase from *A. vinelandii* and *E. coli* were performed. It is shown from these results that the two FAD groups in the isolated dimeric enzyme as well as in the enzyme in the intact complex of *E. coli* are in non-equivalent surroundings. This contrasts with the near equivalence of the FAD groups of both the enzyme and complex isolated from *A. vinelandii*. Reduction of the complex with Mg^{2+}, thiamine pyrophosphate and pyruvate or with NADH enables the attachment of a maleimide analogue specifically to the lipoyl moieties of the transacetylase(s). Spin label (N-(1-oxyl-2,2,5,5,-tetramethyl-3-pyrrolidinyl) maleimide) introduced in such a way proves the existence of at least two different micro-environments around the lipoyl moieties in complex isolated from *A. vinelandii*. EPR spectra of the specifically spin labelled complexes from *E. coli* and *A. vinelandii*, when dissolved in Tricine buffer, show interactions of at least two electron spins with each other, which indicate that the lipoyl moieties are rather close together.

Fluorescent label (N-(1-anilino-naphthyl-4) maleimide) is specifically attached to the lipoyl moiety of the high mol. wt. transacetylase of the freshly isolated complex from *A. vinelandii*. From the large differences in the apparent lifetimes τ_p and τ_m, as detected by phase fluorometry, it is shown that this fluorescent label is distributed in different micro-environments. The differences observed in energy transfer between fluorescent label, attached to the lipoyl moiety of the high mol. wt. transacetylase, indicate different conformations of the complex from *A. vinelandii*. Upon introduction of the label after reduction with NADH a much larger energy transfer, thus a shorter distance is observed between the label and FAD than when reduction is performed with Mg^{2+}, TPP and pyruvate. A similar conformation dependence upon reduction is found for

the pyruvate dehydrogenase complex from *E. coli*. It is thus proposed that the transacetylase of *E. coli* and the high mol. wt. transacetylase of *A. vinelandii* are both non-symmetrically distributed within the complex.

INTRODUCTION

Earlier studies from this laboratory on the flavoprotein lipoamide dehydrogenase from pig heart |1| showed that this dimeric enzyme containing identical peptide chains has different fluorescence lifetimes of its two FAD molecules. This indicates that the two prosthetic groups are in non-equivalent surroundings. The lower symmetry can arise from mutual interaction of the FAD molecules and is then of local nature. It is also possible that the symmetry of the enzyme peptide chains is affected by dimerization. This could be induced by the separation of lipoamide dehydrogenase from the complex. Therefore studies on the intact complex were performed. For this purpose the fluorescence lifetimes were measured of the pyruvate dehydrogenase complexes from pig heart and *A. vinelandii* at two temperatures. It appeared |2| that the lipoamide dehydrogenase from pig heart in the intact complex has also two non-equivalent FAD groups. This proves that the observed asymmetry is not induced during the purification procedure. The observation that the complex from *A. vinelandii* did not show this asymmetry of the FAD groups |2| indicated that the non-equivalence is perhaps typical for eucaryotic species.

In the present studies properties of the lipoamide dehydrogenase in the pyruvate dehydrogenase complexes from *A. vinelandii* and *E. coli* were compared with those of the isolated enzymes from the same sources. It is shown here that in the *E. coli* complex the FAD groups of lipoamide dehydrogenase are also non-equivalent. It is obvious that these results will require reanalysis of the structural model of the pyruvate dehydrogenase complex expressed by Reed |3|, who concluded from electron microscope studies that the *E. coli* complex consists of symmetric aggregates of the different component enzymes.

To further investigate the symmetry relations of the pyruvate dehydrogenase complexes from *E. coli* and *A. vinelandii* spin labels and fluorescent labels were used. These labels are covalently bound to the lipoyl group of the transacetylase components of these complexes. Such a comparative study is of interest in view of the fact that major structural differences exist between the two pyruvate dehydrogenase complexes, *i.e.* there are two different transacetylase components in the *A. vinelandii* complex |4| and there is one such a component in the *E. coli* complex |3| as well as the much lower mol. wt. and FAD-content of the *A. vinelandii* complex.

MATERIALS AND METHODS

The pyruvate dehydrogenase complex from *A. vinelandii* was isolated as described before |5|. The specific activity of the complex was 8-10 μmol NADH $min^{-1}.mg^{-1}$. The complex from *E. coli* was a gift from Dr. Lester J. Reed, Clayton Foundation Biochemical Institute, Austin, Texas and had a specific activity of about 25 μmol $min^{-1}.mg^{-1}$. The lipoamide dehydrogenase from *A. vinelandii* was isolated according to Van den Broek |6|; the enzyme from *E. coli* was a gift from Dr. Charles H. Williams, Veterans Administration Hospital, Ann Harbor, Michigan.

The enzyme and the complexes were dissolved in 50 mM phosphate buffer pH 7.0, unless otherwise stated. Prior to labelling the complex was treated with 1 mM of N-ethylmaleïmide as described previously |4|. Sodium dodecylsulphate gels without staining were used to determine the position of covalently-bound fluorescent label. Gel electrophoresis was performed by the method of Laemmly |7| omitting 2-mercapto ethanol. N-(1-anilino-naphtyl-4) maleïmide was synthesized according to Kamaoka *e.a.* |8|, but ring closure was done at 120°C |9|. Tricine (Sigma) and other chemicals were used as described earlier |4|.

EPR measurements were carried out on a Varian E-3 spectrometer. Sample temperature was controlled with a Varian Variable Temperature Accessory V-4557 within an accuracy of 1°C. Fluorescence spectra were recorded on a Hitachi Perkin Elmer MPF-2A spectrometer. Fluorescence lifetimes were measured on a phase fluorometer, operating at 60 MHz and built analogues to Spencer and Weber |10|. The modulation was measured after separate amplification of both the DC- and AC-signals obtained from the photomultiplier and measuring these on a DANA model 5403 digital voltmeter operating in the ratio mode. A full description of the instrument will be given elsewhere |11|. The instrument has an accuracy of 2% in the phase measurement and 3% upon measurement of the modulation. The excitation light was obtained from a 150 W-Xe source, connected to a Jarrell Ash grating monochromator. Broad band filters (half width 50 nm) were used to isolate the label and flavin fluorescence (Balzer K45, λ_{max} = 450 nm resp. K4, λ_{max} = 550 nm). The cuvet holders were connected to a Lauda kryostat, maintaining temperature control within 0.5°C.

RESULTS AND DISCUSSION

Flavin fluorescence Phase fluorometry was used to detect differences in the micro-environments of the flavin prosthetic groups, bound to the lipoamide dehydrogenase in the complexes isolated from *E. coli* and *A. vinelandii*, as well as

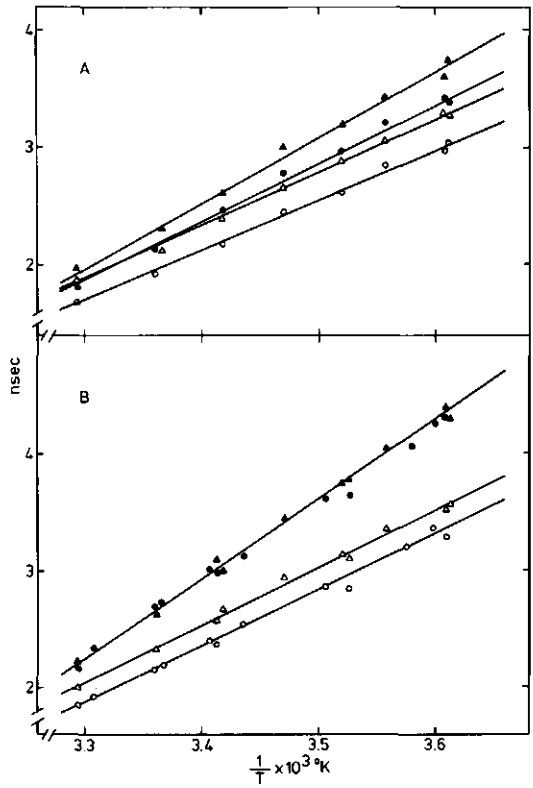

Fig. 1 TEMPERATURE DEPENDENCE OF FLUORESCENCE LIFETIMES OF BOUND FAD. A, isolated from *A. vinelandii*; B, isolated from *E. coli*. (▲-▲), τ_m; (Δ-Δ), τ_p of free lipoamide dehydrogenase. (●-●), τ_m; (o-o), τ_p of pyruvate dehydrogenase complex. The enzymes and complexes are dissolved in 50 mM phosphate buffer pH 7.0.

in the free enzymes. The flavin was excited (at 440 nm) by modulated light. The emitted light is phase-shifted as well as diminished in modulation, both relative to the incident light. From these two parameters apparent lifetimes can be calculated. When only one fluorescent species is present (one lifetime), the lifetime from the phase shift (τ_p) and that from the modulation (τ_m) are identical and reflect the real lifetime. When more than one fluorescent species (different lifetimes) are present $\tau_p < \tau_m$ and are not identical with the real lifetimes [12, 13]. In Fig. 1 these "lifetimes" are presented for the pyruvate dehydrogenase complexes and lipoamide dehydrogenases of *A. vinelandii* and *E. coli*. To ascertain that a mixture of different temperature-dependent conformations was not present, the measurements were made at different temperatures. Also at least two series of measurements on two different enzyme (-complex) preparations were made. The reason to give these data (Fig. 1) in the form of an Arrhenius plot is not directly apparent. For a fluorescence lifetime it is expected that temperature-dependent dynamic processes (solvent interaction etc.) will affect the decay

in such a dependence. As can be concluded from Fig. 1 τ_p and τ_m behave the same and thus mainly dynamic processes are responsible here. From the difference in values it follows, that more than one lifetime, and thus non-equivalently bound flavin molecules are present in the *E. coli* complex and enzyme. This difference, however, is much smaller for the complex and enzyme from *A. vinelandii* and is here in fact so small, that it indicates within the experimental limits about identical surroundings. Of interest is the observation that also the FADs of the *E. coli* complex and enzymes show a tendency to become identical above 30°C. No evidence for a conformational transition is apparent with this method.

Spin label studies Maleïmide spin label (N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl) maleïmide) can be covalently attached to both the complexes from *E. coli* and *A. vinelandii* as already has been reported for *A. vinelandii* |4| and recently by Ambrose *et al.* |14|, in a similar way for the *E. coli* complex. To clarify the observed discrepancy between relative correlation times, calculated directly from the observed linewidth by the method of Stone |15| and those obtained from signal amplitude, as mentioned earlier |4|, the viscosity of a solution containing such spin labelled complex (*A. vinelandii*) was varied. The viscosity was altered by adding glycerol up to 30% and varying the temperature. From these spectra it is clear that at higher viscosity more than one type of spectrum is visible instead of one as was assumed before. This is illustrated in Fig. 2, for the two specifically labelled complexes of *A. vinelandii*, i.e. via pyruvate dehydrogenase (Mg^{2+}, TPP, pyruvate) and via lipoamide dehydrogenase (NADH). Fig. 2A shows a spin label spectrum belonging to a complex labelled via pyruvate dehydrogenase in 30% glycerol. As expected only broadening by the higher macroscopic viscosity relative to the spectrum presented in Grande *et al.* |4| is visible, but at -3°C (Fig. 2A) clearly at least two kinds of species with different correlation times are visible. With the complex labelled via lipoamide dehydrogenase this is already the case at 16.5° (Fig. 2B) and more clearly at lower temperatures. At -21.5°C (Fig. 2A) resp. -16.5 (Fig. 2B) the different types become again almost indistinguishable, because the more mobile species becomes also immobilized and the differences thus much less apparent.

The values derived from these studies are further summarized in Fig. 3. The curves of this figure are obtained by calculating with the formulas derived by Stone |15| from the line width of the +1 line and the -1 line (Fig. 2A, - 3°C) the correlation time for the mobile species. From the distances, indicated in Fig. 2A by a and b, the correlation time of the relative immobile species is obtained by the semi-empirical method of Mensch and Meier |16|. This method correlates such

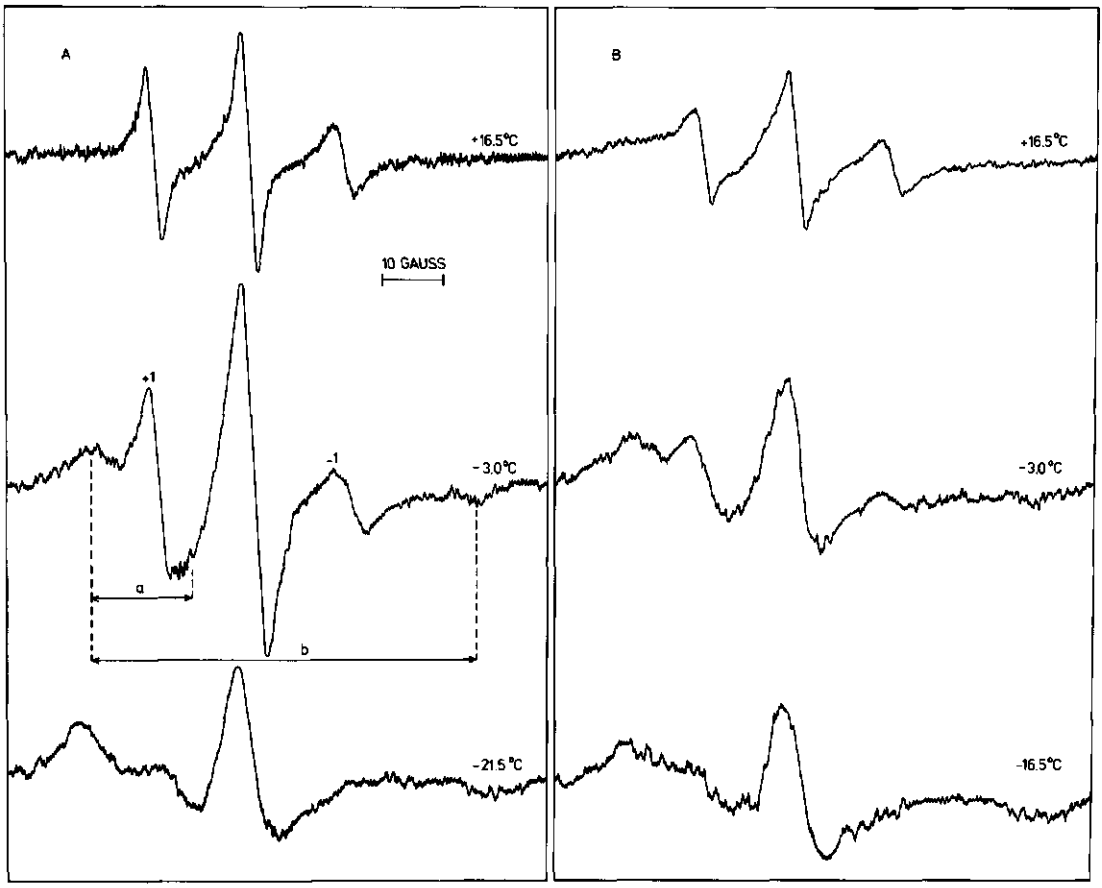

Fig. 2 SPECTRA OF SPIN LABEL BOUND TO PYRUVATE DEHYDROGENASE COMPLEX FROM *A. VINELANDII* IN A 30% GLYCEROL-WATER MIXTURE.
A, specifically labelled via pyruvate dehydrogenase (Mg^{2+}, TPP, pyruvate). The concentration is 8 µM with respect to FAD. B, specifically labelled via lipoamide dehydrogenase (NADH). The concentration is 6 µM with respect to FAD. Instrumental settings at 16.5°C: gain 5×10^5, modulation amplitude 2G; power 50 mW. A, 4 min. scan, time constant 0.3 s; B, 8 min. scan, time constant 1.0 s. All other spectra were recorded at gain 2×10^6, modulation amplitude 1.25 G, power 50 mW, scan time 8 min., time constant 1.0 s.

distances obtained from observed spectra of free spin label in glycerol/H_2O mixtures at different temperatures with rotational correlation times as calculated from the known viscosity. The thus obtained difference between the correlation times calculated from the +1 line and the -1 line (the o-line is due to overlapping of all species in the centre, not suited for calculation) indicates aniso-

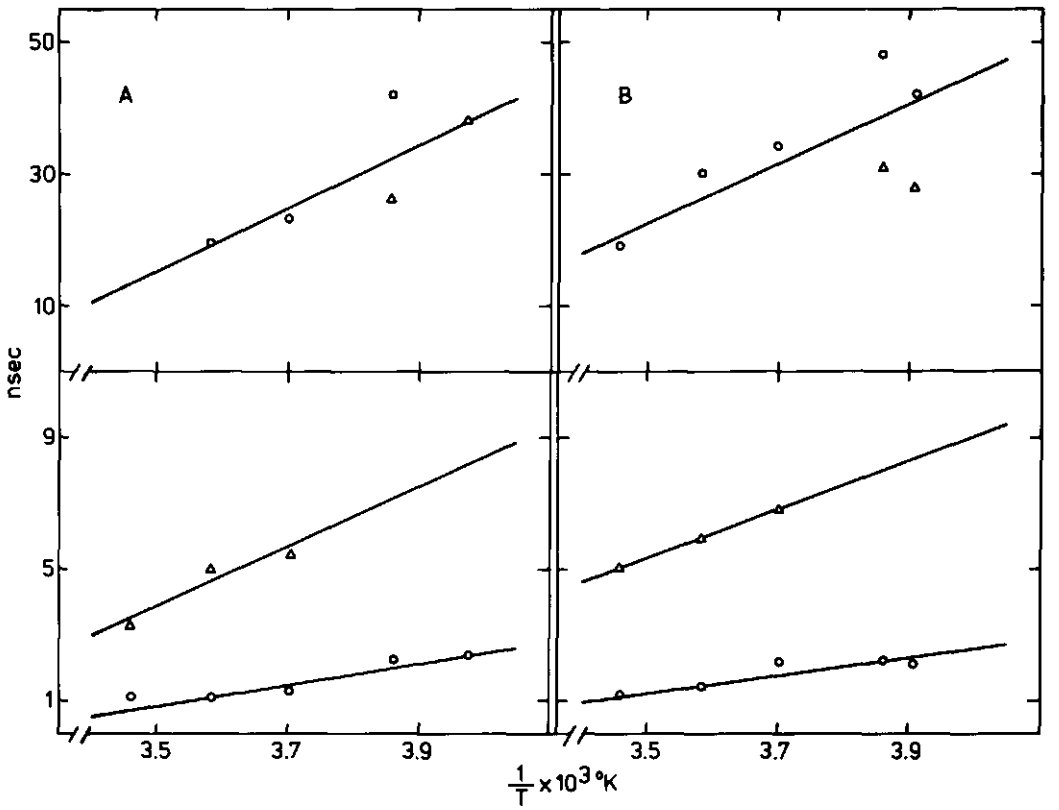

Fig. 3 TEMPERATURE DEPENDENCE OF THE CORRELATION TIMES OF SPIN LABEL BOUND TO PYRUVATE DEHYDROGENASE COMPLEX FROM *A. VINELANDII*.
A and B, see Fig. 2.
Upper curves: Δ, derived from a, o, derived from b. Lower curves; o, derived from +1, Δ, derived from -1. Method of calculation as mentioned in the text.

tropy within one species although even the examples given by Stone [15] show already this kind of deviation. Such an anisotropy seems to be less for the less mobile species. The relatively large spread in the curves of Fig. 3 is due to overlap of the spectra, thus giving rise to an uncertainty in linewidth and distance (a and b) estimated from the spectra. The results show clearly that at least two species are present in 30% glycerol. A mobile one, which is in itself somewhat anisotropic and a less mobile species. The presence of the two species could be induced by a conformational change (by viscosity and/or temperature) or to different surroundings of the lipoyl moieties intrinsically present in the *A. vinelandii* complex. The first possibility can be ruled out because of the

linear relationships between correlation times and the reciprocal temperature (temperature range about 40°C). The presence of different surroundings is more likely and could be related with the presence of two transacetylase species within this complex. On the other hand the fluorescent label is specifically bound to the transacetylase species with the largest mol. wt. in a non-homogeneous way, as indicated by different lifetimes. This implies that the lipoyl moieties of the transacetylase component are distributed itself in different environments, which can also explain the two spin label species observed. Estimates from the data of Fig. 3A indicate that the two species have correlation times in buffer (viscosity 1.002 centipoise) of less than 0.3 ns and about 5 ns. While the same estimate from Fig. 3B leads to a much more anisotropic mobile species relative to 3A with correlation times of resp.0.2 ns (+1 line) and 2 ns (-1 line) and an immobile species with a correlation time of about 9 ns. The fact that the "immobile" species is rotating relatively fast (5 resp. 9 ns) in buffer at room temperature implies strong overlap of the EPR spectra, thus explaining the peculiar line shape of the spectrum reported previously |4|. Differences in amounts present of the different species explain the small differences observed in the spectra of the complex labelled via different sites (*cf.* Fig. 2A and 2B). The observation (Fig. 4A) that a similar peculiar line shape is present in the *E. coli* complex (*cf.* ref. |14|) is as judged from the results reported below, due to a similar phenomenon, despite the fact that the *E. coli* complex contains one transacetylase component.

It was reported |17| that phosphate has a stimulating effect on the activity of the *A. vinelandii* complex in Tris-HCl buffer. In addition a stabilizing effect by phosphate relative to Tris-HCl with respect to denaturation and inactivation is observed. Tricine, which does not stimulate the activity, does show a stabilizing effect. In order to study the influence of phosphate on the conformation of the complex, Tricine buffer was used instead of Tris-HCl buffer.

After dialysis against Tricine buffer (pH 7.0) the pyruvate dehydrogenase complex isolated from *E. coli* was labelled specifically by the same procedure as described for the complex isolated from *A. vinelandii* |4|. The complex is inactivated in less than 10 minutes upon incubation with Mg^{2+}, TPP, pyruvate and excess of spin label as judged by the disappearance of the overall activity. The EPR spectrum obtained from the labelled complex is shown in Fig. 4B which is completely different from the spectrum obtained in phosphate buffer (Fig. 4A). To assure that no new radicals were formed, the complex in Tricine was unfolded with 1% sodium dodecylsulphate and 0.2 M NaOH, resulting in the spectrum shown in Fig. 4C. The spectrum shown in Fig. 4B is not found instantaneously in all cases.

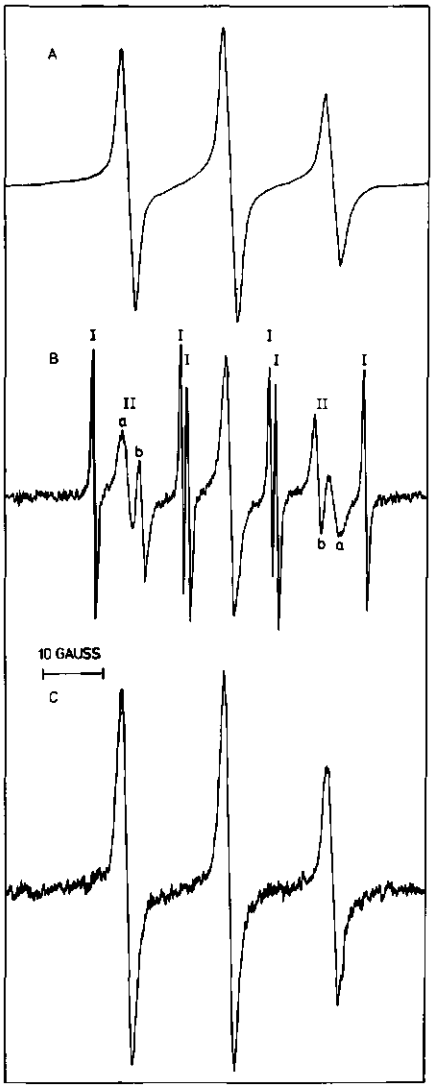

Fig. 4 SPECTRA OF SPIN LABEL BOUND TO PYRUVATE DEHYDROGENASE COMPLEX FROM *E. COLI* IN DIFFERENT BUFFERS. A, phosphate buffer pH 7.0, concentration 4.4 µM with respect to FAD (gain 3.2×10^5, modulation amplitude 2.0 G); B, Tricine buffer pH 7.0, concentration 4.0 µM with respect to FAD (gain 6.2×10^5, modulation amplitude 0.32 G); C, as B, but in the presence of sodium dodecylsulphate (1%) and NaOH (0.2 M), concentration 2.2 µM with respect to FAD (gain 1×10^6, modulation amplitude 2.0 G). The complex was specifically labelled via pyruvate dehydrogenase (Mg^{2+}, TPP, pyruvate).

With the *E. coli* complex in Tricine this new type of spectrum is always present after warming up to 21°C in the EPR cavity. Freezing and thawing of the complex either before or after labelling enlarges the amplitude of the spectrum shown. The *A. vinelandii* complex shows also this type of spectrum in Tricine buffer to some extent, although normally such a spectrum is not completely obtained even after 5 times freezing and thawing and 2 hours at 21°C. Most remarkable is, however, the observation that a complex showing this spectrum in Tricine shows, after dialysis against phosphate buffer again, the normal three-line spectrum.

Also the reverse is true, *i.e.* the complex which is labelled in phosphate buffer shows after dialysis against Tricine buffer also the spectrum of Fig. 4B. The spectrum of Fig. 4B is due to complex which has a different conformation than freshly prepared complex in phosphate buffer. The overall activity of a complex, non-specifically labelled with N-ethylmaleïmide, treated in an analogous way, shows however still more than 50% of the original activity. The spectrum itself is very difficult to explain. It can be described as consisting of at least two types of spectra. One type (I), which has a very narrow linewidth, consists of two series of three lines, with a nitrogen hyperfine coupling of 14.6 G, while the series are separated by 15.6 G and centred around the central g-value. The other type (IIa and b) appears also as two series of three lines with different nitrogen hyperfine coupling centred around the common g-value. The apparent relatively broad (IIa) and narrow lines (IIb) can, however, be ascribed according to G.R. Luckhurst |18| as resulting from very weak interactions between two spins ($a/J >> 1$, *cf.* ref. |18| p. 157). An alternative could be that the relatively broad lines (IIa) reflect the original spectrum in phosphate buffer. This type will always be present to some extent depending on the conditions already mentioned and on the origin of the complex (*e.g. A. vinelandii*). The relatively narrow lines (IIb) have in most cases the same nitrogen hyperfine coupling as the first type (I). The linewidths of these species, however, do not match, *i.e.* about 0.45 G for the first type (I) and around 0.9 G for the second (IIb). In both cases, however, the linewidth is in fact much smaller than that of a nitroxide radical, rotating freely in an aqueous solution (1.32 G at room temperature). This excludes therefore all interactions which are not based on some kind of exchange narrowing, either with an other electron or with a nuclear spin. An exchange-narrowed spectrum of a nitroxide with a I=1 nucleus, leads to three series of three lines, all with the same linewidth. In case of additional asymmetric linebroadening an increase in linewidth upon going to high-field is expected |18|. The presence of a relatively broad series (0.9 G) only in the centre is thus not in accordance with these assumptions. The explanation of the presence of IIa and IIb lines as arising from weak spin-spin interaction seems therefore reasonable.

Even so, one is left with the difficulty of explaining the presence of the series of narrow type I lines, clearly consisting of doublet-split triplets. Accepting the presence of weak spin-spin interaction, one could visualize also the presence of strong spin-spin coupling. Thus a triplet-type or biradical type of spectrum could be expected |18|, both leading to a too large number of lines.

The outer peaks of a triplet spectrum separated by 2D (*cf.* notation according to Luckhurst |18|) are normally not intensive. In case these lines are broadened by exchange, only the innerlines, separated by respectively D+3E and D-3E (*cf.* ref. |18|), should be observed. These two lines can be converted into one line (split into a triplet, due to N-hyperfine coupling), on both sides of the centre by the rapid rotation of the nitroxyl groups around their own long lipoyl arm. Such a situation could be visualized by projecting the direction of each electron spin on two axes, one parallel with and one perpendicular on the lipoyl arm. This arm itself is rotating much slower than the direction perpendicular to it on which also the electron spin is projected. The directions of the spins along the long arm can be projected on a common axis, while in the directions perpendicular to the arms no common axis can be defined due to rapid rotation. Part of the linewidth is determined by hyperfine coupling with the CH_3-proton of the nitroxide group. Projection (thus a decrease) of these couplings on the common axis leads to linenarrowing. This could explain the doublet of three narrow lines. However, since the common axis is fixed to the complex, which itself is slowly rotating, a powderlike spectrum must be expected, which has an asymmetrical line shape. Since also no triplet signal at half-field is present this explanation must be rejected. The only way to prevent the presence of a powder-type spectrum is rapid joint-movement of the two spins, a movement much more rapid than the tumbling of the complex. In view of the existence of the transacetylase core in the complex (*cf.* |3|), this possibility cannot be excluded. Another explanation could be found by assuming a rapid movement from an environment in which a strong proton (or another nucleus with $I=\frac{1}{2}$) interaction exists to this proton interaction. This could be for example visualized by a rapid rotation of one electron spin around the lipoyl arm. During this rotation the elctron passes a fixed single proton. In addition explanations in which interactions between more spins are taken into account cannot be excluded on the basis of these observations.

The following clearly demonstrates that spin-spin interaction is more likely than interaction of a single spin with a nucleus. Pyruvate dehydrogenase complex from *E. coli* in Tricine, aspecifically labelled with N-ehtylmaleimide, was incubated with a 15-fold excess spin label. FAD (10 μM) and EDTA (100 μM) were added and the mixture was photoreduced at 5°C in front of a 150 W Xenon-lamp. Within 4 min. no remaining EPR signal could be detected. To this mixture 1/3 of the amount of the spin label present before photoreduction, of not reduced spin label was added together with Mg^{2+}, TPP and pyruvate. After 6 min. no overall activity was present, similarly as in a control experiment in which the total amount of spin label was not subjected to photoreduction. In the latter experiment the sig-

nal as shown in Fig. 4B was obtained, while in the case of the 1:3 ratio spin label/photoreduced label only a slight amount of this signal is visible together with the normal 3-line spectrum. Such a result is expected in view of the fact that in this case, when electron spin electron spin interaction is responsible for the phenomenon of Fig. 4B, the size of this signal must decline to at least 1/16 of the original size.

In summary: from the experiments with spin labels reported here it appears that in the pyruvate dehydrogenase complexes isolated from *A. vinelandii* and *E. coli* the transacetylase bound lipoyl moieties are present in at least two different environments.

Fluorescence label studies As reported for the complex isolated from *A. vinelandii* |4| N-(1-anilino-naphtyl-4) maleïmide is covalently attached to both the high and low mol. wt. transacetylases upon specific reaction in the presence of Mg^{2+}, TPP, pyruvate or of NADH. Recent studies in our laboratory showed that with the freshly prepared complex fluorescence is found almost exclusively on the high mol. wt. enzyme. A reason for this apparent discrepancy is perhaps the freezing and thawing of the enzyme complex. Indeed such a treatment, repeated five times, resulted in a complex which showed pronounced fluorescence, after specific labelling, on both the transacetylases, but also to some extent on the other enzymes. Control experiments revealed that this freezed-thawed complex is also inactivated by the fluorescent label in the presence of oxygen, Mg^{2+} and TPP, although at a rate much slower than that upon specific inactivation. Fluorescence is then found most pronounced on the pyruvate dehydrogenase component. More detailed studies are in progress to characterize these secondary changes, which do not affect the overall activity. In order to obtain specific incorporation of the fluorescent label complexes which were stored in liquid nitrogen, were used. The complexes stored in this way behave as freshly prepared enzyme which had not been frozen at all. The excitation spectrum for flavin emission at 520 nm of the pyruvate dehydrogenase complex isolated from *E. coli* is shown in Fig. 5A. The spectra given are uncorrected for lamp output or photomultiplier sensitivity. The FAD concentrations were in the order of 3 μM. In order to correct for small variations in concentration and making the samples directly comparable, the flavin emission at 520 nm (λ exc = 467 nm) was amplified to the same recorder deflection; the fluorescent label has no absorption at 467 nm. The emission maximum of the fluorescent label bound to the protein is shifted to 440 nm, as compared with 470 nm of label bound to cysteine in buffer (pH 7.0),

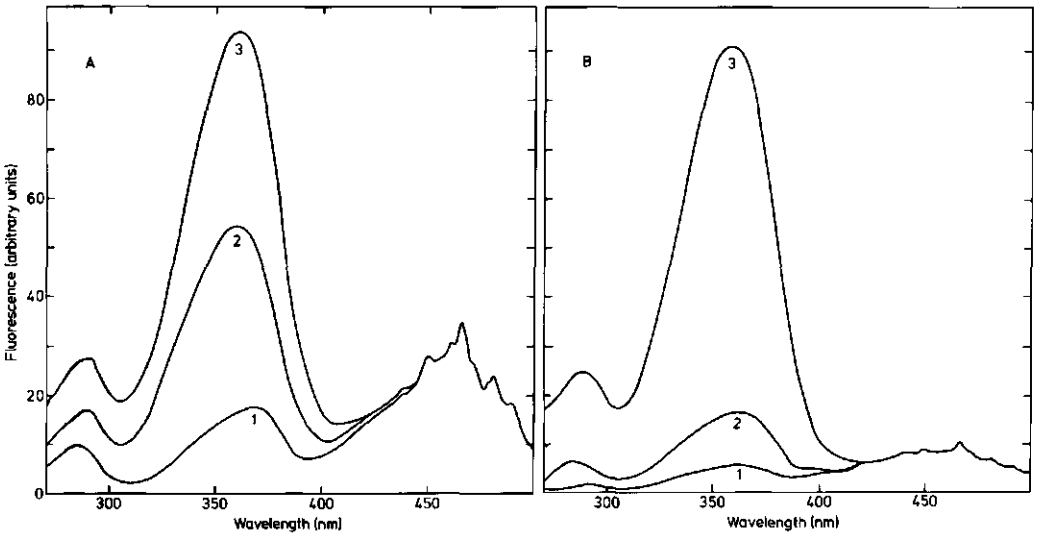

Fig. 5 EXCITATION SPECTRA OF PYRUVATE DEHYDROGENASE COMPLEXES LABELLED WITH N-(1-ANILINO-NAPHTYL-4) MALEIMIDE.
A, isolated from *E. coli*; B, isolated from *A. vinelandii*. Curve 1, N-ethylmaleimide prelabelled complex; Curve 2, specifically labelled via pyruvate dehydrogenase (Mg^{2+}, TPP, pyruvate); Curve 3, specifically labelled via lipoamide dehydrogenase (NADH). The complex is dissolved in 50 mM phosphate buffer pH 7.0, the concentration is about 3 μM with respect to FAD (see text), emission wavelength 520 nm.

which indicates a rather apolar environment. Although the label-fluorescence emission maximum at 440 nm is not shifted for the different labelled complexes its fluorescence intensity is higher by incorporation via lipoamide dehydrogenase (NADH) than via pyruvate dehydrogenase (Mg^{2+}, TPP, pyruvate). Part of the fluorescence emission intensity at 520 nm (λ_{exc} = 360 nm) can be ascribed to a contribution of the tailing of this label fluorescence. Reduction of the FAD of the complex labelled via pyruvate dehydrogenase, with excess dithionite results in a 70-80% decrease, of the signal at 520 nm (excitation at 360 nm). The differences shown in Fig. 5 are, however, so significant that it is apparent that different ways of incorporating the label lead to large differences in spectral properties. The complex isolated from *E. coli* contains just one type of transacetylase, which is labelled via both ways as shown by gel electrophoresis. On the other hand, with the complex from *A. vinelandii* only the high mol. wt. transacetylase is labelled, a rather unexpected result in view of the fact that our spin label results suggest labelling of both transacetylases, at

least if incorporated via pyruvate |4|. The spin label experiments showed that via lipoamide dehydrogenase always less label is incorporated than via pyruvate dehydrogenase.

It is therefore rather unlikely that the large difference in 520 nm emission obtained by 360 nm excitation can be explained by the eventual difference in amount of label incorporated. The spin label experiments |4| argue against this. An obvious conclusion is that in both the complexes from *E. coli* and *A. vinelandii* the conformation induced, depends on the way the label is introduced. This can be due to different micro-environments and thus different quantum yields. In addition it is possible that energy transfer occurs from the bound label to the FAD of lipoamide dehydrogenase, the amount depending on the conformation of the complex. The fact that the largely enhanced (360 nm-excited) 520 nm emission declines, upon reduction with $Na_2S_2O_4$ indicates that this emission is mainly enhanced label-sensitized flavin emission and little residual label emission. It is of interest to mention that especially with the *A. vinelandii* complex labelled via lipoamide dehydrogenase considerably less fluorescence is observed at 520 nm after freezing and thawing. In view of the large overlap between emission of label and absorption of FAD, a fraction of the label molecules located close to the FAD could effectively transfer excitation energy and lead to the spectrum observed. In case only a difference in quantum yield of the label is responsible for the major part of the differences observed, depending on the way the label is incorporated, it can be expected that, apart from the possibility of a shift in fluorescence maximum, the fluorescence lifetimes of the label will depend on the way of its incorporation. None of these are observed, as already stated for the shift of the fluorescence maximum and are illustrated for the lifetimes in Table 1. It is apparent from this Table that the lifetimes measured do not belong

Table 1 FLUORESCENCE LIFETIME OF N-(1-ANILINO-NAPHTHYL-4) MALEIMIDE SPECIFICALLY INCORPORATED IN PYRUVATE DEHYDROGENASE COMPLEXES

Source	Incorporation via	τ_p^a ns	τ_m^a
E. coli	NADH	2.3	4.1
	Mg^{2+}, TPP, pyruvate	2.1	3.6
A. vinelandii	NADH	2.7	4.7
	Mg^{2+}, TPP, pyruvate	2.2	4.4

a Excitation at 360 nm, emission separated by a Balzer K45 (λ_{max} = 450) filter. The temperature was maintained at 21.5°C.

to one fluorescent species. At least one species with a relatively short fluorescence lifetime and one with a longer lifetime must be present. One could argue whether a small amount of FAD-emission passing through the filter could influence this interpretation. In fact a control experiment in which lifetimes were measured before and after reduction of the flavin with $Na_2S_2O_4$ reject this by showing an even larger difference between τ_p and τ_m (label introduced via pyruvate dehydrogenase before reduction τ_p = 4.3 ns, τ_m = 5.3 ns; after reduction τ_p = 4.4 ns, τ_m = 6.7 ns, temp. 11°C). Reduction of FAD shifts its absorption to shorter wavelengths, removing the possiblity of energy transfer from the fluorescent label. The increase in lifetime of this label is in accordance with this observation.

The differences in quantum yield observed, if not due to different amounts of fluorescent label, are at first glance difficult to explain with the data of Table 1. The difference between both τ_p and τ_m of the label incorporated via the two methods is not large, but consistent. It is possible that the lower values of τ_p and τ_m in the case of incorporation via pyruvate is due to the presence of the acetyl groups on the lipoyl moieties. The presence of the acetyl groups could influence the decay of the excited state and thus the quantum yield. On the other hand, energy transfer will also lead to shorter lifetimes, which will especially be important for the label incorporated via NADH. Energy transfer between label molecules can be expected in view of the spin label results, the latter (spin-spin) interactions occurring at shorter distances. Energy transfer between alike molecules will also lead to a decline in lifetime of part of the molecules and thus anisotropy in the lifetimes; the presence of a transacetylase core makes this process possible |3|.

The data can thus be explained to some extent. Part of the fluorescent molecules are in a rather apolar environment (long τ) and are responsible for most of the fluorescence at 440 nm. The others are short living, due to polar environment and energy transfer. In the presence of acetyl groups the fluorescence lifetimes are shorter and therefore the label fluorescence is lower at 440 nm. In the absence of acetyl groups (NADH) the fluorescence lifetimes are longer, giving rise to a higher fluorescence at 440 nm.

It is clear that due to the fact that the label is incorporated in an anisotropic way, that no quantitative calculations with respect to the distance between FAD and fluorescent label are possible, again it can be derived that both groups must be rather close. This conclusion is supported by the fact that in the presence of spin label bound to the transacetylase some quenching (10-15%) of the fluorescence is observed.

The same reservations with respect to quantitative calculations can be applied to the results on pyruvated dehydrogenase complex from *E. coli* of Hammes and coworkers |19, 20|. Due to the fact that τ_p and τ_m do not reflect real lifetimes, but are (instrumental) "averages" of all lifetimes present, measurement of τ_p alone can lead to erroneous interpretations. The fact that already the FAD groups of pyruvate dehydrogenase complex from *E. coli* have different lifetimes (*cf.* Fig. 1) will result in at least two ways of energytransfer and thus non-identical τ_p and τ_m. Also the possibility of non-homogeneous binding of thiochrome diphosphate |19| or 8-anilino-1-naphthalene-sulphonate |20| will further complicate the interpretations.

It is clear that the pyruvate dehydrogenase complexes isolated from *A. vinelandii* and *E. coli* are indeed differently organized in terms of stoethiometry, mol. wts., asymmetry of the two FAD environments of lipoamide dehydrogenase and the presence of one or two transacetylases, but are similar in other aspects. The label experiments indicate that in both complexes the lipoyl moieties of the transacetylase components are distributed in the complex in a non-symmetrical way. Structural changes which depend on the way of incorporating the label can be induced. The similarity of the spin label spectra observed with both complexes in Tricine buffer points toward mutual structural features in the aggregation of the core of the transacetylase components independent of the presence of more than one type of this enzyme. It is clear that, if analysed with the techniques used in these studies, the symmetry of the core complex must be lower than previously derived from electron microscopy |3|. The function of low mol. wt. transacetylase in *A. vinelandii* has not become clear from these experiments. Topological experiments with bifunctional reagents |21| indicate that this component is located near the pyruvate dehydrogenase component, while the high mol. wt. enzyme is located close to the lipoamide dehydrogenase. Our fluorescence studies support the view that the FAD group of lipoamide dehydrogenase and the lipoyl moieties are not too far apart, as indicated by the occurrence of energy transfer. The fact that with freshly prepared complex no detectable fluorescence is found on the low mol. wt. transacetylase, is in accordance with our previous results with 2-^{14}C pyruvate. This could imply that the low mol. wt. form acts as acetyl transferring enzyme between the pyruvate dehydrogenase and the high mol. wt. transacetylase, at least in fresh preparations. The lower activity of the *A. vinelandii* complex compared to the *E. coli* complex thus could be connected by this step. It is of interest to mention in this context, that our kinetic studies support the view (*cf.* ref. |17|) that the acetyl transfer reaction is rate-limiting and determines the cooperative kinetics. The results from this

study support this view. In both complexes, however, the lipoyl moieties of the transacetylases must then be non-symmetrically distributed within the complex and have different reactivities, which in turn depend on the local environments, against sulphydryl reagents in order to explain our label results. It is therefore necessary to extend these studies by the use of reagents which are more selective, while also lower reaction temperatures can help to discriminate between the different sites. It is also apparent from the results obtained with N-(1-anilino--naphthyl-4) maleïmide that this probe is not very well suited for this system. The almost identical excitation wavelength as compared with the second absorption band of FAD, the small but substantial overlap in emission with that of FAD, the very large critical transfer distance and the high affinity for sulphydryl groups present, emphasize the need for a more selective probe, which can be used for selective energy transfer in order to perform distance calculations.

ACKNOWLEDGEMENT

We are thankful for valuable discussion to Dr. A. de Kok, Mr. R.J. Platenkamp and to Dr. W.H. Scouten, who also synthesized the N-(1-anilino-naphthyl-4) maleïmide. Furthermore, we want to thank Dr. L.J. Reed for his gift of pyruvate dehydrogenase complex from *E. coli*, Dr. C.H. Williams for his gift of lipoamide dehydrogenase from *E. coli* and Mr. B.J. Sachteleben for the preparation of the Figures.

This study has been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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IV A detailed ^{1}H-Nuclear Magnetic Resonance study on alloxazines and isoalloxazines

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SUMMARY

1 An easy two-step synthesis of isoalloxazines giving high overall yields is described. The procedure is less time-consuming than previously described ones.
2 A series of models in the neutral and cationic form were investigated in detail by ^{1}H-NMR technique. Unequivocal assignment of all resonance signals was achieved by selective deuteration of some compounds and double resonance technique. The experimentally determined coupling constants were verified by computer simulation.
3 Considerable enhancement of the signals due to CH(9) and CH(6) is found upon decoupling of the methyl group at position 8 and 10 and 7, respectively. These results are compared with those obtained with FAD.
4 The methyl resonance signal of the 7-methyl derivatives is split into a doublet due to coupling with CH(6).
5 The difference in chemical shifts observed upon successive introduction of methyl groups into the benzene subnucleus of (iso)alloxazines indicates that the molecule becomes less planar upon methylation.
6 The pyrimidine ring of (iso)alloxazine does not directly contribute to the ring current except via indirect effects through the carbonyl groups.
7 The experimental data are compared with published MO calculations and discussed.

1 INTRODUCTION

Higher molecular weight derivatives of isoalloxazine play an important role as cofactors in many flavoenzyme dependent biological reactions. The large

progress achieved in biochemical research of flavoproteins in the past few years has stimulated many chemists to investigate the chemistry of flavo-coenzymes and low molecular weight derivatives thereof in order to obtain a better insight into the biological function of flavin-dependent enzymes [e.g. 1,2]. On the other hand, to be able to give a full account of the observed chemical properties of isoalloxazine and to predict the chemical interaction with various substrates, it is necessary to know its submolecular structure, i.e. the σ- and π-electron distribution. A few molecular orbital calculations have been performed [3-5]. These results, however, do not agree well with published experimental work. This is not too surprising considering the complexity of the isoalloxazine molecule. There is, therefore, still a great need to study this molecule by different physical methods which can provide some of the needed information. For this reason we have undertaken a detailed investigation of a series of isoalloxazine derivatives employing different physical methods.

In this paper we report on the ^{1}H-NMR properties of a series of isoalloxazine derivatives and their isomers (alloxazines). The aim of this study was threefold: 1) to characterize these molecules in detail by this technique; 2) to prepare the base for the initiated NMR study on flavoproteins; 3) to correlate the obtained results with theoretical calculations.

2 MATERIALS AND METHODS

The NMR spectra were obtained on a Varian high resolution XL-100 NMR spectrometer. The Fourier transform technique was used to acquire all spectra. Usually the following instrumental conditions were employed: pulse width 20 μsec (90° pulse equals 28 μsec), acquisition time 4 sec., without delay. Most spectra were run at 1000 Hz spectral width, with peakpositions being determined from the computer-generated printout, using TMS as internal standard. The proton magnetic resonance probe temperature was 27 ± 1°C. All samples were dissolved in deuterated CH_3CN (Merck A.G., Darmstadt). The solvent contained 1% TMS. Solvent deuterium was used as an internal lock. The concentration of the samples varied between 5 - 15 mM depending on the solubility of a particular compound. For each compound at least two spectra were recorded from independent samples. The agreement among the different spectra was within 0.5 Hz. Some spectra were recorded on a Bruker HX-360 MHz instrument at the University of Groningen, The Netherlands. Double resonance experiments were conducted by continuous irradiation of the signal to be decoupled. To separate the Nuclear Overhauser from the decoupling effect the irradiation frequency was shut off immediately before accumulation and turned on after acquisition for at least 2 sec.

Simulation of the spectra The experimental spectra, where appropriate, have been fitted with the aid of the computer program LAOCOON 3 |6| and the DEC-10 computer system of the Agricultural University. The original program was obtained from Dr. Hollander, University of Leiden, and was rewritten for the DEC-10 computer. The standard deviations of the calculated parameters were better than 0.05 Hz unless otherwise stated. In fitting the experimental spectra special attention was paid to the fact that always good agreement was achieved between the calculated and experimental spectra with respect both to the frequencies and to the intensities of the lines.

Synthesis The alloxazine derivatives (compounds *IX* - *XIV*, Table 2) have been prepared by condensation of the appropriate aniline derivatives with 1,3-dimethyl-4-amino-uracil as described by Goldner *et al.* |7|. 1,3,6,7-Tetramethyl- and 1,3,7-trimethyl-8-trideuteriomethyl-alloxazine were obtained from the corresponding riboflavin-5'-monophosphates |8| which were degraded by periodate to the corresponding 10-formylmethyl-isoalloxazines |9|. Further oxidation of the compounds thus obtained by the method of Müller and Dudley |10| and alkylation of the products yielded the desired alloxazine derivatives.

The isoalloxazine derivatives (compounds *I* - *VIII*, Table 2) were synthesized following virtually the procedure of Kuhn and Weygand |11| except the preparation of compounds *2*. The needed N-methyl-o-nitro-aniline derivatives *2* were obtained from the corresponding commercially available o-nitro-aniline derivatives *1* analogous to the procedure described by Halasz |12|. The general procedure is as follows: to 1.0 g of *1* (e.g. R_1=R_4=H, R_2=R_3=CH_3) dissolved in 10 ml conc. H_2SO_4 10 ml formaldehyde (37%) was added slowly under stirring during a period of 45 min at 60-70°C. Thereafter the reaction mixture was kept at the same temperature for an additional time which varies between 1-5 hrs depending on the derivative of *1* employed. The reaction can easily be followed by thin layer chromatography by dilution of a sample of the reaction mixture with H_2O and extraction with $CHCl_3$. The chromatogram was developed in isopropylether. The starting material exhibits a yellow colour whereas the product is orange coloured. The product moves ahead of the starting material. After completion of the reaction the mixture was cooled to room temperature and poured on 200 ml ice-water. The precipitate was filtered and washed with H_2O. The filtrate was extracted with $CHCl_3$, the organic phase dried with Na_2SO_4, filtered and evaporated. Crystallization of the combined product from ethanol-H_2O yields red crystals. The yield of *2* (R_1=R_4=H, R_2=R_3=CH_3) was 1.0 g (~ 92% of theory).

The starting materials *1*, where R_1=R_2=R_3=CH_3, R_4=H, and R_1=H, R_2=R_3=R_4=CH_3, were prepared according to Dolinsky *et al.* |13|. The compounds *2* (R_1=R_2=R_3=R_4=H; R_1=R_3=R_4=H, R_2=CH_3) were prepared from the corresponding *1* by acetylation followed by methylation in dimethylformamide in the presence of methyliodide and K_2CO_3 during 2-3 days at room temperature. Hydrolysis of the isolated products at 80°C for 12 hrs in an ethanol-2M NaOH mixture (1:1, by vol) afforded the desired compounds.

Catalytic reduction of 1.0 g of *2* (e.g. R_1=R_4=H, R_2=R_3=CH_3) in 20 ml glacial acetic acid in the presence of Pd/C yields the corresponding phenylenediamine derivative. The colourless solution was filtered into an Erlenmeyer flask containing 10 ml glacial acetic acid, 1.3 g alloxan hydrate and 0.5 g boric acid. Prior to filtration the content of the Erlenmeyer flask was flushed with N_2 for 10 min. The reaction mixture was then kept under N_2 for 12 hrs under stirring. Filtration and extensive washing of the yellow precipitate with ethanol and ether gave 1.25 g of *3* (R_1=R_4=H, R_2=R_3=CH_3) (82% of theory). Methylation of the N(3) position of the isoalloxazine ring was carried out as described elsewhere |14|. 3,7,10-Trimethyl-8-trideuteriomethyl-isoalloxazine was obtained from the corresponding riboflavin-5'-monophosphate analog |8| by periodate degradation |9| and treatment of the compound thus obtained by 0.1 M NaOH for a period of 1 hr at room temperature. Isolation by acidification and methylation of the product led to the desired compound. 9-Deuterio-3,7,10-trimethyl- and 9-deuterio-3,7,8,10-tetramethyl-isoalloxazine were synthesized from the corresponding aniline derivatives as described elsewhere |8|.

Analytically pure alloxazine and isoalloxazine derivatives were obtained by column chromatography. Excellent results were obtained using "Kieselgel Mallinckrodt", 100 mesh (product of Serva, Heidelberg, Germany) as a stationary phase. As mobile phase CH_2Cl_2 was used for the alloxazine and $CHCl_3$ for the alkylated isoalloxazine derivatives. The former compounds were eluted with a mixture of CH_2Cl_2 and $CHCl_3$ and the latter ones with a mixture of $CHCl_3$ and CH_3OH. The composition of the solvent mixtures depends on the compound to be eluted. The purity of the compounds was also checked by thin layer chromatography employing different solvents ($CHCl_3$, CH_3CN, $CHCl_3$ containing 10% methanol).

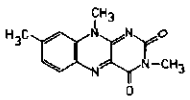
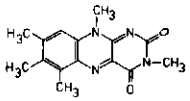
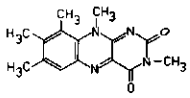
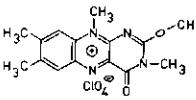
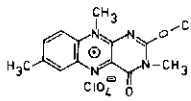
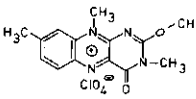
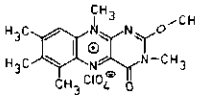
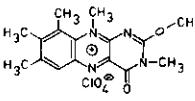
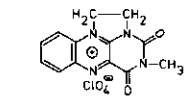
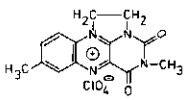
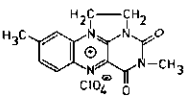
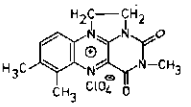
Quarternary isoalloxazines substituted at positions 2α, N(3) or at 2α, 4α (compounds *XXVI* - *XXX*, Table 5) were obtained from the corresponding isoalloxazine derivatives *3*. The procedure followed was that of Dudley and Hemmerich |15| which was, where necessary, modified. Thus, 1.0 g of *3* (e.g.

R_1=R_4=H, R_2=R_3=CH_3) was suspended in a mixture of 20 ml conc. CH_3COOH and $(CH_3CO)_2O$ (1:1, by vol.) and brought to reflux, then small portions of Zn dust were added over a period of 30 min. during which time the yellow solution was bleached almost completely. The reaction mixture was then kept for further 30 min. under reflux during which time occasionally small amounts of Zn dust were added. Filtration, evaporation and suspension of the residue in 25-30 ml H_2O gave 0.80 g of *4* (R_1=R_4=H, R_2=R_3=CH_3). Treatment of 1.0 g of *4* (R_1=R_4=H, R_2=R_3=CH_3) in 25 ml dimethylformamide in the presence of 2.0 g K_2CO_3 with a mixture consisting of 4.5 ml dimethylformamide and 2 ml $(CH_3O)_2SO_2$, which was added slowly over a period of 3 hrs, at 60-70°C during 5 hrs gave a mixture of the corresponding isomers *5* and *6*. The reaction mixture was filtered and evaporated to dryness. The residue was suspended in 25 ml H_2O, the precipitate filtered and air-dried. The filtrate was extracted twice with 20 ml $CHCl_3$, the organic phase dried and concentrated to 10 ml. Addition of isopropylether to the remaining $CHCl_3$ solution and letting it stand at 4°C for 1 day gave another crop of product. The total yield of *5* and *6* was 1.55 g (∿ 70%). It was difficult to separate the isomers *5* and *6* by fractional crystallization as described elsewhere for the diethyl analogs |15|. An easy separation of the isomers was achieved by passing the mixture of the isomers (1.55 g), dissolved in 10 ml CH_2Cl_2, through a Kieselgel column (see above). The isomer *6* was eluted with CH_2Cl_2 whereas the isomer *5* was eluted with $CHCl_3$ containing 1% CH_3OH. Evaporation of the eluates to dryness and recrystallization of the residue from CH_3OH and H_2O gave analytically pure products as judged by thin layer chromatography. 60% of the isolated material consisted of *5* and 40% of *6*. For the chromatography ethylacetate was used as solvent. The spots of the colourless compounds were visualized by exposure of the thin layer plates to iodine vapour. The isomer *6* moved faster than the isomer *5*. The oxidation of *5* and *6* thus obtained to the corresponding quarternary salts *7* and *8* was carried out by dissolving the compounds in CH_3CN, adding a few drops of conc. $HClO_4$ and crystalline $NaNO_2$ until the intermediate orange to red colour had disappeared. Thereafter a few ml of H_2O was added and CH_3CN evaporated under reduced pressure. During this procedure the isoalloxazinium perchlorates crystallized. The crystals were filtered, washed with H_2O and dried with a small volume of ethanol and thoroughly with ether. Recrystallization from CH_3OH/H_2O gave pure products.

The 1,3,10-trimethyl-isoalloxazinium derivatives (compounds *XXII* - *XXV*, Table 5) were synthesized from the corresponding alloxazine derivatives as described by Dudley and Hemmerich |16|.

TABLE 1

ELEMENTAL ANALYSES AND MELTING POINTS OF THE NEWLY SYNTHESIZED FLAVIN DERIVATIVES

Compound	Molecular Formula	Mol. Weight		Elemental Analysis % C	% H	% N	% Cl	M.P. (°C)
	$C_{13}H_{12}N_4O_2$	256.26	calc.	60.93	4.72	21.86	-	281-284
			found	60.61	4.65	21.89	-	
	$C_{15}H_{16}N_4O_2$ [a]	284.31	calc.	63.36	5.67	19.71	-	289-290
			found	63.0	5.6	19.6	-	
	$C_{15}H_{16}N_4O_2$ [a]	284.31	calc.	63.36	5.67	19.71	-	281-283
			found	62.9	5.8	19.3	-	
	$C_{15}H_{17}ClN_4O_6$	384.78	calc.	46.8	4.4	14.56	9.2	221-229 [b]
			found	46.7	4.5	14.6	9.3	
	$C_{14}H_{15}ClN_4O_6$	370.75	calc.	45.3	4.1	15.1	9.56	220-221 [b]
			found	45.2	4.1	15.2	9.7	
	$C_{14}H_{15}ClN_4O_6$	370.75	calc.	45.3	4.1	15.1	9.56	232-240 [b]
			found	45.3	4.2	15.2	9.6	
	$C_{16}H_{19}ClN_4O_6$	398.8	calc.	48.18	4.8	14.05	8.89	> 290
			found	48.0	4.7	14.3	8.7	
	$C_{16}H_{19}ClN_4O_6 \times H_2O$	416.8	calc.	46.20	5.02	13.50	8.55	246-249 [b]
			found	46.4	4.8	13.5	8.6	
	$C_{13}H_{11}ClN_4O_6$	354.71	calc.	44.01	3.13	15.80	10.00	300-302
			found	43.9	3.0	15.8	10.0	
	$C_{14}H_{13}ClN_4O_6$	368.73	calc.	45.59	3.55	15.2	9.62	301-305
			found	45.5	3.5	15.3	9.6	
	$C_{14}H_{13}ClN_4O_6$	368.73	calc.	45.59	3.55	15.2	9.62	313-314
			found	45.4	3.5	15.4	9.6	
	$C_{15}H_{15}ClN_4O_6$	382.76	calc.	47.06	3.95	14.64	-	269-271 [b]
			found	47.0	4.0	14.7	-	

a) contains less than one mole of water.

b) melts under decomposition.

The starting materials for the synthesis of the N(1,10)-ethylene bridged compounds *12* (compounds *XVII* - *XXI*, Table 5) were the corresponding N(10)-(β-hydroxyethyl-)isoalloxazines (*11*) alkylated at N(3). The compounds *11* were obtained by treatment of the corresponding 1,2-dinitro-benzene derivatives (*9*) with ethanolamine in iso-amylalcohol according to the procedure of Chassy *et al.* |17|, except compound *11*, where R_1=R_2=R_4=H, R_3=CH_3. The N(3)-alkylated-10-(β-hydroxyethyl-)isoalloxazine derivatives were transformed to the corresponding N(1,10)-ethylene bridged compounds *12* either by the method of Müller and Massey |18| or by the procedure published by Hemmerich *et al.* |19|. The crude products thus obtained were dissolved in hot water, a few drops of conc. $HClO_4$ added and letting stand at room temperature yielded the desired compounds in analytically pure form. The overall yields were 60-70% (*11* → *12*). Compound *11* (R_1=R_2=R_4=H, R_3=CH_3) was prepared from the corresponding *1* by condensation with glycolaldehyde in abs. ethanol according to the procedure used to synthesize riboflavin |20|. The purity of the isoalloxazinium perchlorates was checked by thin layer chromatography employing CH_3CN as solvent. The analytical data of the new isoalloxazine derivatives are presented in Table 1. 5-Methylphenazinium methylsulphate was purchased from E. Merck A.G., Darmstadt, Germany, and recrystallized from CH_3CN. Its perchlorate analog was prepared by dissolving it in water and adding a small amount of conc. $HClO_4$ upon which crystallization of the perchlorate occurred. N-Methylquinoxalinium methylsulphate has been synthesized according to Smith *et al.* |21| and its perchlorate was prepared as described above for N-methylphenazinium perchlorate.

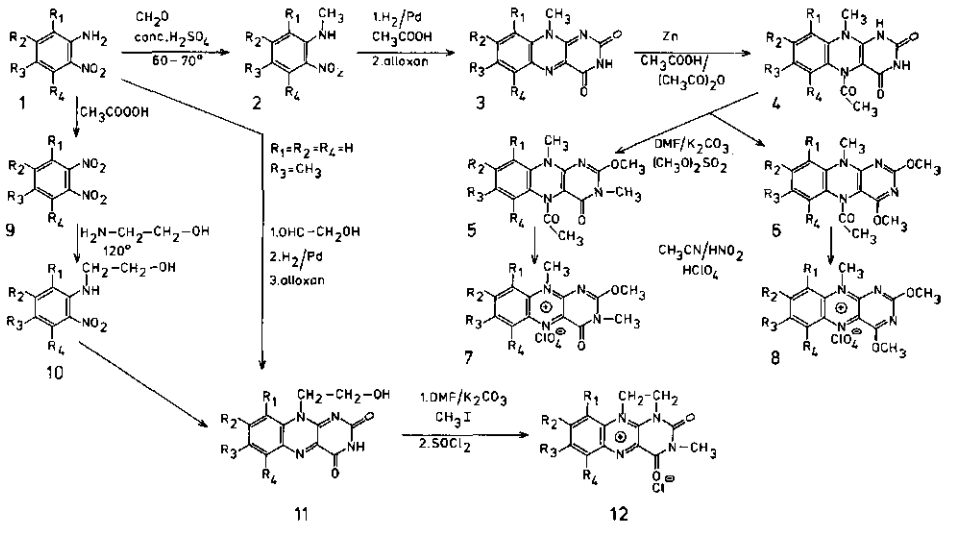
Precoated thin layer plates, Silica Gel 1B2, Baker-flex, have been used. The melting points were determined on an Electrothermal melting point apparatus and are not corrected.

3 RESULTS

Synthesis Most of the new isoalloxazine derivatives used in this study were synthesized virtually according to published procedures. Nevertheless we wish to focus attention on some modifications and improvements in the synthesis of N(10)-methyl-isoalloxazine derivatives *3*. Thus, the two most frequently employed procedures were developed by Kuhn and Weygand |11| and by Tishler *et al.* |22|. As starting materials served o-nitro-aniline derivatives *1* for the former method and aniline derivatives for the latter method. Both methods are suitable for the synthesis of N(10)-methyl-isoalloxazine derivatives modified in the

benzene subnucleus. However, the overall yields obtained by both methods are relatively low (∿ 30%) due to the preparation of many intermediate products, i.e. it takes several days to synthesize e.g. 7,8,10-trimethyl-isoalloxazine (*3*, R_1=R_4=H, R_2=R_3=CH_3) from 3,4-dimethyl-aniline according to the procedure of Tishler *et al.* |22| as described by Hemmerich *et al.* |23|. We have improved the above-mentioned procedures in such a way that starting from o-nitro-aniline derivatives *1* N(10)-methyl-isoalloxazines *3* can easily be obtained in a two-step synthesis with overall yields of 50-70%. Thus the o-nitro-aniline-derivatives *1* (Scheme 1) were converted to the corresponding N-methyl-derivatives *2* by treatment with a high excess of formaldehyde in conc. H_2SO_4 at 60-70°C in analogy to the procedure described by Halasz |12|. The reaction time varies between 2-5 hrs depending on the particular compound used. The yields of *2* were between 80-92% of the theory. The course of the reaction can easily be followed by thin layer chromatography. However, compounds *1* devoid of a substituent at the para position to the amino group (*1*, R_3=H) do not yield the corresponding methyl derivatives *2* under these conditions. The water solubility (pH 7) of the products *2* obtained with *1* (R_1=R_3=R_4=H, R_2=CH_3; R_1=R_2=R_3=R_4=H) indicate that sulphonation had occurred. These products have not been further characterized. Compounds *2*, where R_1=R_2=R_3=R_4=H and R_1=R_3=R_4=H, R_2=CH_3, can be synthesized

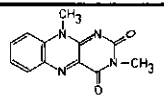
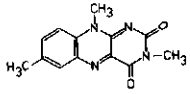
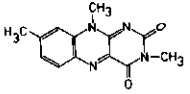
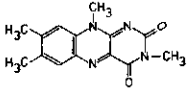
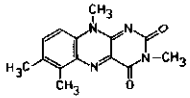
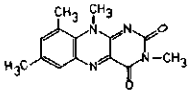
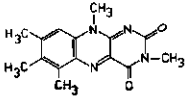
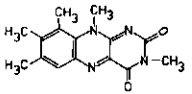
Scheme 1

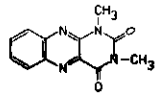
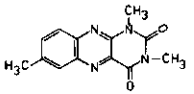
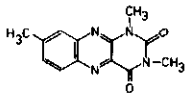
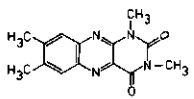
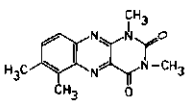
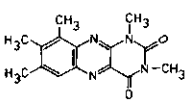
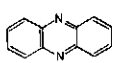


from the corresponding *1* by acetylation, methylation and hydrolysis. Catalytic reduction of *2* followed by condensation of the product thus obtained with alloxan gave the desired N(10)-methyl-isoalloxazine derivatives *3*. Since the intermediate phenylenediamine derivatives are rather sensitive towards molecular oxygen the condensation reaction was performed under exclusion of oxygen by flushing the reaction mixture with N_2. In this way less contaminated products *3* and better yields were obtained. Compounds *3* were methylated at the N(3) position in order to enhance solubility in organic solvents. The isoalloxazinium salts *7* and *8* were synthesized according to the procedure of Dudley and Hemmerich |15| but using $(CH_3O)_2SO_2$ as an alkylating agent. Under these conditions isomer *5* is obtained in 60% and isomer *6* in 40% yield. Using $(C_2H_5O)_2SO_2$ as reagent the isomer distribution is reversed |15|. The derivatives *5* and *6*, in contrast to their diethyl analogs, differ much less in their solubility behaviour and were therefore difficult to separate by fractional crystallization |15|. Easy and quantitative separation of the isomers *5* and *6* was achieved by column chromatography under the condition that the isomeric mixture of *5* and *6* was completely free of polar organic solvents, i.e. using commercial $CHCl_3$ in place of ethanol-free $CHCl_3$ resulted in a very poor resolution of the two isomers. Compound *11*, with the exception of two derivatives (R_1=R_2=H, R_3=R_4=CH_3; and R_1=R_2=R_4=H, R_3=CH_3), have been obtained by treatment of *9* |17| with ethanolamine in isoamylalcohol at 120°C for 24 hrs. The recrystallized *10* were then transformed into *11* in the same way as described above for *2*. Compound *11* (R_1=R_2=H, R_3=R_4=CH_3) was prepared from isoriboflavin by periodate oxidation and BH_4^- reduction of the product thus obtained as described elsewhere |9|. The reaction of *9* (R_1=R_3=R_4=H, R_2=CH_3, and R_1=R_2=R_4=H, R_3=CH_3) with ethanolamine did not yield the two corresponding isomers of *11*, but gave one and the same compound, namely *11* where R_1=R_3=R_4=H, R_2=CH_3, as independently judged by fluorimetry (Visser and Müller, to be published). Compound *11*, R_1=R_2=R_4=H, R_3=CH_3, was therefore synthesized unambiguously from the corresponding *1* by condensation with glycolaldehyde, catalytic reduction of the Schiff's base thus formed which in turn was condensed with alloxan to yield the desired product *11*. The transformation of *11* into *12* was easily achieved using either $SOCl_2$ as sole reagent |19| or a mixture of DMF and K_2CO_3 in the presence of mesylchloride |18|. The first procedure is more efficient and has been used to prepare the derivatives *12*. Methylation, if desired, was carried out prior to the ring closure reaction.

TABLE 2

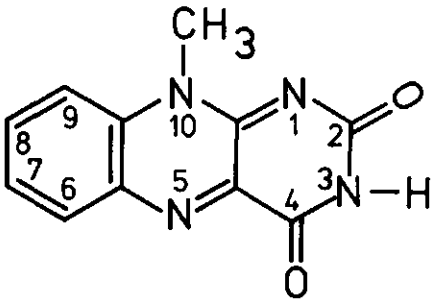
CHEMICAL SHIFTS AND COUPLING CONSTANTS OF VARIOUS NEUTRAL ISOALLOXAZINE AND ALLOXAZINE DERIVATIVES[a)]

Compound	R.P.[b)] No.	Aromatic Proton Resonances: chemical shifts in Hz				coupling constants in Hz						Methyl Resonances: chemical shifts in Hz						
		6	7	8	9	J_{6-7}	J_{6-8}	J_{6-9}	J_{7-8}	J_{7-9}	J_{8-9}	6	7	8	9	10	1	3
	I	811.4	760.3	789.7	777.6	8.3	1.5	0.3	7.2	1.0	8.7	-	-	-	-	402.6	-	336.2
	II	796.7	-	778.7	772.5	-	2.1	0.4	-	-	8.6	-	252.5 251.7	-	-	400.8	-	335.3
	III	802.6	748.8	-	764.7	8.5	-	-	-	1.6	-	-	-	260.7	-	400.0	-	335.1
	IV	791.1	-	-	764.0	-	-	-	-	-	-	-	243.4	253.2	-	400.3	-	335.1
	V	-	-	776.1	756.6	-	-	-	-	-	8.6	273.4	245.7	-	-	400.8	-	336.1
	VI	778.8	-	759.6	-	-	2.1	-	-	-	-	-	245.2	-	282.2	415.0	-	335.0
	VII[c)]	-	-	-	748.5	-	-	-	-	-	-	277.4	237.7	255.5	-	402.5	-	337.5
	VIII	775.3	-	-	-	-	-	-	-	-	-	-	244.2	242.3	259.7	398.1	-	334.6

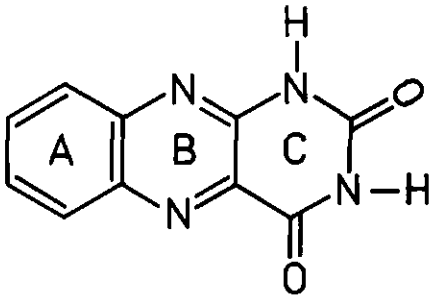
	IX	820.1	779.6	794.0	801.4	8.6	1.4	0.6	7.0	1.1	8.6	-	-	-	-	-	371.6	345.6
	X	798.7	-	780.0	791.8	-	2.1	0.3	-	-	8.8	-	259.3 258.5	-	-	-	370.4	345.0
	XI	809.0	767.0	-	781.0	8.6	-	0.7	-	2.0	-	-	-	261.9	-	-	370.8	344.9
	XII	794.4	-	-	777.2	-	-	-	-	-	-	-	249.8	252.7	-	-	368.7	344.1
	XIII	-	-	776.7	765.2	-	-	-	-	-	8.7	277.8 277.1	252.8 252.0	-	-	-	371.0	346.0
	XIV	784.8	-	-	-	-	-	-	-	-	-	-	253.1	248.2	273.8	-	374.1	345.1
	XV[d]	808.3	779.9	779.9	808.3	8.4	1.4	0.5	6.8	1.4	8.4	-	-	-	-	-	-	-
	XVI[d]	821.9	789.6	789.6	821.9	8.8	1.5	0.3	6.3	1.5	8.8	-	-	-	-	-	-	-

a) Solvent is deuterio-acetonitrile.
b) R.P. = Ring Position.
c) Due to low solubility some chloroform was added to the sample.
d) For convenience the ring numbering is analogous to that of isoalloxazine.

Scheme 2



iso-alloxazine



alloxazine

Nuclear magnetic resonance data The results described in this paper can be divided into two parts, namely data obtained from isoalloxazines and their isomers, the alloxazines, and the cationic species of these compounds. For comparison also results obtained from other heterocycles are included which are related to our system, i.e. quinoxaline and phenazine and their cationic species. The structures of the isoalloxazine and alloxazine molecules are shown in Scheme 2 together with the ring numbering as recommended by IUPAC-IUB |24|.

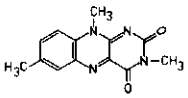
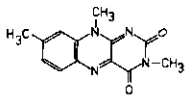
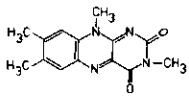
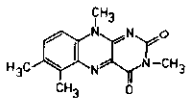
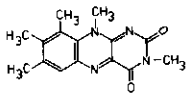
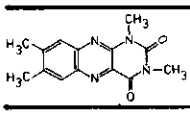
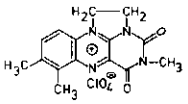
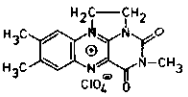
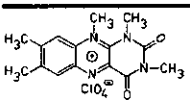
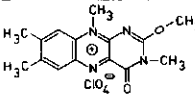
The results obtained with the neutral forms of isoalloxazine and alloxazine derivatives are summarized in Table 2. The N-CH_3 resonances of the isoalloxazines appear in the frequency range of about 335 to 400 hz (Table 2, *I - VIII*). The signal at lower field is assigned to the N(10)-CH_3 group. The correctness of this assignment is supported by the observation that a significant enhancement of the signal at 764.0 Hz of *IV* is observed when the signal at 400.3 Hz is irradiated or *vice versa* in double resonance experiments. No such enhancement was found upon irradiating the signal at 335.1 Hz of *IV*. In fact all isoalloxazine derivatives show these effects, which are due to Nuclear Overhauser Effect (NOE). The enhancements of signals with some compounds observed by double resonance technique are given in Table 3. In performing selective NOE experiments it was observed that the decoupling effect was many orders of magnitude larger than NOE. For this reason in Table 3 the NOE and the decoupling effects are not discriminated.

The methyl group attached to ring A resonate in the frequency range between 235 and 280 Hz. Comparison of the methyl resonances of *II* and *III* with those of *IV* indicates that introduction of a methyl group in ortho position to the origi-

TABLE 3

PERCENTAGE ENHANCEMENT[a)] OF SIGNAL INTENSITIES OBSERVED IN DOUBLE IRRADIATION EXPERIMENTS.

The arrows indicate the signal irradiated and the values give the enhancement of the signal due to the indicated position of the (iso)alloxazine ring.

Compound	No.	Ring Position				
		6	7	8	9	10
	II	40 ↑	↑ 70	b)	b) 10	↑
	III		50	↑	40	
	IV	80 ↑	c) ↑ 30	↑ c) 30	60 ↑ 15	10 ↑
	V	↑ c)	c) ↑	↑ <10 30 d)	30 30 <10 ↑ 20	10 ↑
	VIII	↑	30			
	XII	↑	40	40	↑	
	XXI	↑ c)	c) ↑ 10	50 ↑ d)	40 d) ↑ 20	b) ↑
	XX	↑	40	30	↑ 20	b) ↑
	XXIV	↑	30	20	↑ 10	<10 ↑
	XXVIII	↑	40	80	↑ 10	<10 ↑

a) The enhancements are both due to decoupling and to NOE. Since it is difficult to separate both effects quantitatively, only the total enhancement is given and the stated values have to be considered qualitatively rather than absolutely. As blanco served the intensities of a spectrum in which a position was irradiated where no absorptions occur. The power used was 95 db.

b) The enhancement is spread over the multiplets and thus small.

c) Due to the width of the irradiating field the neighbouring signal is affected too.

d) The original doublet is transformed into a singulet.

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nally present methyl group causes an upfield shift of about 8 Hz. These effects are summarized in Table 4. Based on this observation the resonance at lower field of *IV* is assigned to the 8-CH_3 group. This assignment is in agreement with published results |8|. It should be noted that the methyl resonance of *II* is split into a doublet due to coupling with the proton at position 6 as verified by decoupling experiments (cf. Table 3). In fact only the monomethyl analogs *II*, *X* (Table 2), *XVIII*, *XXII*, *XXVI* (Table 5), show this doublet, but not the isomers *III*, *XI* (Table 2), *XIX*, *XXIII*, *XXVII* (Table 5). The methyl resonances of *V*, *VI* and *VII* have been assigned on the basis of the above-mentioned shift induced by methylation (Table 4). For *VIII* it was difficult to assign the two high field resonances only on the basis of the additivity rule. However, irradiation of the signal due to CH(6) gives an enhancement at 244.2 Hz proving that the signal at this frequency originates from CH_3(7) (Table 3).

The signals due to the aromatic protons have been assigned in a similar way as mentioned above. The low field resonance of *IV* at 791.1 Hz has been assigned unequivocally to the proton at position 6 by selective deuteration at position 9 |8|. This assignment is in agreement with the decoupling data given in Table 3. It is noteworthy to mention that the enhancement upon decoupling is larger for the 6,7-H-CH_3 pair than for the 8,9-CH_3-H pair (Table 3).

The low field part of the spectrum of *III* consists of an AB part with a single line in its centre. The doublet of the AB system at higher field and the single line are quite broadened as compared to the doublet at lower field. Irradiation of the methyl resonances at 260.7 Hz, thus decoupling the 8-CH_3, resolves the high field doublet of the AB system into two doublets and the single line becomes also a doublet. This splitting of the signals is due to the meta-coupling between the protons at positions 7 and 9 and thus allowing the assignment of the high field part of the AB system to the proton at position 7. With this information on hand the spectrum was simulated using the frequencies

TABLE 4 RELATIVE SHIFTS[a] (in Hz) OF AROMATIC METHYL RESONANCES DUE TO METHYL SUBSTITUTION

compound	Ring Position: ortho shift due to[b] 6	7	8	9	meta shift due to[b] 6	7	8	9
IV	-	-7.5	-8.7	-	-	-	-	-
V	-6.4	-	-	-	-	-	-	-
VI	-	-	-	-	-	-	-	-6.9
VII	-5.7	-	-8.0	-	2.3	-	4.0	-
VIII	-	-	-1.0 / -22.5[c]	-10.9	-	-	-	0.8
XII	-	-9.2	-9.1	-	-	-	-	-
XIII	-6.5	-	-	-	-	-	-	-
XIV	-	-	-	-4.5	-	-	-	3.3
XX	-	-9.5	-10.0	-	-	-	-	-
XXI	-7.4	-	-	-	-	-	-	-
XXIV	-	-8.6	-9.2	-	-	-	-	-
XXV	-	-	-	-9.5	-	-	-	1.1
XXVIII	-	-8.2	-9.6	-	-	-	-	-
XXIX	-6.3	-	-	-	1.8	-	-	-

a) Negative shifts are to high field.

b) Shifts observed upon addition of methyl group(s) at the indicated position to originally present methyl group(s).

c) The 8-methyl is thought to be added to compound VI, thus giving ortho shifts at 7 and 9 positions (7/9).

obtained from the CH_3(8)-decoupled spectrum. The low field resonances of *II* were assigned in a similar way. The spectrum is, however, complex and consists of two parts. The low field part shows one broad signal, superimposed on it some fine structure can be observed. The high field part consists of four strong lines. In order to be able to assign the signals unequivocally *II* was selectively deuterated at position 9. This compound yields a spectrum consisting of two broad lines which exhibit some fine structure. This proves that the four lines at high field are due to the protons at positions 8 and 9. Upon irradiation of the 7-methyl resonance the broad signal at lowest field is split into a doublet whereas the signal at higher field is only to a minor extent better resolved. The doublet at low field originates from the meta-coupling between CH(6) and CH(8). These results prove that the signal at low field belongs to CH(6). This

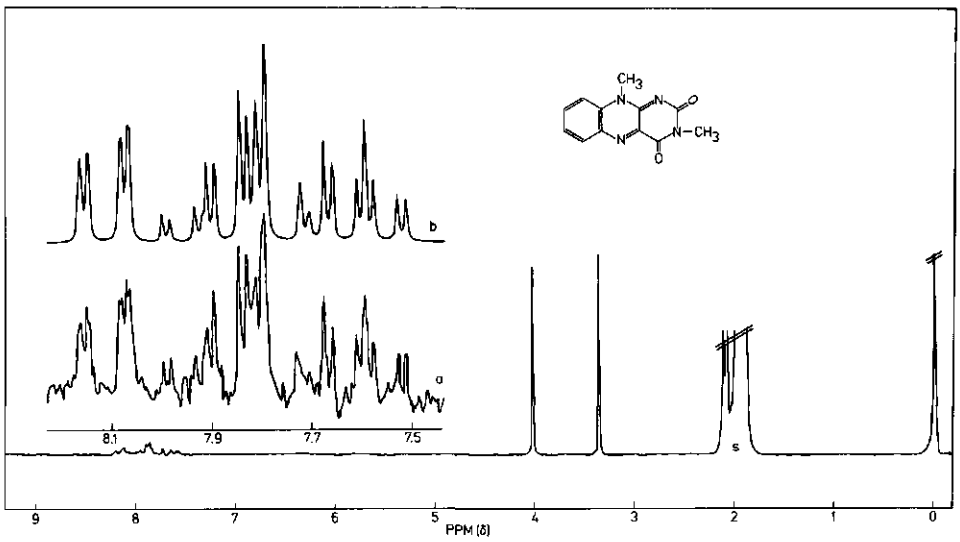


Fig. 1 ^{1}H-NMR SPECTRUM OF 3,10-DIMETHYLISOALLOXAZINE (*I*) in CD_3CN.
a: low field part of the spectrum expanded by a factor of five in the horizontal as well as in the vertical direction;
b: calculated spectrum corresponding to a; s = resonances due to the solvent.

is in agreement with double resonance experiments conducted with the non-deuterated compound which shows one sharp line upon irradiation of the high field part of the spectrum. By fitting the spectrum of *II* the starting values for iteration were taken from the deuterated compound decoupled at 252 Hz. The spectrum of *I* is very complex and is shown in Fig. 1. In Fig. 1a the low field part of the spectrum of *I* is also shown in an expanded scale. It was not possible to obtain derivatives of *I* selectively deuterated at one or two atoms[1)],

1) Treatment of 3,4-dimethylaniline hydrochloride in D_2O leads to the exchange of the two protons in ortho position to the amino group. From this compound 9-deuterio-7,8,10-trimethylisoalloxazine has been prepared |8|. When the corresponding N,3,4-trimethyl-ortho-phenylene-diamine dihydrochloride was treated in D_2O under anaerobic conditions, the protons were exchanged almost quantitatively yielding, after condensation with alloxan, 6,9-dideuterio-7,8,10-trimethylisoalloxazine. The deuterium content of this compound at both the 6 and 9 positions is at least 95% as judged by NMR. Therefore, this procedure is much more efficient than that described previously |25, 26|. Similarly starting from N-methyl-ortho-phenylene-diamine dihydrochloride 6,7,8,9-tetradeuterio-N(10)-methylisoalloxazine was obtained. The deuterium content of this compound was greater than 95% of the theory.

which would have simplified the spectrum. The only assumption made by the simulation of the spectrum of Fig. 1a, was that the CH(6) appears at lowest field. This is a very reasonable assumption considering the results obtained with the other derivatives (Table 2). Fig. 1a shows that the low field part consists of an AB system. The rest of the spectrum is very complex due to meta and para couplings. The best fit found in an iterative way is shown in Fig. 1b. Only lines well above the noise level were used for iteration, the frequencies of the lines were read off the computer listing of the experimental spectrum. The computer listing contains 32 lines from which 20 lines with a calculated intensity of more than 0.1 were used.

In performing double resonance technique it was found that *V* behaved unexpectedly. Thus irradiation at 273.4 Hz (CH_3(6)) yields a large signal enhancement at 756.6 Hz (CH(9)) and a very small increase of the intensity of the signal at 776.1 Hz (Table 3). By which mechanism the strong enhancement of the para position as compared to the meta position is achieved is not yet clear to us but must in part be due to NOE.

The resonances of the alloxazine derivatives were assigned in analogy to those of the isoalloxazines. The influence of methylation on original present methyl groups for the class of alloxazines follows that described above for the isoalloxazine derivatives (Table 4). Decoupling experiments (Table 3) were consistent with the assignments. The aromatic protons were assigned by both decoupling experiments and using selectively deuterated compounds. All assignments were then, where appropriate, verified by computer simulation. The results are also presented in Table 2. The experimental spectrum of 1,3,7-trimethylalloxazine (*X*) is given in Fig. 2. The low field region of the spectrum, expanded by a factor of five, is also shown in Fig. 2a. The NMR pattern shown in Fig. 2a was obtained by decoupling the resonance signal of the 7-methyl group in order to achieve a better resolution of the low field part of the spectrum. Fig. 2b shows the computer fit to this spectrum. As illustrated in the stick spectrum of Fig. 2 the high field part represents an AB system due to the protons at 8 and 9 positions, while the signal of the 6 proton is distorted due to overlap with the low field AB pattern. The low field part of the spectrum of *XI* consists also of an AB system and has been analysed in the same way as described above for *X*. To simulate the experimental spectrum of compound *IX* it was assumed, as for *I*, that the CH(6) resonance lies at lowest field. In contrast to all other compounds studied the aromatic methyl resonances of *XIII* are split into doublets (Table 2).

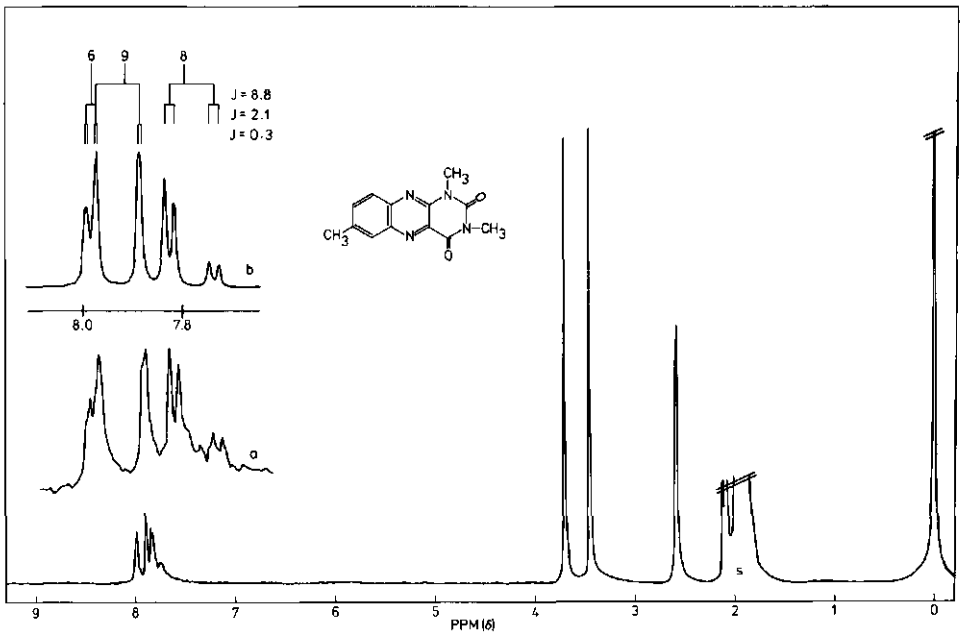


Fig. 2 ^{1}H-NMR SPECTRUM OF 1,3,7-TRIMETHYLALLOXAZINE (*X*) IN CD_3CN.
a: expanded low field part of the spectrum (as in Fig. 1); this spectrum was obtained by decoupling the resonance due to the CH_3(7);
b: calculated and stick spectrum corresponding to a; s = as in Fig. 1.

For comparison the spectra of quinoxaline and phenazine dissolved in acetonitrile were also recorded. The published spectra of these compounds were obtained in other solvents than acetonitrile[2]. These spectra were simulated using the frequencies of our experimental spectra. The coupling constants thus obtained are in agreement with published data |27|. The frequencies of CH(1) and CH(2) of *XV* are not reported here, because they are not relevant to our study.

Protonation of isoalloxazine or alloxazine removes the structural difference present in the neutral molecules. In our cases the proton is replaced by a methyl (methylene) group which offers the possibility to study isomeric species of the cations, which are otherwise not available in organic solvents.

2) The published data were obtained from CH_2Cl_2 solutions |27|.

Due to the positive charge of the molecules it can be expected that the chemical shifts of the cations are located at lower fields than those of the corresponding neutral molecules. The results are collected in Table 5. The $N(3)CH_3$ resonance of the cations appears at about 350 Hz and so are only slightly shifted as compared with those of the neutral species (*IX* - *XIV*). The methyl

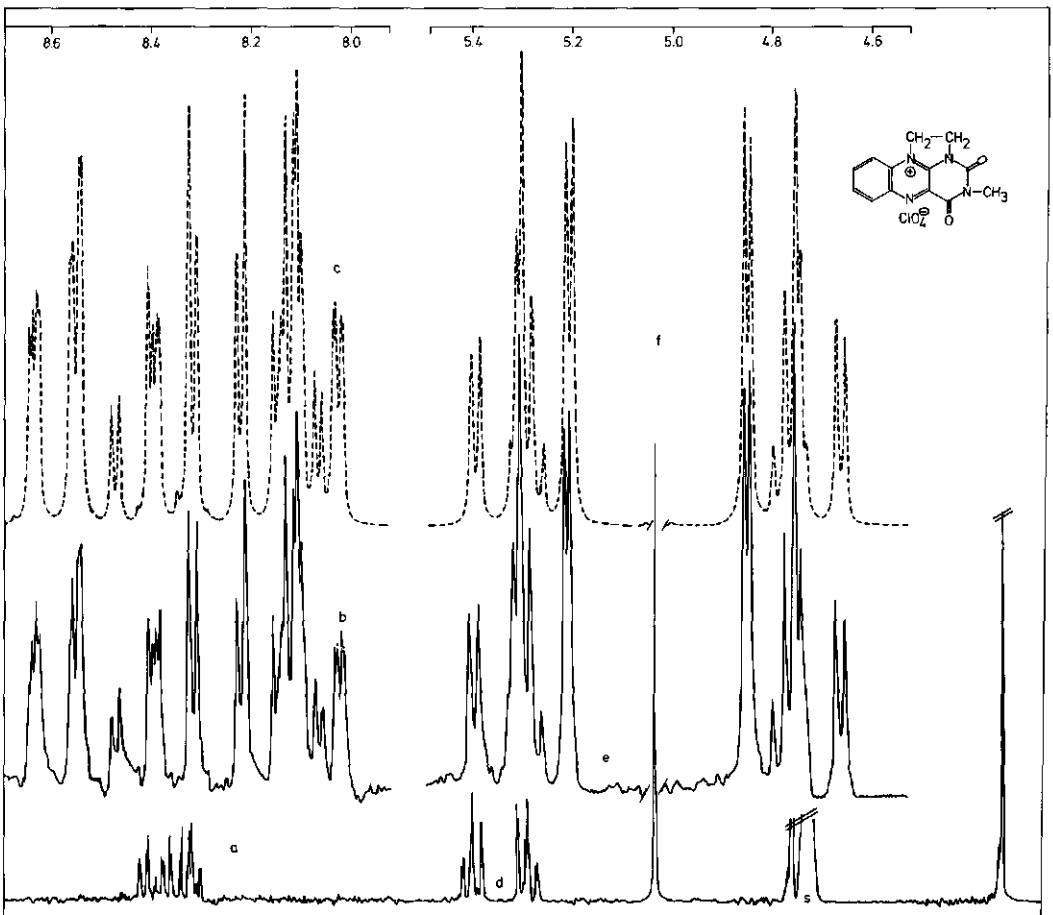
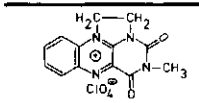
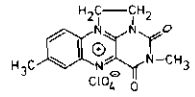
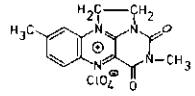
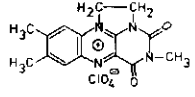
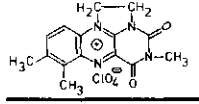
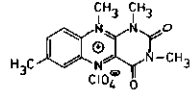
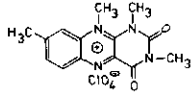
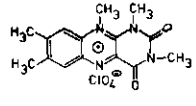
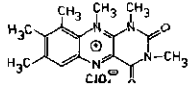
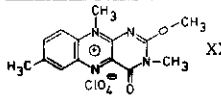
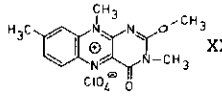
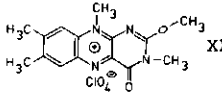
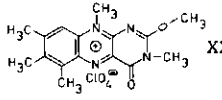
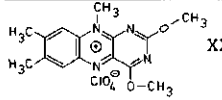
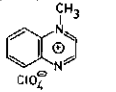
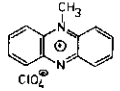


Fig. 3 ^{1}H-NMR SPECTRUM OF 1,10-ETHYLENE-3-METHYLISOALLOXAZINIUM PERCHLORATE (*XVII*) IN CD_3CN. b and e: expanded parts of the spectrum (as in Fig. 1) corresponding to a and d, respectively; c and f: computed spectra belonging to b and e, respectively, s = as in Fig. 1.

TABLE 5

CHEMICAL SHIFTS AND COUPLING CONSTANTS OF VARIOUS ISOALLOXAZINIUM PERCHLORATE DERIVATIVES[a)]

Compound	R.P. No.[b)]	Aromatic Proton Resonances: chemical shifts in Hz 6	7	8	9	coupling constants in Hz J_{6-7}	J_{6-8}	J_{6-9}	J_{7-8}	J_{7-9}	J_{8-9}	Methyl(methylene) Resonances: chemical shifts in Hz 6	7	8	9	10	1	2α	3
	XVII	858.7	814.9	838.1	806.9	8.6	1.3	0.5	7.3	1.2	8.5	-	-	-	-	N = 19.0[c)] 529.0	476.1	-	347.7
	XVIII	838.8	-	823.9	797.6	-	1.8	0.6	-	-	8.7	-	269.5 268.8	-	-	N = 19.0 526.3	473.7	-	347.5
	XIX	845.0	799.3	-	790.0	9.0	-	0.7	-	1.4	-	-	-	277.6	-	N = 18.9 521.9	473.3	-	346.8
	XX	833.0	-	-	789.5	-	-	-	-	-	-	-	259.1	268.1	-	N = 19.0 521.8	471.9	-	346.1
	XXI	-	-	820.6	780.6	-	-	-	-	-	8.7	286.5	261.8	-	-	N = 18.5 523.6	473.1	-	347.7
	XXII	827.8	-	816.8	817.3	-	1.6	0.8	-	-	7.6	-	268.2 267.3	-	-	438.8	377.7	-	346.6
	XXIII	835.6	797.3	-	809.2	8.6	-	0.9	-	1.6	-	-	-	277.6	-	436.4	377.7	-	346.5
	XXIV	822.8	-	-	807.2	-	-	-	-	-	-	-	258.4	269.0	-	436.6	376.7	-	345.7
	XXV	809.8	-	-	-	-	-	-	-	-	-	-	259.5	259.5	273.0	423.0	383.5	-	347.2

	XXVI	832.6	-	822.8	823.4	-	2.0	0.5	-	-	8.0	-	269.5 **268.5**	-	-	452.2	-	439.1	355.6
	XXVII	840.6	798.1	-	815.5	8.7	-	0.5	-	1.7	-	-	-	279.2	-	449.8	-	439.0	355.2
	XXVIII	827.5	-	-	813.7	-	-	-	-	-	-	-	259.4	271.0	-	450.1	-	437.7	354.8
	XX$_1$	-	-	-	800.2	-	-	-	-	-	-	290.8	253.1	272.8	-	448.3	-	437.6	355.8
	XXX	830.7	-	-	822.7	-	-	-	-	-	-	-	262.8	275.7	-	462.2	-	439.0[d]	-
	XXXI[e]	852.8	829.1	834.1	845.8	8.4	1.4	0.7	7.0	1.1	9.1	-	-	-	-	468.2	-	-	-
	XXXII[e]	864.1	829.3	850.4	865.1	8.8	1.4	0.6	6.8	1.0	9.4	-	-	-	-	492.3	-	-	-

a) Solvent is deuterio-acetonitrile
b) R.P. = Ring Position.
c) See text.
d) 4a-methyl resonates at 433.3.
e) For convenience the ring numbering is analogous to that of isoalloxazine.

resonances of ring A are more sensitive to the positive charge than those of the $N(3)CH_3$ group and are now located in the frequency range of 260 to 290 Hz. The methyl substitution effect is similar to that found for the neutral molecules (Table 4). The resonances of the methyl groups attached to ring A have thus been assigned in an identical manner as described above, i.e. by additivity and double resonance techniques. In addition compound *XX* has been selectively deuterated at $CH_3(8)$ |8| to support our assignment. The experimental spectrum of *XVII* is shown in Fig. 3. The complex parts of the spectrum (Figs. 3a and d) are also shown in an expanded scale (Figs. 3b and e, respectively). Double resonance technique unequivocally showed that the low field part of the multiplet (Fig. 3e) is due to the $N(10)-CH_2$ group, i.e. irradiation at about 530 Hz resulted in an increase of the intensity of the signal at about 807 Hz and *vice versa* (Table 3). Furthermore, decoupling of the low field part of Fig. 3e converts the multiplet at higher field into a badly defined triplet. The two CH_2-groups behave as an AA'BB' system and have been characterized by their frequencies and the sum of their J_{AB} coupling constants denoted by N as obtained by computer fitting (Table 5). These values were derived from the actual spectrum by the method described by Bovey |28|. Thus the values were calculated by the analytical expression for the first line on the left wing in combination with the second line on the right wing of the low field part multiplet (Fig. 3e). Since the spectrum is centrosymmetric the same procedure can be applied to calculate the high field part of the multiplet. Since only a few distinct lines are available, extensive iteration would yield very crude values and was therefore not performed. As seen in Fig. 3f the frequencies of the various lines could, however, be simulated quite well whereas some of the intensities are not in complete agreement with the actual spectrum. It should be noted that some asymmetry in the coupling constants appeared upon iteration of $J_{AA'}$ and $J_{BB'}$, when the frequencies were kept constant. For the other ethylene-bridged isoalloxazinium salts the values given in Table 5 were calculated in the same way.

The aromatic protons of the cations exhibit in general the same NMR properties as their neutral forms. Compound *XVIII* yields a very good resolved ABX type spectrum, in contrast to *XIX*, giving rise to an ABC system which could be simulated easily. The calculated spectrum of *XVII* shown in Fig. 3c was obtained assuming again that the signal at lowest field belongs to the CH(6). This is a reasonable assumption considering the unequivocally assigned frequencies of the CH(6) resonances of the other compounds (*XVIII*, *XIX*, *XX*). The very good

fit (Fig. 3c) obtained supports this assumption. Double resonance experiments with the cations show similar enhancements as found with the neutral molecules (Table 3). Compound *XXI* showed the same peculiar behaviour as its neutral analogs (*V*, *XIII*) (Table 3).

The resonances of *XXII* - *XXV* lie, and this is especially true for the aromatic proton signals, at higher field than those of *XVII* - *XXI*. To identify the resonances belonging to N(1) and N(3) we used 1,7,8-trimethylalloxazine |10|. The cationic species of this compound was prepared in a mixture of CD_3CN and trichloro-acetic acid. For comparison *XXIV* was dissolved in the same solvent mixture. The spectra thus obtained exhibit the $N(CH_3)$-resonances at 450.0, 360.0 and 391 Hz, respectively, for the latter compound while the 1,7,8-trimethylalloxazinium salt exhibits the $N(CH_3)$ resonance at 385.0 Hz. Comparing these values with those given in Table 5 it is seen that the resonances in this solvent mixture are shifted to low field as compared with those of *XXIV* in acetonitrile. Therefore, the resonance at about 377 Hz of *XXII* - *XXV* has been assigned to the N(1)-methyl group. The frequencies due to the protons at positions 8 and 9 of *XXII* differ only slightly. Nevertheless, the low field part of the spectrum of *XXII* could be calculated despite the fact that the coupling constant between the protons at positions 8 and 9 could be determined only approximately. The assignments of the various resonances of *XXVI* - *XXIX* has been done as described above for the other quarternary derivatives. Some uncertainty existed with respect to the assignment of the resonance due to 2α-CH_3. One is tempted to assign the resonance at lower field (450 Hz) to the OCH_3 group. However, irradiation of the signal at 814 Hz (CH(9)) gives an enhancement of the signal at 450 Hz. This proves that the resonance at higher field must be assigned to OCH_3. This is also supported by NMR data obtained from *XXVIII* in the presence of methoxide yielding an adduct |29| which showed that the signal intensity at 438 Hz decreased slowly with time due to slow exchange of the 2α-OCH_3 group with bulk CD_3O^-. The low field part of the spectrum of *XXVI* is quite complex. Due to the large overlap of the lines no accurate values can be determined for the frequencies. Specific deuteration at position 9 of *XXVI* allows, however, to estimate the frequencies of the signals due to CH(6,8). The coupling constants, on the other hand, are somewhat less accurate ($\pm$ 0.1 Hz), than those determined for the other compounds. For the computer fit the coupling constants were estimated from the spectrum obtained in a mixture of CD_3CN/CD_3OD (1:3 by vol.), in which a better resolved spectrum was obtained.

The spectra of methyl-quinoxalinium perchlorate (*XXXI*) and methyl-phenazinium perchlorate (*XXXII*) (Table 5) were also taken and simulated for reference. To our knowledge no spectra of these compounds are published. As with quinoxaline the resonances due to CH(1) and CH(2) are not given (in Table 5) for *XXXI*. These resonances form a complex pattern and are centred at 954.2 Hz (CH(1)) and 908.5 Hz (CH(2)). It was very difficult to fit the spectrum of methyl-quinoxalinium perchlorate, therefore, also a 360 MHz spectrum was recorded. The spectrum thus obtained was much better resolved and could easily be simulated. The calculated parameters for the 360 MHz spectrum, appropriately corrected for the frequencies, did not yield the 100 MHz spectrum. It was, therefore, necessary to fit the coupling constants for the 100 MHz spectrum using the corrected frequencies of the 360 MHz spectrum. It was, however, *not completely* possible to optimize both the 100 MHz and the 360 MHz spectra with the same optimal parameters. Also here it was assumed that "CH(6)" is at lowest field.

With the data presented in Tables 2 and 5 at hand it is reasonable to check these values also on their dependence on concentration and solvent polarity. Inevitable the choice of a particular solvent introduces always some perturbation of the ground state. The choice of a particular solvent in this study was mainly determined by the solubility of the compounds investigated, thus allowing a direct comparison of the various derivatives of isoalloxazines, alloxazines and their cationic species. The concentration dependence was studied in detail employing *IV* which is soluble in CH_3CN over a wide range of concentration. The results show that only the resonances due to CH(6) and N(3)-CH_3 are affected by concentration. Thus the signal due to CH(6) shifts from 805.5 Hz to 807.5 Hz at infinite dilution whereas the signal due to N(3)-CH_3 shifts from 335.0 Hz to 332.5 Hz. These values were derived by extrapolation. The solvent polarity, on the other hand, affects the resonances much more. To illustrate this effect *IV* and *XII* were investigated using mixtures of $CDCl_3$ and CD_3OD. The results are given in Table 6. The data demonstrate that the resonances due to the aromatic protons and the N(3)-CH_3 are more influenced than the other resonances by the solvent polarity. Moreover, the influence of the polarity on the aromatic protons is different for the isoalloxazines and alloxazines, i.e. for the former compounds the resonance of the CH(9) is influenced much more than that of the CH(6) whereas this effect is reversed for the latter compounds.

Table 6 THE INFLUENCE OF THE POLARITY OF THE SOLVENT ON THE RESONANCES (in Hz) OF (ISO)ALLOXAZINES

As solvent served a mixture of chloroform and methanol. No corrections were made for dilution

3,7,8,10-tetramethyl-isoalloxazine (IV)

% CD_3OD	Aromatic Proton Resonances		Methyl Resonances			
	Ring position 6	9	7	8	3	10
1.2[a)]	806.8	743.8	245.6	255.8	352.8	412.6
11.1	806.5	754.0	248.0	258.7	351.8	414.1
29.8	806.1	762.2	249.7	260.7	351.4	416.0
50.0	803.1	769.2	249.2	260.7	350.0	416.3
68.6	798.7	774.0	248.2	259.8	347.7	414.7
88.0	798.2	772.2	248.0	259.5	346.2	413.8

1,3,7,8-tetramethyl-alloxazine (XII)

% CD_3OD	Aromatic Proton Resonances		Methyl Resonances			
	Ring Position 6	9	7	8	3	1
1.2[a)]	806.4	779.7	250.8	253.4	359.4	380.8
11.1	803.6	782.0	252.1	254.9	358.6	381.6
29.8	801.3	782.7	253.3	255.3	357.6	380.9
50.0	799.5	783.0	254.0	256.8	357.1	380.7
68.6	793.3	777.7	251.8	254.0	354.2	375.7
88.0	792.7	778.2	252.0	254.3	352.7	375.0

a) For stabilization of the $CDCl_3$ solution a small amount of CD_3OD was added.

4 DISCUSSION

Comparing the frequencies of the resonances of aromatic protons of the compounds described in this paper it is seen that the resonance of CH(6) appears at lowest field. From this one could conclude that this position of the isoalloxazine ring system possesses the lowest electron density. However, some doubt exists in the literature concerning the assignment of the resonances of CH(6) in flavin-adenine dinucleotide (FAD) and in riboflavin-5'-monophosphate (FMN) |30-32|.

Table 7 COMPARISON OF EXPERIMENTAL AND CALCULATED CHEMICAL SHIFTS (in Hz) OF AROMATIC PROTONS OF 3,10-DIMETHYL ISOALLOXAZINE (I)

Position	Calculated[a)]	Observed	Difference
6	795.8	811.4	- 15.6
7	784.1	760.3	+ 23.8
8	781.2	789.7	- 8.5
9	759.4	777.6	- 18.2

a) Using formula I and electron densities as obtained by Fox et al.[3)]. The theoretical standard deviation as calculated from the standard deviation of the correlation coefficients is 21 Hz.

Part of the reasoning to assign a certain resonance to CH(6) was based on published π-electron calculations |33|. Comparing our results with such calculations |33| we find that hardly any correlation exists between calculated π-electron density and chemical shift. On the other hand it can be expected, and in fact it has been shown by Sterk and Holzer |34|, that a much better correlation is obtained when the total (π- and σ-) electron density is taken into account. The results given in Table 7 are obtained taking the (total) electron density as calculated by Fox *et al.*[3)] and employing the correlation equation (1) given by Sterk and Holzer |34|:

$$\delta_H = 28.511 - 19.198q^H - 2.687q^C \qquad (1)$$

where q^H is the electron density at the proton and q^C the electron density at the carbon atom under consideration. The correlation coefficients of equation (1) were derived from MINDO/2 calculations |36|. Although the agreement between experimental and calculated values seems poorly, the calculated values are, with the exception of that due to position 7, within the theoretical standard deviation of 21 Hz. It should be noted that the use of slightly different correlation coefficients, which would be obtained for MINDO/3 calculations because of the somewhat different parametrization with respect to MINDO/2, would probably improve the agreement between calculated and experimental values. The results of Table 7 support the assignment of the resonance at lowest field to CH(6). Fur-

3) Personal communication; the data were obtained by an all valence electron molecular orbital calculation within the MINDO/3 formalism |35|. The theoretical values (CNDO calculations) as published by Grabe |5| yield a lesser degree of correlation than those of Fox.

thermore, the agreement between experimental and calculated values, as given in Table 7, could probably be improved if the dependence of the resonances on the solvent polarity would also be considered.

As shown in Table 6 the polarity of the solvent has a large influence on the resonance position whereas the concentration affects the resonances only to a minor extent (see above). In context with the dependence of the resonances on the solvent polarity it should be mentioned that isoalloxazines interact in a specific manner with H_2O |37|. Therefore, the observed effects (Table 6) using methanol as a solvent could partially be due to a specific interaction between solute and solvent. However, it is evident from Table 6 that the difference between the resonance frequency of CH(6) and that of CH(9) in apolar solvents is much larger than in polar solvents, i.e. in going from apolar to polar solvents the two signals move towards each other.

The different behaviour of the CH(9) resonance of 3,7,8,10-tetramethyl-isoalloxazine (*IV*) as compared to that of 1,3,7,8-tetramethyl-alloxazine (*IX*) suggests that the electron distribution in both molecules is affected differently by the solvent polarity (Table 6).

Theoretical calculations |3-5| show that a high electron density is located on the carbonyl groups. With increasing polarity of the solvent an even higher electron density can be expected on these carbonyl groups. In the case of the isoalloxazines this can lead to a shift of electron density from positions 9 and 9a towards the two carbonyl groups and/or withdrawal of electron density from positions 6 and 8. The first possibility leads to some decrease of the ring current, due to loss of electron density in the rings A and B, which is partially compensated by additional delocalization of the N(10) π-electron pair. This would result in a downfield shift of CH(9) and an upfield shift of CH(6), which is observed. The second possibility is more unlikely, because it would be expected that both a decrease of the ring current (no additional delocalization) and of the electron density at the 6 and 8 positions should lead to an upfield shift of the resonances due to positions 7 and 9, and a downfield shift of the resonances due to positions 6 and 8 caused by the further reduced electron density at these positions. In fact a reversed effect is observed. This interpretation is further supported by the calculations of Fox[3)] who found a relatively high excess of electron density at C(9) and C(7), while the C(8) and C(6) atoms are positive. Since the carbon atoms at positions 6 and 8 are already positive it is, therefore, reasonable that in polar solvents electron density is withdrawn from the C(9) atom by the now even more polarized carbonyl group at position 2.

In the case of alloxazines the influence of the carbonyl groups seems to be less important than in isoalloxazines. Electron density withdrawal via both carbonyls will affect the electron densities at C(6) and C(8) to some degree. From Table 6 it might be concluded that with increasing polarity resonances of the isoalloxazines become more alike to those of the alloxazines, as expected from the delocalization of the N(10) π-electron pair in the series of isoalloxazines. Additional support of the influence of the carbonyl groups is obtained by the comparison of the isoalloxazinium salts. Thus in going from *XXIV* to *XXVIII* and to *XXX* the aromatic proton resonances move gradually to lower field. This illustrates that the original present carbonyl groups (*XXIV*) withdraw electrons from the A and B ring and lower thus the ring current. The same effect is shown even more pronounced by the N(10)-CH_3 resonances of these compounds (Table 5) which also reflect alteration in the ring current.

In the literature there exists a contradiction concerning the assignment of the two aromatic protons of FAD |30-32|. The results given in Table 6 suggest that in even more polar solvents than methanol the resonance of CH(6) and CH(9) might cross over. This effect could further be influenced by the formation of the intramolecular complex |37, 38|. To remove the above-mentioned apparent contradiction we synthesized 8-trideuteriomethyl-FAD[4]. The spectrum of this compound (0.05 M in 0.1 M phosphate buffer, pD 7.04[5]) exhibits the CH_3(8) resonance (231.5 Hz) (decreased intensity) at lower field than the CH_3(7) as expected. Double irradiation experiments conducted with a sample of FAD, not pretreated with deuterium oxide, but dissolved in the same buffer and identical pH and concentration conditions as above, revealed that CH(9) is at 749.7 Hz and CH(6) at 739.7 Hz. An identical experiment performed with FAD in pure deuterium oxide (pD 5.60[5]) was conducted to check the influence of pH and buffer on the position of the resonances. Also here the CH(9) was located at lowest field, however, the difference of the chemical shifts between CH(9) and CH(6) being smaller, i.e. 750.2 Hz *vs* 742.3 Hz. Upon addition of solid FAD to this sample it was noticed that the signal due to CH(6) gradually moved to lower fields with in-

4) CD_3(8)-FAD was prepared in deuterium phosphate buffer in analogy to the procedure published for CD_3(8)-FMN |8|. However, the exchange reaction for CH_3(8) of FAD is much slower than that for CH_3(8) of FMN. To achieve an exchange of about 70% a refluxing time of 6 hrs was necessary. As could be expected some hydrolysis of the internucleotide pyrophosphate linkage occurred during this time. Fluorimetrical analysis of the reaction mixture according to the method of Wassink and Mayhew |39| revealed that 15% of the total FAD had been hydrolyzed.

5) pH meter reading, the values are thus not corrected for the deuterium activity.

creasing concentration and was finally located at lowest field. In addition it is remarkable that the enhancement found with this sample in a double irradiation experiment is only a few percent of that expected according to the results reported in Table 4, but in agreement with published data [30]. Apparently some of the decoupling power is quenched by the presence of the adenine ring in FAD. Thus these results indicate that the position of the resonance due to CH(6) and CH(9) is determined by concentration, pH of the solution, the polarity of the solvent and the intramolecular complexation.

The shifts induced on the aromatic proton resonances upon methylation of ring A are summarized in Table 8. These results show that the effects are much more symmetric for the alloxazines than for the isoalloxazines and their salts. The substitution effects are not additive and are in fact somewhat smaller than the sum of the ortho and meta effects. The para effects, however, are extremely high; e.g. *V*, *VI*, *VII*, *VIII*, *XIII*, *XIV* and *XX*. This indicates a more bended conformation of the aromatic ring of these compounds. This conclusion is further

Table 8 RELATIVE SHIFTS (in Hz) OF THE AROMATIC PROTONS DUE TO METHYL SUBSTITUTION

The shifts are to high field relative to 3,10-dimethyl-isoalloxazine (I), 1,3-dimethylalloxazine (IX) and 1,10-ethylene-3-methylisoalloxazinium perchlorate (XVII), respectively

Compound	Ring Position			
	6	7	8	9
II	14.7	-	11.0	5.1
III	8.8	11.5	-	12.9
IV	20.3	-	-	13.6
V	-	-	13.6	20.7
VI	32.6	-	30.1	-
VII	-	-	-	29.1
VIII	36.1	-	-	-
X	21.4	-	14.0	9.6
XI	11.1	12.6	-	20.4
XII	25.7	-	-	24.2
XIII	-	-	17.3	36.2
XIV	35.3	-	-	-
XVII	19.3	-	14.2	9.3
XVIII	13.7	15.6	-	16.9
XIX	25.7	-	-	17.4
XX	-	-	17.5	26.3

supported by the enhancement observed at position 9 upon irradiation of the CH_3(6) in *V*, *XXIII* and *XXI*. A bended conformation is expected for the trimethylated ring A but this is, to a lesser degree, already the case for the disubstituted compounds.

The influence of added methyl groups on originally present methyl groups in ring A is for all compounds studied almost additive but non-symmetric (Table 4). An exception to this rule are *VI* and *VIII* (Table 2) and *XXV* (Table 5), where a larger shift is observed upon methylation. This effect is caused by the mutual influence of the methyl groups at positions 9 and 10 and is reflected either in the resonance position of the N(10)-CH_3 or in that of the CH_3(9). From Table 4 it is also apparent that methylation at positions 6 and 7 induces less shift as compared with that at 8 and 9 position. The relative low shift values observed for the isoalloxazines (Table 4), compared with the other compounds, indicate less aromaticity. This is also apparent from the large difference between the resonances of the 7 and 8 methyl groups of isoalloxazine as compared with those of alloxazines.

The results given in Table 2 indicate that the aromaticity of alloxazines is higher than that of isoalloxazines (cf. e.g. *I* and *IX*). Moreover, comparison of (iso)alloxazine with quinoxaline and phenazine clearly shows that (iso)alloxazine resembles much more quinoxaline (*XV*) than phenazine (*XVI*). From this it can be concluded that ring C of the (iso)alloxazine system does not directly contribute to the ring current, as expected, but excerts indirect effects via the carbonyl groups on rings A and B as mentioned above. The same can be said for the cations (Table 5) where the N(3)-CH_3 group is, as compared to that of the neutral molecules (Table 2), influenced only little upon introduction of a positive charge into the molecule (*XXII* - *XXV*). Only when the N(1) position is unsubstituted (*XXVI* - *XXX*), and thus allowing for conjugation with ring C, a larger shift of the N(3)-CH_3 resonance is observed. In addition the comparison of the N(1)-CH_3 resonance of *VIII* - *XIV* with those of the cationic analogs *XXII* - *XXV* reveals that the charge at N(1) is not much altered.

On the other hand, in contrast to expectation, the frequency difference between the two methylene groups of the bridged compounds *XVII* - *XXI* (Table 5) is smaller than the difference between the frequencies of the N(1)- and N(10)-methyl groups of the cations *XXII* - *XXV* (Table 5). This indicates that in the bridged compounds the positive charge is distributed between N(1) and N(10), while in *XXII* - *XXV* the positive charge is mainly localized on N(10). This would explain the relative high field position of the CH(9) resonance in the bridged compounds

as compared to that of the other quarternary salts (Table 5), because in the latter compounds the electron withdrawal from the CH(9) position is more effective. Support that this is a reasonable interpretation comes also from other results. The cationic isoalloxazines given in Table 5 form addition products with methoxide, leading to a neutral molecule. Methoxide adds to the 9a position of the isoalloxazine molecules |29|. Thus addition of methoxide to the ethylene bridged compounds (*XVII - XXI*) leads to a large upfield shift of both methylene groups (about 70 Hz), while with the N(1)-methylated compounds (*XXII - XXV*) addition of methoxide results also in an upfield shift of the N(10)-methyl resonance (to 372 Hz), but the N(1)-methyl resonance is affected only to a minor extent. Furthermore, if we now compare the resonances of the aromatic protons of *XVII* (Table 5) with those of quinoxalinium (*XXXI*) or phenazinium (*XXXII*) perchlorate it is seen that *XVII* is more alike to *XXXI* than to *XXXII*. However, in *XVII* the CH(7) resonance appears at relative high field, thus revealing again the relative high electron density in ring A and B while the CH(6) and CH(8) resonances are shifted to relative low field due to the mentioned influence of the carbonyl groups.

It is evident from these results that proton nuclear magnetic resonance experiments do yield in part only the required information necessary for the elucidation of the molecular structure of (iso)alloxazines. Although the results give valuable information concerning the conformation of the molecule more accurate information about the charge distribution in the molecule can be obtained from ^{13}C spectra. For this reason such a study has been undertaken. The results will be published soon.

ACKNOWLEDGEMENT

We are thankful to Prof.Dr. J.L. Fox, University of Texas, Austin (U.S.A.), for the communication of the MINDO/3 calculations prior to publication, to Mr. J.S. Santema for the preparation of some compounds, to Mr. R.J. Platenkamp for the help in the calculation of the theoretical values and valuable discussion, to Mr. B.J. Sachteleben for the preparation of the Figures, and to Miss A.H.W. Trip for the patience in preparing the Tables. We are indebted to Ciba-Geigy A.G., Basel (Switzerland), for the performance of the elemental analyses.

This study has been carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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V ^{13}C-NMR study on isoalloxazine and alloxazine derivatives

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SUMMARY

1 A series of isoalloxazine and alloxazine derivatives have been investigated.
2 The synthesis of selectively ^{13}C-enriched derivatives made it possible to assign unambiguously the signals due to the quarternary carbon atoms at position 4, 4a and 10a of the isoalloxazine ring system.
3 The assignment of the other resonances was ensured by the use of selectively deuterated and chemically modified compounds as well as by decoupling techniques.
4 The assignments given in this paper differ in part from those published by Breitmaier and Voelter on FMN and FAD (Eur.J.Biochem. (1972) 31, 234).
5 The solvent dependence of the resonances has been studied in dioxan/water mixtures.
6 The experimental data are compared with published MO calculations and discussed.

1 INTRODUCTION

In context with started ^{1}H- and ^{13}C-NMR studies on flavoproteins it is of importance to characterize the prosthetic group of these proteins by this technique. In a previous paper |1| we have described the proton nuclear magnetic resonance characteristics of alloxazines and isoalloxazines. Breitmaier and Voelter |2| have conducted a ^{13}C-NMR study on flavin-adenine-dinucleotide (FAD) and riboflavin-5'-monophosphate (FMN), the natural constituents of flavoproteins. These authors could, however, not assign all resonances unambiguously. Furthermore, preliminary ^{13}C-NMR results obtained in our laboratory indicated that our

assignments were not in accordance with those published by Breitmaier and Volter |2|. We have, therefore, investigated this problem in detail and found that an unequivocal assignment was only possible employing chemically and isotopically modified flavin (isoalloxazine) derivatives. The results are presented in this paper.

2 MATERIALS AND METHODS

The ^{13}C-NMR spectra were taken on a Varian XL-100 spectrometer operating at 25.2 MHz. The instrument is equipped with a 16 K Varian 620-L computer. All spectra were acquired in the Fourier transform mode using 12 mm sample tubes. Proton noise decoupling was used in all experiments, except those in which single frequency decoupling was employed to aid peak assignments. The following instrumental conditions were used: acquisition time 0.8 sec, pulse width 10 µsec ($\pi/2$ = 28 µsec), spectral width 5000 Hz. The peak positions were determined from the computer generated printout, using TMS as internal standard. The chemical shifts given for a particular compound agreed well with values determined independently at different times and are within 3 Hz of each other. The sample temperature was 26°C. The compounds were dissolved in deuterated chloroform (99.8 atom %, Merck, Germany) containing 10% tetradeuterio-^{12}C-methanol (99.5 atom % D, 99.95 atoms % ^{12}C, Merck Chemical Comp., Germany). It was necessary to add methanol to the chloroform solution to prevent a slow destruction of the (iso)alloxazines. The number of transients accumulated varied between 20000 and 70000 depending on the solubility of the compound under investigation. Always saturated solutions were used resulting in concentrations varying from 0.1 M to 0.01 M.

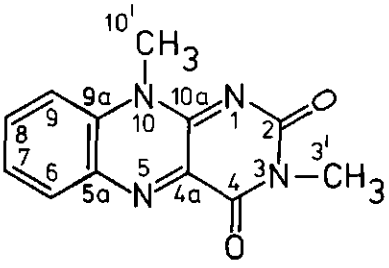
The synthesis of the compounds used in this study has been described elsewhere |1| and references therein. The selectively ^{13}C-labelled barbituric acids (starting materials for the isoalloxazine synthesis) were obtained by condensation of urea with ^{13}C(2)- or $^{13}C_2$(1,3)-diethylmalonate (90 atom %, Prochem, England) in the presence of sodium ethoxide according to Murray |3|. Fieser and Fieser |4| have suggested that an excess of ethoxide, in place of an equimolar amount of ethoxide with respect to urea and malonate as usually employed, might speed up the reaction. We have explored this suggestion and found that a twofold excess of ethoxide does not only shorten the reaction time but also gives higher yields of barbituric acid. Thus a mixture of 1.0 g ^{13}C-labelled diethylmalonate, 0.336 g urea, 0.4 g sodium and 8 ml absolute ethanol gave 0.71 g barbituric acid (91%, lit. 72-78%) with m.p. 254-255°C (not corrected, lit. 254°C) after 5 hr refluxing and work up of the reaction mixture.

$^{13}C(4a)$- and $^{13}C_2(4,10a)$-N(10),7,8-Trimethylisoalloxazines were obtained by condensation of the appropriate barbituric acids with N,3,4-trimethyl-6-(p-carboxyphenylazo)-anilin in a mixture of glacial acetic acid and butanol as described by Tishler et al. |5|. The yield was 92% (lit. 88%). The compounds were then methylated at N(3) and purified as described elsewhere |1|.

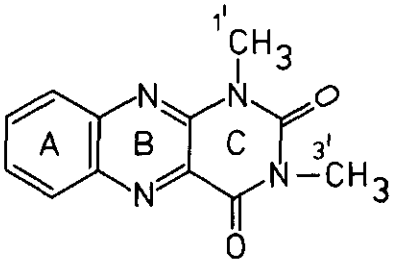
3 RESULTS

The structure of the isoalloxazine and alloxazine molecules are shown in Scheme 1 together with the internationally accepted ring numbering |6|. The

Scheme 1



iso-alloxazine



alloxazine

assignment of the resonances of (iso)alloxazines is based mainly on the comparison of spectra of various derivatives. The correctness of the so assigned resonances was then further checked by taking spectra under off resonance conditions and also employing isotopically substituted (deuterium, ^{13}C-enriched) compounds.

It is often a problem to assign unambiguously resonances to the quarternary carbon atoms in a molecule, since these resonances exhibit often very weak intensities in noise decoupled spectra. In the isoalloxazine molecule at least six quarternary carbon atoms are present, namely at position 2, 4, 4a, 5a, 9a and 10a. The resonances due to 5a and 9a in a spectrum can be recognized by indirect effects on these resonances, i.e. by methyl substitution in ring A. On the other hand, the resonances due to the carbon atoms of the two carbonyl groups can be expected to appear at lowest field. The only two resonances, which will be more difficult to assign, are those due to the 4a and 10a carbon atoms. For this reason

compounds enriched with ^{13}C at these particular positions were synthesized. In fact derivatives of these compounds were prepared for use in ^{13}C-NMR experiments on flavoproteins.

Fig. 1 shows the spectra of the selectively ^{13}C-enriched isoalloxazines. The spectrum of the compound enriched in position 4a is given in Fig. 1A where also

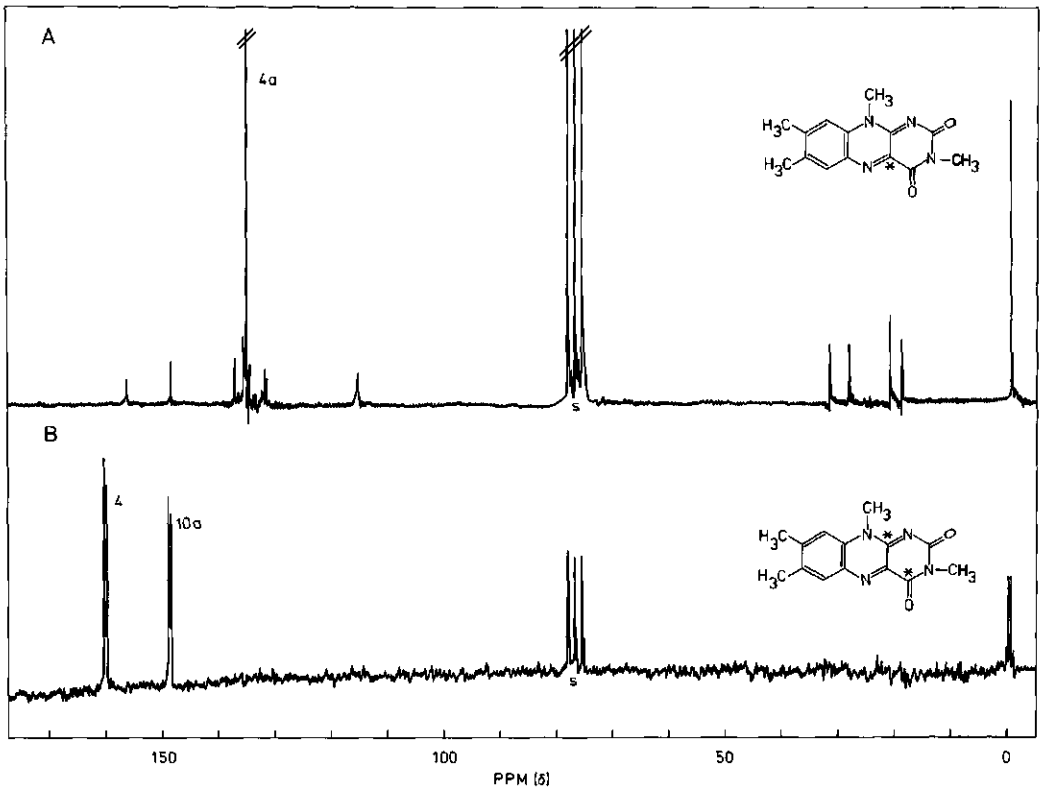


Fig. 1 ^{13}C-ENRICHED (90 ATOM %), PROTON NOISE DECOUPLED ^{13}C SPECTRA OF 3,7,8,10-TETRAMETHYLISOALLOXAZINES IN $CDCL_3/CD_3OD$ (9:1 BY VOL.). The asterisk indicates position of ^{13}C-enrichment in the isoalloxazine ring system. s = resonances due to the solvent.

some of the resonances due to the natural abundance ^{13}C-atoms are seen. The spectrum of the 4,10a-$^{13}C_2$-enriched compound is shown in Fig. 1B. The through-bond coupling between the two carbons is 0.4 ppm. This has been confirmed by ^{13}C decoupling experiments. To reach an unequivocal assignment we took a ^{1}H-NMR spectrum of this compound. The proton spectrum exhibits two doublets due to coupling of the ^{13}C-atoms with the N-methyl groups. Decoupling the ^{13}C resonance at lowest field (Fig. 1B) transformed the doublet of the N(3)-methyl resonance signal in the

^{1}H-NMR spectrum into a singulet. Similarly, decoupling the other ^{13}C resonance gave a singulet of the resonance signal due to the N(10)-methyl in the proton resonance spectrum. The proton resonance frequency of the N-methyl groups are known from a previous study |1|. These results unambiguously show that the resonance signal at lowest field in Fig. 1B is due to C(4) and the resonance signal at higher field due to C(10a). In comparing the spectrum of Fig. 1B with that of Fig. 1A it becomes evident that in the former spectrum the natural abundance resonance signal due to the C(4) atom is not observed whereas the resonance signal due to C(10a) is clearly seen. The other resonances have been assigned based on the influence of methyl substitution of ring A which affects the position as well as the intensity of the other resonances in the ring. The results obtained from isoalloxazines are summarized in Table 1. These data show that the resonance at about 156 ppm is independent on substitution of ring A. This fact and the low field position of this resonance makes it feasible to assign it to the C(2) atom. The resonances due to 5a and 9a will be influenced by methyl groups placed in ortho, meta or para position to them. The methyl substituent effects are summarized in Fig. 2. Thus introduction of a methyl group into position 7 of I leading

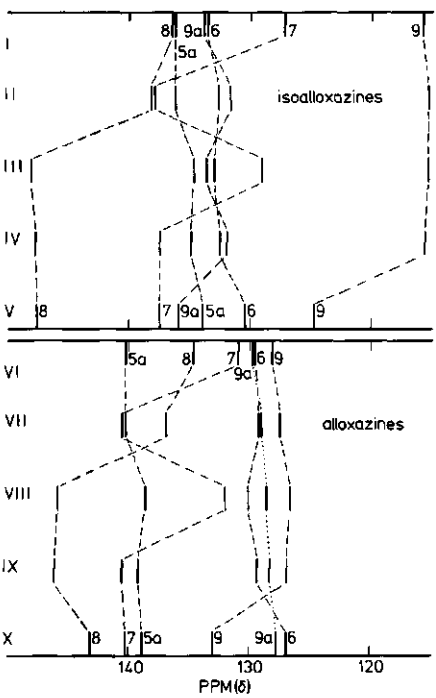
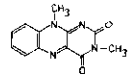
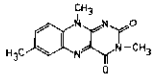
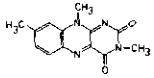
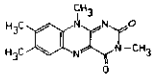
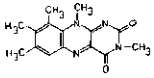
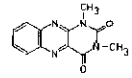
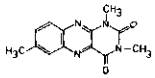
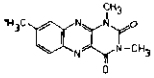
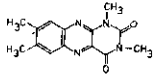
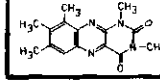


Fig. 2 CORRELATION DIAGRAM OF ^{13}C-NMR CHEMICAL SHIFTS OF ISOALLOXAZINE AND ALLOXAZINE DERIVATIVES. The Arabic numbers refer to the (iso)alloxazine ring numbering (cf. Scheme 1). The Roman numbers refer to the compounds given in Table 1.

TABLE 1

^{13}C CHEMICAL SHIFTS (in ppm) OF ISOALLOXAZINE AND ALLOXAZINE DERIVATIVES[a]

Compound No. / R.P.[b]	2	4	4a	5a	6	7	8	9	9a	10a	1'	3'	7'	8'	9'	10'
 I	156.6	160.3	n.o.[c]	136.2	133.5	127.2	136.4	115.8	133.8	149.7	-	28.8	-	-	-	32.3
 II	156.5	160.3	136.7	136.2	132.6	137.9	138.2	115.4	131.7	149.3	-	28.8	20.9	-	-	32.3
 III	156.7	160.5	135.7	134.7	133.0	129.1	149.1[d]	115.4	133.7	149.1[d]	-	28.8	-	23.0	-	32.2
 IV	156.7	160.6	135.5	135.0	132.6	137.5	148.8	115.7	132.0	149.2	-	28.8	19.5	21.6	-	32.2
 V	156.8	160.6	134.9	134.0	130.5	137.5	148.7	124.8	136.0	151.3	-	28.7	19.9	20.7	18.0	41.7
 VI	151.2	160.3	145.7	140.3	129.7	131.0	134.7	128.2	129.8	143.8	29.8	29.4	-	-	-	-
 VII	151.1	160.4	145.2	140.3	129.3	140.4	136.9	127.6	129.2	142.2	29.6	29.2	21.8	-	-	-
 VIII	151.1	160.3	145.6	138.7	130.2	132.1	145.9	126.8	128.7	143.8	29.6	29.2	-	22.4	-	-
 IX	151.2	160.6	145.3	139.3	129.4	140.6	146.2	127.1	128.5	142.8	29.6	29.2	20.3	20.9	-	-
 X	151.8	161.4	n.o.[c]	139.0	127.1	140.3	143.3	133.2	128.0	143.3	29.5	29.1	21.5	17.2	13.4	-

a) Spectra were obtained in the solvent mixture $CDCl_3/CD_3OD$ (9:1 by vol.).
b) R.P. = Ring Position.
c) n.o. = not observed.
d) Too intense to be only 10a.

to II influences the resonance position of C(9a) (para position) to a greater extent than that of C(5a) (meta position). As expected a reversed effect is observed on these resonances in employing III. These assignments are in full agreement with results obtained with IV and V. In addition the resonances due to C(7,8,9) are easily assigned on the basis of the large C-I shift upon methyl substitution as shown in Fig. 2. Furthermore the results obtained from V support the assignment of the resonance signal at about 132 ppm to C(6) (para shift) (Fig. 2).

The high field resonances, all due to methyl groups, can be divided into two groups (Table 1). Comparing the resonances of I with those of II-V it is obvious that the resonances at about 20 ppm are due to methyl groups bound to C-atoms and those at about 30 ppm due to methyl groups bound to N-atoms. Mere inspection of the data presented in Table 1 leads already to the right assignments of the C-CH_3 resonances. In order to support further these assignments and to confirm at the same time the assignment of the other resonances some off--resonance spectra were recorded and some selectively deuterated compounds have been employed. The proton noise decoupled ^{13}C-NMR spectrum of II is shown in Fig. 3A. The spectrum shown in Fig. 3B was obtained under single frequency off resonance conditions |7|. An irradiation frequency of 250 Hz (from TMS) in the proton spectrum was employed. This frequency corresponds almost to the resonance position of the CH_3(7) in the proton spectrum |1|. From this experiment the following is expected: a) the ^{13}C resonances due to quarternary atoms will not be influenced, i.e. they will appear as singulets; b) depending on the number of protons C-atoms bearing protons will split up in multiplets; c) the closer the proton resonance position of a particular group to the irradiating frequency the more reduced become the proton-^{13}C couplings in the ^{13}C spectrum. In the proton resonance spectrum, in going from high to low field, the resonance signals due to the following groups appear in the order: N(3)-CH_3, N(10)-CH_3, CH(9), CH(8), CH(6) |1|. At the high field part of Fig. 3B two quartets and a broadened singulet are seen which are due to the 3 methyl groups of II. The broadened signal, at highest field, exhibits in fact a small residual splitting and is thus closest to the irradiation frequency. This signal must, therefore, be assigned to CH_3(7). The quartet centered at 28.8 ppm shows a smaller residual splitting than that centered at 32.3 ppm. This proves, knowing the proton resonance frequencies, that the ^{13}C signal of N(3)-CH_3 is located at higher field than that of N(10)-CH_3 (Table 1). The same conclusion can be derived in comparing the results obtained from isoalloxazines with those obtained from alloxazines. The low field part of the spectrum given in Fig. 3B, except the doublet due to CH(9), is shown in an expanded

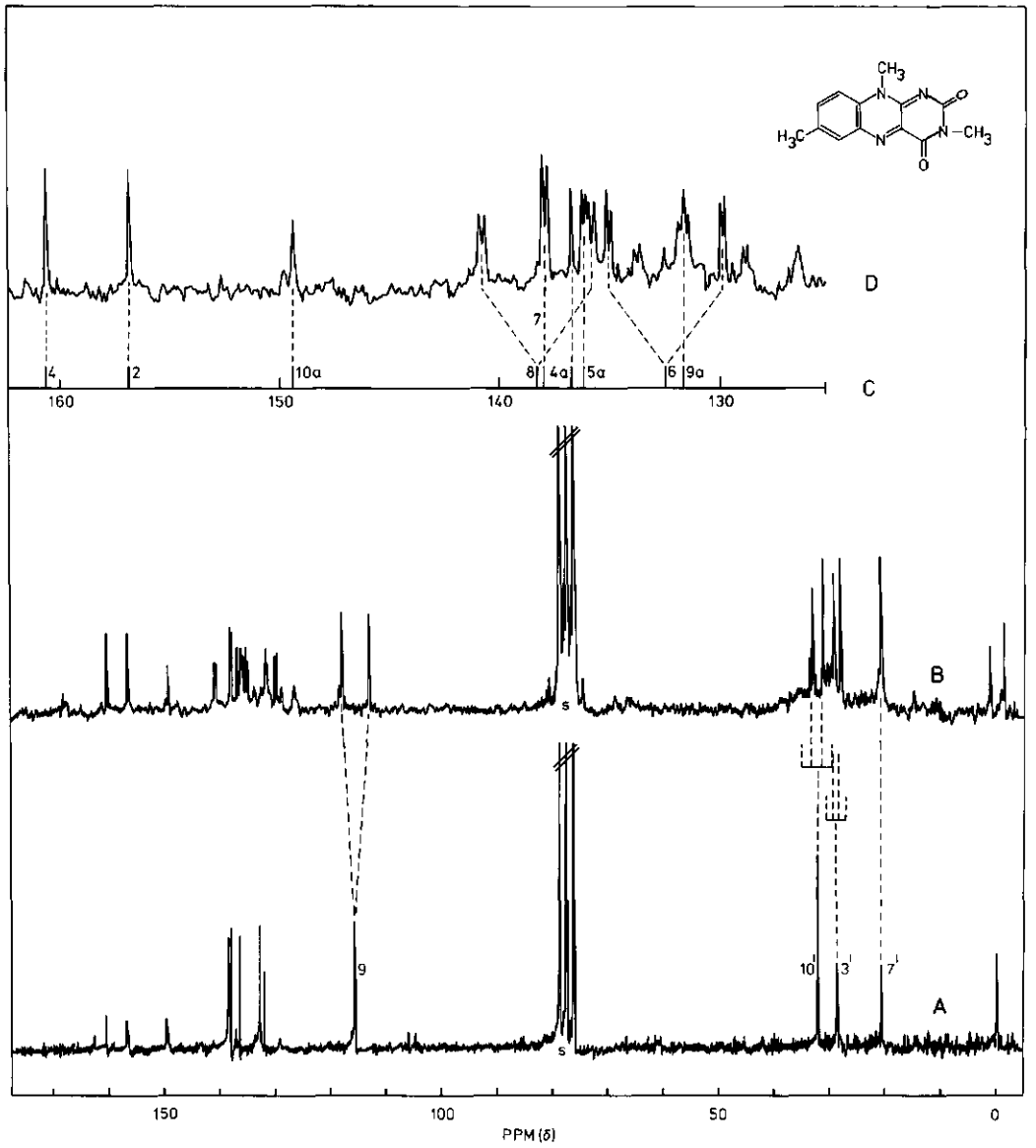


Fig. 3 NATURAL ABUNDANCE ^{13}C SPECTRA OF 3,7,10-TRIMETHYLISOALLOXAZINE (II) IN $CDCL_3/CD_3OD$ (9:1 BY VOL.). A: proton noise decoupled spectrum; B: spectrum obtained under off resonance conditions, the irradiation frequency was at 250 Hz from TMS; D: expanded low field part of the spectrum shown in B; C: stick spectrum corresponding to frequencies observed under noise decoupling conditions. s = as in Fig. 1.

scale in Fig. 3D and the corresponding stick spectrum is given in Fig. 3C. The correctness of the aromatic CH assignments has been checked by a similar experiment but using an irradiation frequency of 810 Hz from TMS, low field to CH(6) resonance, in the proton spectrum |1|. The residual splittings and NMR pattern found in this experiment were in full accord with the assignments given in Table 1. It should be noted that in Fig. 3 some small splittings are observed which are due to coupling with protons other than those directly bound to a particular carbon atom. Furthermore, selective deuteration of IV at position 9 results in a large decrease of the intensity and a small shift of the signal at 115.7 ppm as expected. Similarly selective deuteration of CH_3(8) in IV confirmed the assignment of the resonance at 21.6 ppm.

The signals observed with alloxazine derivatives have also been assigned on the basis of the substituent effects (Fig. 2). In addition the results obtained from isoalloxazines were used where possible to achieve an unambiguous assignment since with alloxazines no enriched compounds were available. The quarternary carbon atoms at positions 2, 4, 4a and 10a have been assigned on the observation that the resonances due to these carbon atoms are not affected upon methylation of ring A as also has been observed with isoalloxazines. Furthermore, from a structural point of view with respect to isoalloxazines, the resonance at lowest field was assigned to the CO(4). This assignment is not really dubious considering that in ^{13}C-enriched 2a,3,7,8,10-pentamethyl-isoalloxazinium perchchlorate |8| the CO(4) resonates also at about 160 ppm, i.e. going from the neutral to the cationic species the resonance due to C(4) is affected only minor. Furthermore the resonance signal due to the C(10a) can easily be recognized on its much larger amplitude than that of C(4a) because of the methyl group in the neighbourhood of C(10a). The only remarkable difference between the quarternary carbons of isoalloxazines and those of alloxazines is that the intensities of the signals due to C(7,8) in VII, VIII and IX are much more intense in noise decoupled spectra than those of the corresponding isoalloxazines. The intensities are in fact comparable with those of CH resonances.

Similarly as with the isoalloxazines the assignments were confirmed by single frequency (at low and high field of the spectra) off resonance experiments. Some of these data are given in Table 2. The only assignments not completely sure are those due to the carbons at position 5a and 9a since the methyl substituent effects (Fig. 2) are less informative for these positions than those for isoalloxazines. Thus the para shift in VII on C(9a) is only -0.6 ppm with respect to VI, while the meta shift in VIII is even larger (Fig. 2). Also the summed effects are small (IX). Furthermore, the resonance signal due to C(9a) in VIII is

Table 2. OFF-RESONANCE DECOUPLING EXPERIMENTS ON 1,3,7,-TRIMETHYLALLOXAZINE (VII)[a] AND 1,3,8-TRIMETHYLALLOXAZINE (VIII)[a]

VII R.P.[b]	^{13}C chemical shift (in ppm)[c]	^{1}H chemical shift (in ppm)[d]	^{1}H irradiation frequency 810 Hz	830 Hz
			e)	e)
6	129.3	7.98	0.2	0.6
7	140.4	-	s[f]	s[f]
8	136.9	7.79	0.7	1.3
9	127.6	7.91	0.4	0.9

VIII R.P.[b]			820 Hz	850 Hz	371 Hz
			e)	e)	e)
6	130.2	8.08	0.2	0.8	5.3
7	132.1	7.66	1.1	1.6	4.7
8	145.9	-	s[f]	s[f]	s[f]
9	126.8	7.80	0.9	1.5	5.0
1'	29.6	3.70	4.5[g]	4.7[g]	0.0[g]
3'	29.2	3.45	4.6[g]	4.7[g]	0.4[g]
8'	22.4	2.62	4.3[g]	4.4[g]	1.7[g]

a) The solvent is CDl_3/CD_3OD (9:1 by vol.)
b) R.P. = Ring Position.
c) Noise decoupled.
d) Taken from ref. |1|.
e) Residual splittings (in ppm) observed upon irradiation at the indicated frequency.
f) s = small quartet splitting of about 0.2 ppm due to CH_3.
g) Quartet splittings.

difficult to recognize in the spectrum. In the noise decoupled spectrum there is a definite but very narrow and weak signal at 128.7 ppm. In off resonance spectra also a very weak signal is observed at 128.7 ppm. The intensity of this signal is very weak as compared to those of C(5a,4,2) and C(4a).

The dependence of the ^{13}C resonances on the polarity of the solvent has been investigated using tetra-acetylriboflavin which is very soluble in pure deuterated dioxan and D_2O as well as in mixtures thereof and allows accumulation of spectra within a reasonable time. The data are collected in Table 3. For comparison also

Table 3. THE DEPENDENCE OF ^{13}C CHEMICAL SHIFTS (in ppm) OF TETRAACETYLRIBOFLAVIN ON THE POLARITY OF THE SOLVENT USING MIXTURES OF DIOXAN AND WATER

R.P.[a] \ D_2O	0 %	1%	5 %	10 %	30 %	FMN[b]	IV[c]
4	159.8	n.o.[d]	n.o.[d]	n.o.[d]	161.2	160.2	160.6
2	155.7	155.7	-	-	158.0	157.6	156.7
10a	151.7	151.6	151.5	151.6	151.6	149.6	149.2
8	146.4	146.5	147.2	147.5	148.9	150.8	148.8
7	136.4	136.5	136.9	137.3	138.2	139.6	137.5
4a	n.o.[d]	n.o.[d]	n.o.[d]	n.o.[d]	137.1	-	135.5
5a	134.6	134.6	134.7	134.8	135.0	134.2	135.0
6	132.8	132.8	132.7	132.6	132.4	130.0	132.6
9a	132.0	132.0	132.0	132.1	132.2	131.4	132.0
9	116.5	116.5	116.6	116.6	116.8	117.2	115.7

a) R.P. = Ring Position.
b) Taken from ref. |2|. The solvent was pure D_2O.
c) These values are given for comparison. The solvent is $CDCl_3/CD_3OD$ (9:1 by vol.), see Table 1.
d) Not observed.

the data obtained from IV in $CDCl_3$ are given. The values given for FMN are those published by Breitmaier and Voelter |2| except that the assignment of the resonances is according to Table 1. The data of Table 3 show that the resonances due to the carbon atoms of ring B are not influenced by the polarity of the solvent. On the other hand the ^{13}C resonances due to positions 7, 8, 2 and 4 are most influenced by the polarity of the solvent.

4 DISCUSSION

It is well known that ^{13}C resonances reflect the electron density distribution in a molecule in a much higher degree than the proton resonances. Therefore, it can be expected that the large chemical difference between alloxazine and isoalloxazine will be recognized in the ^{13}C spectra. It is interesting to compare the experimental data described in this paper with data obtained by theoretical calculations. Unfortunately only calculations on isoalloxazines but not on alloxazines have been published |9, 10|. In comparing our data with π-electron density calculations as conducted by Grabe |9| and Fox et al. |10| it is found that little correlation exists. This is in contrast to published data |11, 12| where a good correlation is

found between the calculated π-density and experimental data on aromatic protons and carbons. However Gawer and Daily |13| observed that such a correlation does not, or to a much lesser degree, exist for protons in heterocycles. A similar trend was observed on ^{1}H-NMR data obtained from (iso)alloxazines |1|. It could thus be expected that also ^{13}C-data obtained from heterocycles would show less agreement between calculated and experimental data. This would indicate that the σ-π separability conditions |14| do not hold for heterocycles, as is apparent from the high polarisation of the σ-core of e.g. pyrrole and pyridine as shown by ab initio calculations |15|. In calculating the charge with PPP-SCF methods, this polarization is not accounted for which leads to incorrect total electron densities |cf. 16|.

Table 4. COMPARISON OF CALCULATED NET CHARGE ^{13}C CHEMICAL SHIFTS (in ppm) OF QUINOXALINE, 3,7,8,10-TETRAMETHYLISOALLOXAZINE (I) AND 1,3-DIMETHYLALLOXAZINE (VI)

	Quinoxaline		I		VI
Ring Position	net charge[a)b)]	chemical shift[b)]	net charge[a)c)]	chemical shift	chemical shift
8	0.009	129.1	0.042	136.4	134.7
7	0.009	129.1	-0.051	127.2	131.0
9	-0.013	129.0	-0.075	115.8	128.2
6	-0.013	129.0	0.021	133.5	129.7
9a	0.087	142.4	0.103	133.8	129.8
5a	0.087	142.4	0.009	136.2	140.3
10a	0.060	144.7	0.264	149.7	143.8
4a	0.060	144.7	-0.099	136.7	145.7
2	-	-	0.705	156.6	151.2
4	-	-	0.624	160.3	160.3

a) The net charge is given as a difference between the total amount of valence electrons (4 for carbons) and the calculated density.
b) Taken from ref. |17|. The published chemical shifts are relative to benzene (127.7 ppm). The given chemical shifts were calculated relative to TMS.
c) See footnote 1).

In table 4 the net electron densities and the experimental values of quinoxaline, 3,10-dimethyl-isoalloxazine (I) and 1,3-dimethyl-alloxazine (VII) are compared. Electron densities, obtained by CNDO/2-calculations, are resonances for quinoxaline, which are given for comparison, are taken from Pugmire et al. |17|. The net electron densities for 3,10-dimethyl-isoalloxazine (I) were calculated by Fox[1)].

1). Personal communication; the data were obtained by an all valence electron molecular orbital calculation within the MINDO/3 formalism.

To correlate the distortion of the (twofold) symmetrical charge distribution with the resonance position the symmetric pairs with respect to quinoxaline are compared in Table 4. From Table 4 it follows that only a rough correlation is obtained. For quinoxaline the carbons with the lowest electron density are indeed less shielded, but the smaller differences in the calculated electron density are not reflected by the resonances. In isoalloxazines the C(8) is less shielded and thus somewhat positive, while C(7) is somewhat more shielded, both in accordance with the calculation of Fox[1]. The resonance of the carbon atom at position 9 is shifted quite far upfield, thus being negative, while that of C(6) is located at relative low field, also as predicted by Fox. The resonances due to C(9a) and C(5a) are located at high field but reversed in the order as predicted by the calculated values. On the other hand the resonance position of C(4a) (at high field) and C(10a) (at low field) are in agreement with the calculated relative charges. Furthermore the very low field position of the carbonyls is in agreement with the calculated low electron densities on these carbons (they miss almost 3/4 electron). The order however is again in disagreement with the prediction. From this it can be concluded that the calculations predict, if compared with the symmetric quinoxaline, approximately the shifts, but the absolute values of the electron densities cannot be trusted[2]. This conclusion is best illustrated by the fact that C(4a) resonates at about the same frequency as C(8) and C(5a) but C(4a) possesses the largest calculated negative charge whereas both C(8) and C(5a) carry positive charge. The results suggest that the 6, 8 and 10a carbons are positive. This means that isoalloxazine is already in apolar solvents strongly conjugated from the 8 and 6 positions via 10a to the 2 carbonyl group. It can be expected that in polar solvents the carbonyl groups tend to be more electron withdrawing, thus polarizing the molecule to a larger extent. Due to the fact that 6 and 8 are already positive it is reasonable to predict that these electrons then have to come from positions 9 and 9a. The ring current will not be altered much because the electron withdrawal and the π-delocalisation will compensate each other. This should result in a shift of the electron towards C(10a) causing the molecule to become more alike alloxazine. From Table 3 this effect is not very apparent except for the downfield shift of *both* resonances due to C(7) and C(8). The large solvent induced downfield shift of the resonance due to C(7) is quite unexpected. This may

2). We assume that there is a direct correlation between charge density and resonance position. There are however also indirect effects, such as ring currents, charges on neighbouring atoms etc., which are usually not considered.

indicate that this carbon atom is not as isolated in the isoalloxazine system as usually assumed. The solvent effects are however much better reflected in the proton NMR spectra of (iso)alloxazines |1|. The reason why these effects are less apparent in the ^{13}C spectra could be ascribed to the higher total valence electron density at the carbon atoms (4 electrons) as compared to that at protons (1 electron) and also to the polarization of the molecule by the carbonyl groups exerting their influence via the π-system.

The ^{13}C-NMR data obtained from alloxazines, when compared with those obtained from quinoxaline and isoalloxazines, can yield valuable information for future calculations on these molecules. From Table 4 it is apparent that alloxazine resembles much more quinoxaline than isoalloxazine. This conclusion is in agreement with that drawn from data obtained by ^{1}H-NMR |1|. Thus the twofold symmetry observed with quinoxaline is decreased to a much lesser degree in alloxazine than in isoalloxazine. In fact the lower symmetry in alloxazine, as compared to quinoxaline, is reflected most obviously only by the resonances due to the carbons at position 5a and 9a. In addition only the resonance due to C(8) in alloxazines appears at significant lower field than that of the corresponding C-atom in quinoxaline indicating the involvement of this atom in the former compounds in the conjugation with the carbonyl groups. Moreover the fact that the ^{13}C-chemical shifts of the C(4a) and C(10a) of alloxazines are more alike than those of isoalloxazines suggests that in the former compounds both carbonyl groups and in the latter compounds mainly the carbonyl group at position 2 are involved in the conjugation of the (iso)alloxazine ring system. The relative high field position of the C(2) resonance of alloxazines, as compared to that of isoalloxazines, suggests delocalisation of the lone electron pairs particularly of N(1) and also of N(3) towards the C(2) atom. This interpretation is in agreement with acidity constants showing that N(1)H and N(3)H are more acidic by about two orders of magnitude than the N(3)H of isoalloxazine |18|.

It is noteworthy to mention that the substituent effects observed with V and X differ from those of the other compounds (Fig. 2). Remarkable is the large asymmetry observed on the resonances due to the carbon atoms in ortho position to the $C(9)CH_3$ in V i.e. 4.0 ppm on the resonance due to C(9a) and -0.1 ppm on the resonance due to C(8) of V relative to IV. A similar but smaller effect is observed with X. These effects indicate that the mutual influence of the methyl groups causes a distortion of the planarity of the isoalloxazine ring. The same effect has been observed on the ^{1}H-NMR spectra |1|.

Comparing the resonance assignment presented in this paper with those published |2| it is seen that five out of the twelve resonances exhibited by the iso-

alloxazine ring were differently assigned by Breitmaier and Voelter |2|. Since we used an apolar solvent instead of water the different assignment could be due to the difference of the polarity of the solvent. This is refuted by the results given in Table 3 which clearly show that the ^{13}C chemical shifts are not influenced drastically by the polarity of the solvent. In addition the observed intensity of the lines is also much more compatible with the assignment presented in this paper. Thus the resonance assigned to C(4a) is a rather intensive line in the published spectra whereas in our spectra this line exhibits a weak intensity as could be expected for a quarternary carbon atom having no adjacent protons. Only the fact being in possession of the enriched compounds allowed us to assign this weak line unambiguously in most of the spectra. In addition it was observed that even the resonance of C(4a) in $^{13}C(4a)$-enriched FMN in aqueous solution, as compared to organic solvents, was very difficult to detect in the ^{13}C spectrum[3]. This result suggests that water interacts probably quite specific with the C(4a) atom of the isoalloxazine ring system, which drastically changes the line shape of the C(4a) resonance.

The data presented in this paper show that only a rough correlation exists between calculated and experimental values. To gain a better understanding of the chemical entity of isoalloxazines, the natural constituents of flavoproteins, more accurate theoretical values are required. We believe that better theoretical results could be achieved starting by the calculations with the more symmetrical molecule alloxazine and employing the methyl substituent effects. The results thus obtained could then form the basis for calculations on isoalloxazines. Such an approach requires extensive calculations on at least a (low cost) MINDO-level with a different parametrisation on each of the derivatives. We have, however not the means to perform such calculations at the moment in our laboratory.

ACKNOWLEDGEMENT

We are grateful to Prof.Dr. J.L. Fox, University of Texas, Austin (U.S.A.), for the communication of the MINDO/3 calculations prior to publication, to Mr. R.J. Platenkamp for valuable discussion, to Mr. B.J. Sachteleben for the preparation of the Figures, and to Miss A.H.W. Trip for preparing the Tables.

This study has been carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

3) C.G. van Schagen and F. Müller, unpublished results.

observed, which indicates a large difference of binding of the thus formed quarternary complex. All these observations will be studied in more detail on the instrument, now available in Wageningen. An observation of interest which caused us to stop measuring at the University of Groningen has to be mentioned in this context. For this type of Mn^{2+}-binding studies it is necessary to work in a buffer, which does not bind Mn^{2+}, as phosphate does. Therefore the PDC was dialysed against Tris-HCl buffer (pH 7.0), in Wageningen frozen in liquid nitrogen and then transported to Groningen. The complex showed after this treatment only a residual activity of 1-2 µmol NADH $min^{-1}.mg^{-1}$ instead of the usual 8-10 µmol NADH $min^{-1}.mg^{-1}$. If the plots, obtained from EPR-measurements, are compared with the one shown in the second article, it appears that in particular the first strong binding site differs. The data of EPR, T_1- and T_2-measurements could only be made graphically identical if instead of one strong binding site only *half* such a binding site for Mn^{2+} relative to FAD was present. This observation suggests that this strong binding site is connected with a site which is essential for the overall activity and not with the PDH-components. Another point of interest is that the data obtained from EPR, T_1 and T_2 cover each other *only* when *two* types of independent Mn^{2+}-binding sites, a strong and a weaker one, are assumed, thus excluding negative cooperativity of Mn^{2+} binding.

One additional experiment must be mentioned. It was tried to obtain information about the distance between the PDH and the lipoamide moieties. For this purpose spin-labelled PDC was titrated with Mn^{2+}. It is expected according to J.S. Leigh Jr. ((1970) Journal of Chemical Physics *52*, 2808-2612) that paramagnetic quenching of the spin label occurs when the spins are not too far apart. Quenching started to be observable at a 10-fold excess of Mn^{2+} relative to PDC, which is far above the concentration needed to occupy the specific sites (*cf.* Fig. 4, second article). Furthermore Mg^{2+} shows, although at about a 60-fold excess relative to PDC, a similar influence on the spin label spectrum. From this it can be concluded that no specific spin quenching is observed and that the distance between Mn^{2+} and spin label is thus rather large. Presumably even an extra group between TPP and the lipoyl moiety is needed to explain these data. Experiments with fluorescent and spin label analogues of TPP are now in progress to study these effects in more detail. The fact that Mn^{2+} does not quench the FAD fluorescence, but a spin label attached to the lipoyl moiety does quench, indicates that the distance between the lipoyl moiety of LTA and FAD is much smaller than the distance between the PDH-active site and the lipoyl moiety.

The fluorescence properties of the prosthetic FAD group of lipoamide dehydrogenase is also of interest. In the third article it is shown that its proper-

ties vary considerably for the different sources from which the enzyme is isolated. Particularly the observation of non-identical binding of the FAD groups in a dimeric enzyme with identical peptide chains is of interest. Knowledge about the flavin molecule is therefore also of importance. As shown in the fourth and fifth articles a large variety of isoalloxazine (the major part of FAD, determining the fluorescence properties) -analogues have been studied. Especially the cationic species are of interest because they can be visualized as stable analogues of intermediates normally occurring in reactions, catalysed by flavoproteins. The detailed information obtained from our NMR studies thus adds considerably to our understanding of the catalytic properties of the isoalloxazine. It is apparent from articles IV and V, that the theoretical description of the flavin molecule lacks still accuracy to explain the differences observed between flavin molecules as well as the different types of reactions catalysed by flavoproteins. It is clear from this thesis that molecular physical methods can be used on these large enzyme complexes and that valuable information can be obtained. With a lot of patience and carefulness much more will be learned.

Samenvatting

Dit proefschrift beschrijft enige structurele eigenschappen van het pyruvaat dehydrogenase complex (PDC). Dit complex is opgebouwd uit drie verschillende enmen die niet covalent met elkaar verbonden zijn. Samen voeren zij een serie reacties uit die, doordat deze enzymen verbonden zijn tot één geheel, veel effectiever verlopen dan wanneer de reacties op willekeurige plaatsen in de cel zouden plaatsvinden. Ten einde deze vrij zwakke hydrophobe bindingen tussen de enzymen niet te verbreken, is gebruik gemaakt van niet destructieve molucuul-fysische technieken. De technieken gebruikt zijn: Electron Spin Resonantie, Kern Spin Resonantie en Fluorescentie. Tevens werden deze technieken toegepast op enkele kleinere moleculen, die van belang zijn bij de reacties gekatalyseerd door het complex. De verkregen resultaten zijn verzameld in vijf artikelen, waarbij tevens in de discussie enige nog niet gepubliceerde resultaten zijn vermeld.

Het eerste artikel beschrijft de conformatie van het Mn^{2+}.TPP complex. Zowel magnesiumionen, die vervangen kunnen worden door mangaanionen, als thiamine pyrofosfaat (TPP) zijn essentieel voor de reactie uitgevoerd door het eerste enzym, de pyruvaat dehydrogenase component (PDH) van het PDC. In de eerste plaats wordt aangetoond dat Mn^{2+} en TPP inderdaad samen een (1:1) complex vormen, waarbij tevens de associatieconstante wordt bepaald. Verder wordt afgeleid dat TPP, dat uit twee ringen verbonden door een CH_2-groep bestaat, waarbij aan de ene ring nog een "staart" zit, zich niet in een open vorm in oplossing bevindt, maar juist vrij sterk gevouwen is.

Het tweede artikel beschrijft de opbouw van het pyruvaat dehydrogenase complex geïsoleerd uit de bacterie *Azotobacter vinelandii*. Dit complex is de kleinste van de tot nog toe gevonden pyruvaat dehydrogenase complexen. Het bevat relatief veel PDH ten opzichte van lipoamide dehydrogenase. Tevens is het opmerkelijk dat twee soorten transportenzymen aanwezig zijn, de ene soort met een molecuulgewicht overeenkomend met dat van de normaal in andere complexen voorkomende dihydrolipoyl transacetylase, de ander met een lager molecuulgewicht.

In het derde artikel worden enige eigenschappen van pyruvaat dehydrogenase complex uit *A. vinelandii* vergeleken met die uit *E. coli*. Aangetoond wordt dat zij sterk verschillen wat betreft de symmetrie van het derde enzym, de lipoamide dehydrogenase component. Verder blijkt ook dat de transporteiwitten, de transacetylase-componenten niet symmetrisch binnen de complexen verdeeld zijn. Verschillende conformaties afhankelijk van de reductietoestand van de lipoyl S-S-brug en van de aanwezigheid van fosfaat worden aangetoond.

Het vierde artikel is een gedetailleerde studie aan (iso)alloxazine en analogen daarvan. Het isoalloxazine is het voor katalyse belangrijkste deel van het FAD, een onderdeel van de lipoamide dehydrogenase component van het PDC. Behalve een eenduidige toekenning van de protonsignalen (^{1}H) in kernmagnetische resonantie wordt ook een vergelijking met elektronen dichtheidsberekeningen gemaakt.

Het vijfde artikel is ook een kernmagnetische resonantie studie aan isoalloxazine en alloxazine analogen. Hier echter is gekeken naar de koolstof (^{13}C) resonanties. Aangetoond wordt dat de toekenningen zoals in de literatuur zijn beschreven een groot aantal fouten bevatten. Tevens wordt, omdat koolstof nauwkeuriger de elektronen dichtheid reflecteert, weer een vergelijking gemaakt met gepubliceerde elektronen dichtheidsberekeningen.

Curriculum Vitae

De schrijver van dit proefschrift is geboren op 3 november 1944 in Haarlem. In 1962 behaalde hij het HBS-B diploma aan de Gemeentelijke HBS te Hilversum. In datzelfde jaar begon hij zijn studie chemie aan de Universiteit van Amsterdam, en in 1966 werd het kandidaatsexamen behaald.
Het doctoraalexamen omvatte: hoofdvak fysische chemie bij prof.dr. J.D.W. van Voorst, bijvakken chemische fysica en groepentheorie; het werd met goed gevolg afgelegd in mei 1970.

Vanaf 15 mei 1970 tot heden is de auteur werkzaam bij het Laboratorium voor Biochemie van de Landbouwhogeschool te Wageningen. Gedurende de eerste twee jaar van zijn aanstelling werden colleges in Chemische Binding, Quantummechanica en Molecuulspectroscopie door hem gegeven.

Dit geschiedde in een periode dat hij aan het Laboratorium voor Biochemie was verbonden als wetenschappelijk medewerker. Een plaats, toegewezen door de Stichting Scheikundig Onderzoek Nederland (S.O.N.) en gesubsidieerd door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.).