The Canavanine-Pentacyanoamine Ferrate Complex Formation

(canavanine|kinetics|thermodynamic parameters|activation energy)

WALTER BRUNE[1], JOSÉ DOMINGOS FABRIS[2], and SALIM JOSÉ[1]

[1]Departamento de Química, Universidade Federal de Viçosa, 36570 Viçosa and [2]EMBRAPA/CNPMS, 35700 Sete Lagoas, MG

INTRODUCTION

The non-proteic amino acid canavanine (Cav[*]), 2-amino-4-guanidinoxy-butyric acid, occurs in rather large amounts in seeds of *Canavalia* spp and of some other leguminous species (Bell, 1958; Fearon & Bell, 1955; Rosenthal, 1977). Cav is toxic to most beetle larvae which feed upon those seeds. The notable exceptions are some species which are able to circumvent its incorporation into their body-specific proteins (Rosenthal, 1983) by way of a t-RNA (Arg), specific only for Arg but not for Cav. This is of interest because both amino acids are similar in structure. Furthermore, Cav exhibits a host of physiological effects on a large variety of organisms (Bell, 1972) which renders its determination in crop screening desirable.

The reagent most commonly used for Cav analysis is the so-called 'photoactivated PCAF', supposedly a stable mixture of the ferrous and ferric forms of the pentacyanoamine iron anion (Fearon & Bell, 1955; Herington, 1953). PCAF, $[Fe(CN)_5NH_3]^{3-}$, reacts with the guanidinoxy moiety of Cav, for which it is considered a specific

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[*] Abreviation used: Cav, Canavanine; PCAF, Pentacyanoamine ferrate; PMQ II, Zeiss spectrophotometer; 552 A, Perkin Elmer spectrophotometer.

reagent when compared to other amino acids commonly integrating proteins (Fearon & Bell, 1955).

Data from literature concerning the reaction of Cav with PCAF appear insufficient. Therefore, we felt bound to obtain information about specifc thermodynamic and kinetic properties of this reaction. To probe its specificity we applied a series of spot tests to various compounds, mostly amino acids.

The formation and stability of the Cav/PCAF complex have also been studied in this paper by means of light-absorption measurements. Besides, the possible role of previous photoactivation of the PCAF solutions for reaction with Cav has been explored.

From the beginning of our current Cav evaluations we felt urged to confront an apparently incomprehensible fact from the literature; namely, that the period and intensity of daylight on exposed PCAF solutions does not seem to affect the molar absorption coefficient of the Cav/PCAF complex. To check on the putative photoindependency of PCAF, the stoichiometric ratio of the components of the reaction was determined. These observations were followed by the evaluation of the reaction enthalpy, entropy, and Gibbs energy changes, and activation energy. To complement these data, the temperature stability of the Cav/PCAF complex has hereinafter been described thermodynamically.

MATERIALS AND METHODS

REAGENTS

For all experiments, both Cav and PCAF were used either as plain aqueous or as buffered solutions. The buffer consisted of a 10 mM sodium phosphate solution, pH 7.0, containing 0.1 mM sodium azide, while Cav, L(+) canavanine.H_2SO_4 (Sigma), was used as received. PCAF was used in the form of its ammonium, disodium salt $Na_2NH_4[Fe(CN)_5NH_3].5H_2O$ (Aldrich) as received. Unless otherwise specified an aqueous PCAF stock solution of 10 mg mL^{-1} was exposed to daylight (Fearon, 1946) for at least one hour before being stored in a brown-colored bottle at room temperature. For use the PCAF stock solution has been conveniently diluted.

ABSORPTION MEASUREMENTS

Either PMQ II, a Zeiss, or a 552 A, a Perkin Elmer, spectrophotometers, according to availability were used. With the exception of the spectrum, absorbances were read at 520nm, only.

PHOTOACTIVATION AND OXYGEN REQUIREMENT

An aqueous PCAF solution was prepared in light as dim as feasible either with previously boiled or aerated water at 22°C. A portion of each solution was then exposed to daylight for one hour. Subsequently each of the resulting PCAF solutions received an equal amount of an aqueous Cav solution, also prepared with previously boiled or aerated water. The resulting mixtures were identical in Cav and PCAF concentrations but differed in their water environment. Absorbances were read from time to time on PMQ II. Seventy minutes after mixing, the "boiled-water samples" received H_2O_2, while the "aerated-water samples" were mixed with ascorbic acid, both to yield 0.1 M solutions. Their absorbances were then ascertained again.

ANALYTICAL COMPARISON OF CAV WITH OTHER COMPOUNDS

Aqueous solutions of various amino acids were applied individually as spots on Whatman Number 1 paper chromatography sheets. Their spots were developed by spraying the sheets with buffered PCAF (Hunt & Thompson, 1971) or, to assure identifiable concentrations, by ninhydrin solution. In cases of color perception through PCAF spraying, this reaction was also performed in solution. Into these 'solution tests' two non-amino acids were included, namely histamine and indol-acetate.

TIME-STABILITY, STOICHIOMETRY, AND FORMATION KINETICS OF THE CAV/PCAF COMPLEX

The time-stability of the complex was studied in a mixture of 2.55 mM aqueous PCAF and 0.232 mM aqueous Cav.

The stoichiometric ratio of the reactants of the Cav/PCAF complex was investigated by the 'continuous-variation' method (Job, 1928; Schaeppi & Treadwell, 1948). This was performed by reading absorbances on PMQ II as response to mole fractions of the two-component system, maintaining a total level of 0.628 mM. The absorbances were read about one hour after mixing.

The formation kinetics of the Cav/PCAF complex as a function of time and temperature was followed on 552 A using a thermostatically controlled device (±0.05°C). The buffered PCAF solution (mixture or blank) was fixed at 1.37 mM. 4.6 mmol Cav was introduced in the form of 50µL droplets and the mixtures were rapidly homogenized. The quality of homogenization was checked by reproducibility of the results during short time spans.

THERMODYNAMIC DATA

Buffered Cav and PCAF solutions were mixed at 0.314 mM concentrations of either com

ponent keeping the mixture at a definite temperature until reaching a stable absorbance value through PMQ II readings. The test was repeated at various temperatures. Readings were corrected against those of plain PCAF solution.

RESULTS

TIME-STABILITY

The rate of Cav/PCAF complex formation and its stability during long periods can be fol-
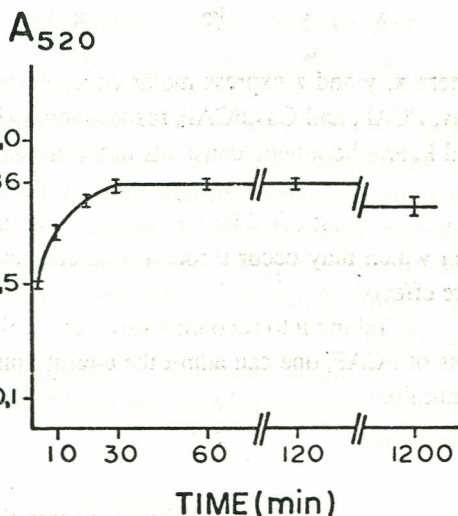

Fig. 1 — Time-stability of the Cav/PCAF complex at 22°C, Molar ratio of PCAF to Cav: 11.

lowed (Fig. 1). According to these results, stability is assumed, for analytical purposes, between half an hour and two hours at least (at 22°C). The color fades less than 10% during 20 h. This conclusion was supportive for further absorbance readings at room temperature.

ABSORBANCE

The absorbance spectrum (Fig. 2) of the Cav/PCAF complex evidence three maxima within the visible and near UV range, i.e. at 520, 425 and 264 nm, while non-complexed PCAF, when run under analogous conditions, exhibits a
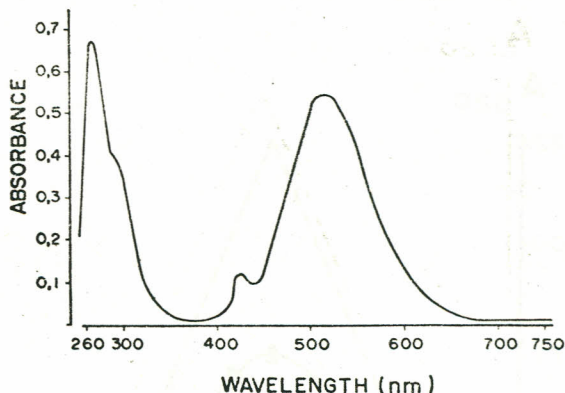

Fig. 2 — Absorption spectrum of the Cav/PCAF complex Soltion is of the same composition as used for time-stability measurements, 1 h after mixing and conveniently diluted.

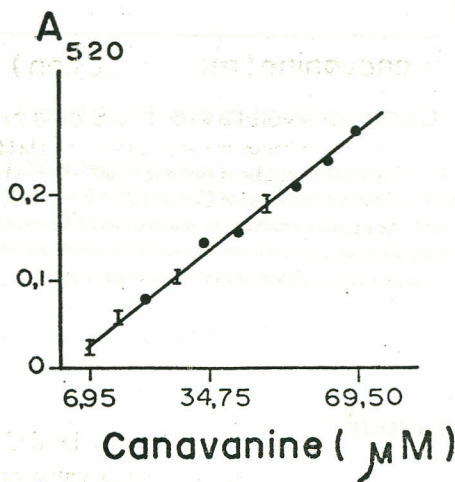strong band at 255 nm. Thus absorbances were measured at 520 nm.

Fig. 3 — Absorption of the Cav/PCAF complex. Samples at 23°C containing an excess of 1.78 mM PCAF and read 1 h after mixing.

By plotting absorbance *vs*. Cav concentration and assuming validity of Beer's law, one can rely on readings between the limits shown (Fig. 3). The entire data yield a correlation coefficient of r^2 = 99.7%.

The molar absorptivity resulted in ε = 3.87 mM^{-1}cm^{-1}.
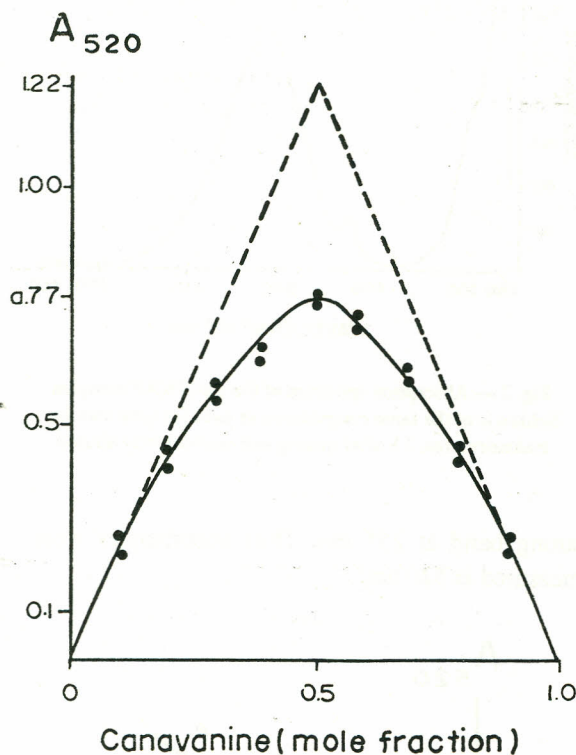
A 520

Fig. 4 — Continuous variation analysis of the Cav/PCAF complex. Joint concentration of Cav and PCAF was held at 0.628 mM. Absorption readings (solid line) at 23°C one hour after mixing. Dotted line indicate extrapolated absorbances at a large excess of one of the reaction partners.

STOICHIOMETRY

The stoichiometric ratio may be deduced and has been corrected for the small value of unreacted PCAF (Fig. 4). According to these data, Cav and PCAF, when complexed, reveal an absorption maximum through their mole fraction of 0.5. Thus the complex consists of equimolar amounts of Cav and PCAF. The extrapolated absorbance of a hypothetical, undissociated complex of a 0.314 mM concentration is 1.22. In consequence, the equilibrium constant at 23°C is calculated as

$$K = 12.1 \pm 0.7 \text{ mM}_{-1}$$

FORMATION KINETICS

1. Reaction Order: To consider the reaction order of the Cav/PCAF complexation, we assumed tentatively a first-order reaction for Cav in the presence of a reasonably high excess of PCAF. More correctly, this reaction may be characterized as pseudounimolecular.

This complexification is in consequence described kinetically as:

$$Cav + PCAF \underset{k_2}{\overset{k_1}{\rightleftarrows}} Cav.PCAF$$
$$x \qquad y \qquad\qquad z$$
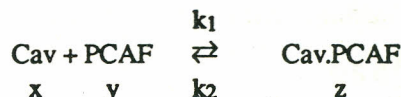
where x, y and z express molar concentrations of Cav, PCAF, and Cav.PCAF, respectively, while k_1 and k_2 are the kinetic constants in the forward and backward modes (Laidler & Meiser, 1982). This model does not consider any decomposition reaction which may occur through time and temperature effects.

Taking into account a sufficiently high excess of PCAF, one can admit the equilibrium concentrations:

$$y = M_0 \qquad x = m_0 - z$$

where M_0 and m_0 are the initial concentrations of PCAF and Cav respectively. Thus the time course of complexation obeys the equation:

$$dz/\{z(k_1.M_0 + k_2) - k_1.M_0.m_0\} = -dt$$

yielding the relation:

$$z/m_0 = \{k_1.m_0/(k_1.m_0+k_2)\}.\{1-\exp[(k_1.M_0+k_2).t]\}$$

To verify the likelihood of this formal treatment, the regression coefficient of the data was estimated as $r^2 = 99.9\%$.

2. Rates of complex formation: Rates of complex formation were observed at fixed temperatures (Fig. 5). Admitting first-order kinetics, their k_1-values were estimated to yield data for activity calculations (Fig. 6)
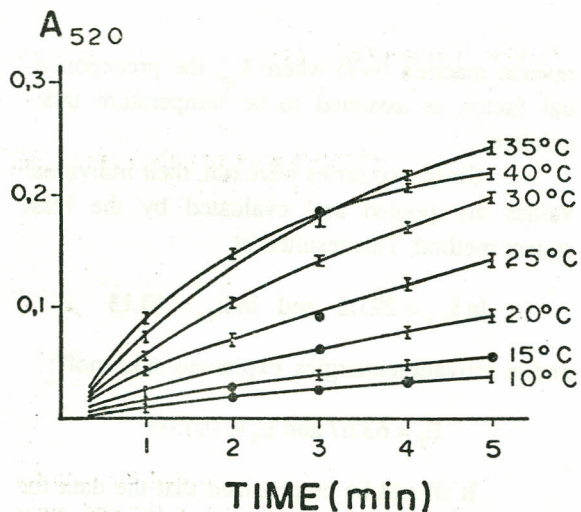
THERMODYNAMIC PARAMETERS

Fig. 5 — Speed of complex formation as temperature function. See text for additional informations.
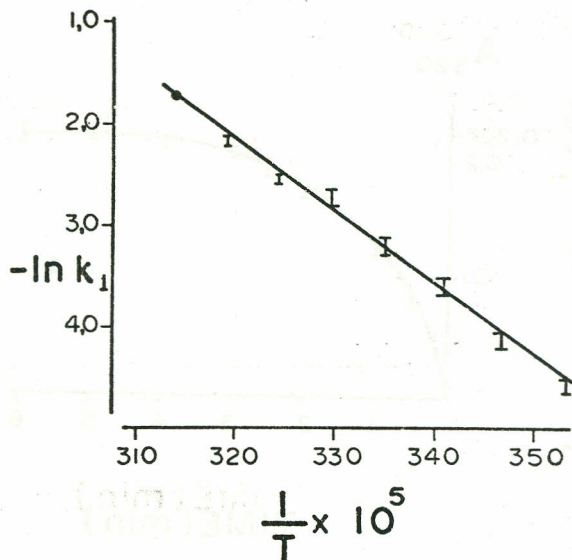

Fig. 7 — Equilibrium constants as function of temperature. Joint solutions of Cav and PCAF, either 0.314 mM at mixing, are held at a definite temperature until achieving stable values. Equilibrium constants are expressed in the association sense.

logarithmic K values *vs* reciprocal temperatures are given (Fig. 7).

Admitting a constant enthalpy within the experimented temperature range, the following standard enthalpy and Gibb-energy changes, expressed in $kJ\ mol_{-1}$, are:

$$\Delta H° = -32,6 \pm 5,1$$

$$\Delta G° = -23,5 \pm 3,7$$

The entropy change is estimated as

$$\Delta S° = -30,8 \pm 4,8\ J\ mol_{-1}K_{-1}$$

ACTIVATION ENERGY

The kinetic data permit a thermodynamic evaluation of the transition-state formation by applying the Arrhenius law (Fig. 5). A pertinent computer program was based on the assumptions that (a) the experimental molar ratio $M_0/m_0 = 17,9$ turns out to be sufficiently high for suiting pseudo first-order kinetics and (b) no decomposition be evidenced within the experimented temperatures and time intervals. In fact, the data (Fig. 8) make a
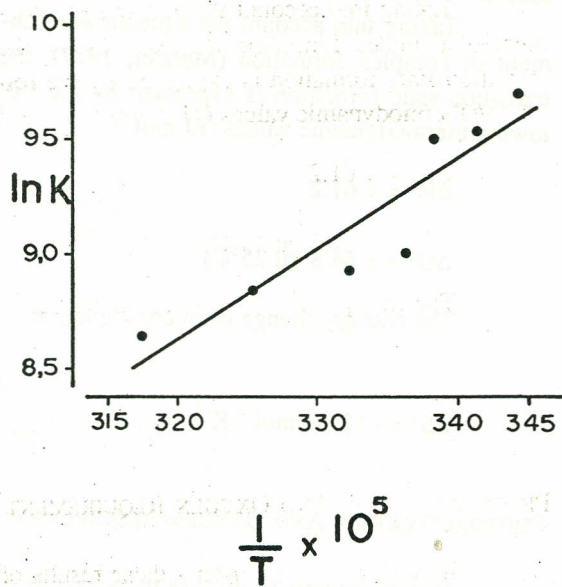

Fig. 6 — Thermodynamic constants as a function of temperature. Reaction-rate constants are determined assuming first-order reaction kinetics. k_1 values result from data of Figure 5.

The Van't Hoff isochore relates enthalpy and entropy changes of chemical reactions:

$$\ln K = -\Delta H°/RT + \Delta S°/R$$

where K is, as fixed before, the equilibrium constant in its association sense. Plots of

A_{520}

TIME (min)
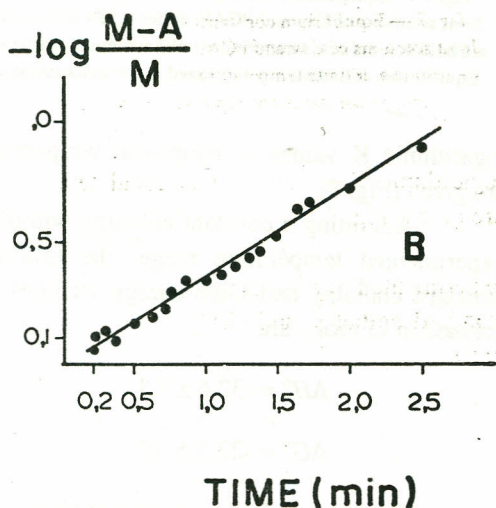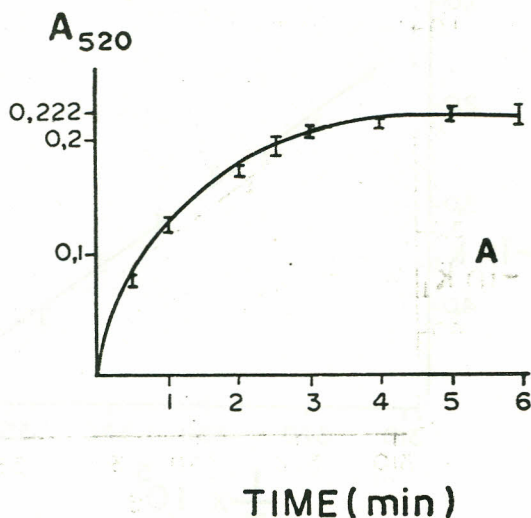
$-\log \dfrac{M-A}{M}$

TIME (min)

Fig. 8 — Reaction-order kinetics of Cav/PCAF complex formation. Temperature 45°C. For a clearer survey only some of the readings are marked. See text for further details. A: Absorption vs time. B: First-order probing, assuming an equilibrium value of M = 0.222. A: individual absorptions at 520 nm.

first-order reaction much more likely than a zero-order (r^2 = 93.6%) or a second-order (r_2 = 93.0%) one.

The Arrhenius law

$$k_i = k_{i,o} \exp(-E_i/RT) \qquad i = 1, 2$$

applies in the present context to the complex-formation (i=1) parameters, as well as to those of the reverse reaction (i=2) where $k_{i,o}$, the preexponential factor, is assumed to be temperature independent.

Since two series were run, their individual values are pooled and evaluated by the least square method. This resulted in

$$\ln k_{1,o} = 29.02 \quad \text{and} \quad \ln k_{2,o} = 40.13$$

and in activation energies, expressed in kJ mol⁻¹:

$$E_1 = 63.67 \text{ and } E_2 = 107.08$$

It should be emphasized that the data for the reverse reaction are poorly estimated since equilibrium levels at low temperatures are only sluggishly approached and thus not satisfactorily ascertained.

Taking into account the aqueous environment of complex formation (Metzler, 1977), the transition-state formation is expressed by the following thermodynamic values (kJ mol⁻¹):

$$\Delta H^{\ddagger} = + 61.2$$

$$\Delta G^{\ddagger} = + 64.8 \text{ (at 25°C)}$$

The entropy change is, in consequence:

$$\Delta S^{\ddagger} = - 12.1 \text{ J mol}^{-1} \text{ K}^{-1}$$

PHOTOACTIVATION AND OXYGEN REQUIREMENT

It is of interest to discuss these results of stoichiometric analysis with those of others, e.g. Fearon and Bell's (1955), namely, that the PCAF solution, when photoactivated, contains Fe in its oxidation levels II and III but in an uncertain ratio.

The stoichiometry of complexation according to our data discloses plain molar integers of the reaction partners. This result was unexpected because the putatively photoactivated PCAF, with its ill-defined Fe(II) and Fe(III) levels should not represent a proper reagent for continuous-variation analysis in order to establish the component ratio.

TABLE I
Effect of the Aqueous Medium and Light-Exposure on the Absorbance[a] of the Cav.PCAF Complex

Reaction time (min)	PCAF solution			
	light-exposed		non light-exposed	
	boiled[b]	aerated[c]	boiled[b]	aerated[c]
2	15	22	14	15
11	19	26	18	25
20	20	28	20	28
30	20	30	22	31
50	21	35	22	35
70	21	36	21	36
> 70; H_2O_2 added	90	–	93	–
> 70; ascorbate added	–	04	–	05

[a] relative values;
[b] water boiled for 5 min and cooled before preparing the PCAF solution;
[c] water air-bubbled for 10 min before preparing the PCAF solution.

TABLE II
Reaction of PCAF with Various Compounds, Performed as Spot Test and in Solution[a]

Compound[b]	Color of reaction products and maximum-absorbtion wavelength in the visible spectrum		
	as a spot	in solution	λ max (nm)
Abrine[c]	pale green	slightly green	700
Arg	none	none	–
Cav	red	red	520
Histamine	pink	pink	470
His[d]	pink	pink	470
Pro	none	none	–
Indol-3-acetate	green	bluish-green	410; 700
Lys	none	none	–
Trp[e]	green	green	700

[a] Another set was developed with ninhydrin instead of PCAF to assure identifiable concentrations of the tested amino acids;
[b] Other amino acids experimented as spot test only: Ala, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Met, Orn, Phe, Pipecolic acid (2-Piperdine carboxylic acid), Ser, Thr, Tyr, Val. None of these exhibited a perceptible color when sprayed with PCAF solution;
[c] Abrine: αN-Methyl-tryptophan;
[d] Histidine required, for color development at room temperature, about a one-day period;
[e] Tryptophan required, for color development at room temperature, about 6 to 10 h.

On the other hand, oxygen may well affect the molar absorption coefficient of the complex (Herington, 1953). To check this assumption the complex was formed in (a) a recently boiled solution, to expel oxygen, and (b) non-light-exposed PCAF solution, to maintain the Fe(II) level of the original reagent unchanged, in confrontation with non-boiled and light-exposed solutions (Table I).

ANALYTICAL COMPARISON OF CAV WITH OTHER AMINO ACIDS

The reaction of Cav with PCAF yields red color as a spot on chromatographic paper as well as in solution. The possibility of further reactions was investigated using a variety of compounds (Table II). When testing the same compound, colors were similar in the spot test as well as in solution. With histamine, His, indol-3-acetate and Trp, the presence of PCAF led to colored complexes.

DISCUSSION

The current results exhibit a similar time-stability as documented (Rosenthal, 1977), i.e., absorbance stability from 40 to 60 min.

The current molar absorption coefficient of $\varepsilon = 3.87$ mM^{-1} cm^{-1} also agrees reasonably well with Rosenthal's, and Fearon and Bell's (1955), which are 2.6 mM^{-1} cm^{-1} and 3.71 mM^{-1} cm^{-1}, respectively.

As a corollary, the lower limit of reliability of Cav concentration is 200 µg with a standard error of 7%. The smallest Cav amount tested by Rosenthal was 0.1 µmol, a quantity about 7 times lower, while Fearon and Bell observed 1 µmol as the lowest limit. But neither these reports nor the current work traced the lowest identifiable limit.

Continuous-variation analysis data are indicative or a 1:1 ratio of the complex partners. This infers, as a first hint, that PCAF represents only one, definite structure. In fact, light-exposed PCAF solutions, though prepared frequently, exhibited the same results, independent of prevailing light intensity, exosure time or wavelength composition.

Since there was no known previous information on this type of analysis for the Cav/PCAF reaction, data of experiments on the 1:1 ratio basis for complex formation were further investigated.

To evaluate the activation energy, a mathematical model was used on first-order kinetics in regard to Cav in the presence of a molar excess of PCAF of about 17.9 times. In accordance with the stoichiometric ratio, deduced from the continuous-variation method, the data (Fig. 7) are fitted on the basis of the mathematical model as stated above, using a least-square fitting program.

The activation energy is calculated through the Arrhenius expression for k_1 and k_2.

The data (Table I) give no indication of photoactivation. On the other hand, a shortage of oxygen depresses absorbance of the Cav.PCAF complex significantly. This depression is easily overcome with H_2O_2 but intensified by ascorbate as also has been reported (Fearon and Bell, 1955). The data evidence an effect of H_2O_2 which goes well beyond the one caused by molecular oxygen in solution. This may be due to various reasons such as concentration levels and oxidation potentials. However, depression of absorbance by ascorbate, which is much more pronounced than that by mere lack of oxygen, suggests an unknown oxidative function. Such a funtion nevertherless does not affect the Fe(II) level of PCAF.

The hypothesis of integers of the reaction partners corroborates our results (Table I). In conclusion, it is proposed that complexation by PCAF occurs in its Fe(II) form, as supplied by the manufacturer, as well as an unknown function of oxygen, enhancing absorbance.

The color exhibited by the Cav.PCAF complex (Bell, 1958; Rosenthal, 1977) might be considered specific with regard to other amino acids tested. It is advisable to take into account however the pink color of the reaction product between PCAF and His (Rosenthal & Dahlman, 1982). Thus, Natelson (1985) mentions, as result of staining His with 'pentacyanoferrate' on paper a 'light pink on long standing'. The color of the reaction product of Trp with PCAF was unex-

pected. No literature references were found on this occurrence. Both amino acids, His and Trp, are now subject of further special studies in this laboratory.

ACKNOWLEDGEMENTS

SUMMARY

The complexation of pentacyanoamine ferrate with canavanine is not a specific one. Other amino acids, especially tryptophan and histidine may also react with proper analytical features. So also do the indolyl and imidazolyl moieties of non-amino acids.

Photoactivation does neither seem to occur by exposure of the pentacyanoamine ferrate reagent to daylight, nor is it a prerequisite for a more sensitive response of canavanine complexation. On the other hand, oxidants and reducers may well interfere with the absorbance of the complex.

In addition molar absorbance, stoichiometric ratio of the complex, and reaction-order kinetics were determined. By means of temperature programming equilibrium constants and reaction kinetics, enthalpy, Gibbs energy, entropy values, and activation anergy of complex formation were stablished.

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