THE ELECTROCHEMISTRY OF PROTEINS AND RELATED SUBSTANCES

PART II. INSULIN

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ABSTRACT

The electroreduction of insulin in pH 7.4 solution was studied at the hanging mercury drop electrode by cyclic voltammetry and at a mercury pool electrode by controlled potential coulometry. The proposed mechanism involves reduction of an adsorbed monolayer of insulin (maximum coverage of 10 μC cm⁻²) in a four-electron reaction at about −0.6 V vs. SCE resulting in breakage of two of the three disulfide bonds in the molecule. Fast reoxidation leads to recovery of much of the reduced species. At longer time (ca. 100 s) a steady state is reached where the reductions and reoxidation involve reversible two-electron reactions.

INTRODUCTION

The adsorption and reactions of proteins at a mercury electrode are of interest with respect to the blockage of the electrode surface for other electrochemical reactions, in the preparation of modified electrodes, and in studied of the conformation and redox chemistry of the proteins themselves. A number of papers in recent years have been concerned with this area (see refs. 1–6 and references therein). Previous studies of the electrochemistry of cystine (RSSR) at a mercury electrode [7] demonstrated strong adsorption and reduction of the disulfide linkage in this compound. In connection with studies of the behavior of enzymes adsorbed on mercury electrodes [8], the effect of reduction of the disulfide bond in proteins on the molecular configuration and chemical properties of proteins was of interest. In this paper we discuss the behavior of insulin, a relatively low molecular weight (5730) and simple protein of known structure. Insulin (Fig. 1) is composed of two polypeptide chains called the A and B chains [9]. The molecule contains three disulfide bonds; two of these bonds are interchain (the A7B7 and the A20B19) while the third is an intrachain bond (A6A11). The X-ray crystallographic structure, determined by Blundell et al. [10], shows that the A7B7 bond is the most accessible to chemical reaction, since it is located directly on the surface of the insulin molecule. The A20B19 disulfide is partially shielded from the molecular surface, but is still accessible. The A6A11 bond is folded into the molecule in a hydrophobic pocket and should be the least reactive. The area occupied by an insulin molecule is 6.9 × 10⁻¹⁴ cm² from the crystallographic data [10]. This value is close to the value found by surface tension measurements for insulin in solution,
Human insulin: two chains and three bridges

A chain
H Gly-Ile-Val-Glu-Gln-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn OH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

B chain
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Fig. 1. Insulin.

7.24 × 10⁻¹⁴ cm² [11]. The isoelectric point of insulin is 6.15 [12]. At pH 8.0 the molecule has a net negative charge of 3.2.

There have been several studies of the bulk electrolytic reduction of insulin. Markus [4] reported that in the reduction at a constant current of 10 mA at a stirred mercury pool in a solution of pH 7.5 to 8.5 two disulfide bonds were reduced quickly, with the reduction rate of the third disulfide bond being much slower. Cecil and Weitzman [5] carried out controlled potential electrolysis of insulin at a mercury pool in a solution of pH 1. When the electrode was held at −1.35 V vs. SCE, four −SH groups were produced after 1.5 h of electrolysis and the product was insoluble. Reduction at −1.8 V vs. SCE produced six −SH groups and a soluble product. Similarly Zahn and Gattner [6] found that reduction at −1.35 V vs. Ag/AgCl (pH = 1) caused reduction of all of the A7B7 bonds; 65% of the A chains were cleaved from the B chains and 45% of the intrachain A6A11 bonds remained intact. Indirect electroreduction of insulin using mediators (e.g., pterine or thiol compounds) and direct chemical reduction of the disulfide bonds (e.g., with sulfite) have also been reported [3,13].

Cecil and Weitzman [5] also examined the polarography of insulin and observed two reduction waves in pH 7.1 solution ($E_{1/2} = −0.65$ V and $−1.02$ V vs. SCE). The first wave, which increased in height with insulin concentration up to 0.012 mM and then levelled off, appeared to be an adsorption wave. The second wave appeared at a 0.012 mM concentration and increased in height up to 0.024 mM insulin. Addition of surfactants caused the second wave to disappear, but not the first. Reduction of the interchain disulfide bonds by treatment with bisulfite caused both waves to disappear. Lee et al. [14] studied insulin reduction at a slow dropping mercury electrode (DME) and reported (pH 7) two reduction waves at $−1.05$ and $−1.65$ V vs. Ag/AgCl.

The use of d.c. polarography and the DME to study proteins presents some problems, however, because of the limited time scale available in the experiment. Since the solution concentrations of proteins are usually very low, complete adsorption equilibrium cannot be attained during the life of a drop. Moreover the anodic processes following disulfide bond reduction cannot be studied easily using conventional polarographic techniques. A hanging mercury drop electrode (HMDE) on the other hand allows much longer times for adsorption equilibration to occur and produces larger faradaic reduction currents (because of greater amounts of adsorbed protein) and a smaller charging current contri-
bution (because the electrode area remains constant). We describe here voltammetric studies of insulin at a HMDE as well as controlled potential coulometric studies at a large mercury pool electrode and propose a mechanism for the electrochemical behavior of insulin.

EXPERIMENTAL

The electrochemical cell, electrodes, apparatus and techniques have been described previously [7] and details of experimental procedures and complete listings of data are available [15]. Recrystallized beef pancreas insulin was obtained from Research Plus of Calbiochem (B-grade) and was used without further purification.

RESULTS

Cyclic voltammetry of insulin

A typical cyclic voltammogram (C.V.) for a 0.1 M phosphate buffer, pH 7.4 containing 0.077 mM insulin at a HMDE, with a fresh mercury drop and pre-equilibrated for one minute with stirring, is shown in Fig. 2. A C.V. of the buffer alone at this scan rate (v) showed a flat charging current of ca. 0.2 μA until background discharge at −1.5 V vs. SCE. Addition of the insulin obviously depressed the charging current (i.e. decreases the double layer capacity) and causes background reduction to occur at −1.4 V. A plot of the cathodic peak current (i_{pc}) of the symmetrical reduction wave (E_{pc} = −0.66 V) against v is

![Cyclic voltammogram of 0.078 mM insulin in pH 7.4 phosphate buffer at HMDE. (a) First scan; (b) with the potential cycled past the first wave until steady state was reached. Scan rate, 200 mV s\(^{-1}\).](image)
linear with zero intercept for scan rates of 0.02 to 0.5 V s\(^{-1}\). These results suggest that this wave can be attributed to reduction of adsorbed insulin. Upon scan reversal following this wave a double oxidation wave (\(E_{pox} = -0.63\) and \(-0.48\) V) occurs. With repeated cycling around these waves (i.e. between \(-0.3\) and \(-0.9\) V), both the cathodic and anodic currents decrease until a steady state (Fig. 2b) is attained. The \(E_{pc}\) for the steady state (ss) wave was at \(-0.63\) V and \((i_{pc})_{ss}\) was about one-half that of the first scan. Similarly the integrated area under the cathodic peak (\(Q_s\)) for the ss was about one-half of that of the first scan \((10 \pm 0.5\) µC cm\(^{-2}\)). A plot of \((i_{pc})_{ss}\) vs. \(v\) was linear with a zero intercept. The anodic peaks became less well-defined upon repeated cycling, finally merging into a single broad wave. The integrated area of this wave (\(Q_a\)) was difficult to determine because of uncertainty in the anodic charging current level and the fact that the anodic current decayed slowly with potential, becoming essentially constant and merging with background. For first scans \(Q_s/Q_c\) was about 0.75 at 0.2 V s\(^{-1}\) and 0.85 at 0.5 V s\(^{-1}\). At steady state the ratio was somewhat smaller, i.e. \((Q_a)_{ss}/(Q_c)_{ss}\) was 0.3 to 0.4. The number of scans required to attain steady state depended upon \(v\) and the potential limits in a way that 85 to 100 s were always required.

When the scan was continued beyond the first cathodic wave, at this concentration a second reduction wave at \(-1.23\) V was observed (Fig. 3). Upon rever-

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**Fig. 3.** Cyclic voltammogram of 0.078 mM insulin at the HMDE, scanned over the second wave. (a) First scan; (b) steady state. Scan rate, 200 mV s\(^{-1}\).

**Fig. 4.** Cyclic voltammogram of 0.17 µM insulin solution, pH 7.4. (a) HMDE pre-equilibrated with solution with stirring for 1 min; (b) fresh HMDE, not pre-equilibrated with solution; (c) steady state current for pre-equilibrated HMDE.
sal an anodic wave at ca. −0.9 V was present. On continued cycling between −0.3 and −1.4 V, this cathodic peak shifted to −1.32 V and $i_{pc2}$ decreased slightly while the anodic peak current increased. For very dilute solutions of insulin (e.g. 0.17 μM, Fig. 4) these waves were absent or very small compared to the first wave. A plot of $(i_{pc2})_a$ vs. $u^{1/2}$ was linear and passed through the origin. The results generally suggest that the reduction wave at −1.23 V and the oxidation at −0.9 V are primarily attributable to dissolved reactants, although some adsorbed reactant must also participate. For the first scans $i_{pc2}$-scan rate plots showed neither clear $v$ nor $u^{1/2}$ dependence and this wave height was associated with that of the first wave at low concentrations (Fig. 4).

Further information on the nature of the reduction processes was obtained by studying the cyclic voltammetric behavior of insulin solutions with concentrations of 0.05 to 60 μM. In these experiments a fresh mercury drop of the HMDE was pre-equilibrated with the solution with stirring for one minute before the C.V. was obtained, to provide sufficient mass transfer of insulin to the electrode surface to approach attainment of adsorption equilibrium. The integrated reduction current for the first reduction peak of the C.V. of a 0.17 μM insulin solution (Fig. 4) was 9.7 μC cm$^{-2}$, a value almost as large as that found for solutions containing four hundred times more insulin. As with more concentrated solutions the peak currents decreased on repeated cycling until at steady state $(Q_c)_{ss}/(Q_c)_{initial} = 0.5$. Furthermore $(Q_a)_{ss}/(Q_a)_{ss} = 0.8$ at this low concentration. Typical data for $Q_c$ and $Q_a$ over the whole concentration range are shown in Table 1. While there is considerable scatter in the data, the

<table>
<thead>
<tr>
<th>Concentration/μM</th>
<th>First scan</th>
<th>Steady state</th>
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<tbody>
<tr>
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<td>$Q_c^{b}$ μC cm$^{-2}$</td>
<td>$Q_a^{c}$ μC cm$^{-2}$</td>
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<td>60</td>
<td>10.9</td>
<td>2.4</td>
</tr>
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$^a$ Solution was pH 7.4; fresh HMDE was equilibrated with the solution with stirring for 1 min.

$^b$ First reduction peak ($E_{pc} = −0.63$ V vs. S.C.E.).

$^c$ Reversal oxidation peak ($E_{pa} = −0.60$ V vs. S.C.E.).

$^d$ Total anodic current (peaks at −0.6 and −0.9 V).
general form of $Q_c$ vs. concentration dependence is suggestive of an adsorption isotherm rising at about 0.05 $\mu\text{M}$ and attaining a saturation coverage of insulin of about 10 $\mu\text{C cm}^{-2}$. The values of $(Q_a)_{ss}/(Q_c)_{initial}$ were about 0.5 over this concentration range. $(Q_a)_{ss}/(Q_c)_{ss}$ decreased from 1 at low concentrations to about 0.6 at higher ones. If a C.V. was obtained immediately on a fresh mercury drop without allowing time for equilibration, the first scan showed very small peaks which first grew upon repeated cycling and then decreased to the steady state values. The wave at $-1.2$ V was very small and parabolic-shaped in these dilute, ($<1.6$ $\mu\text{M}$) solutions; this wave grew and took on the characteristics of a diffusion-controlled wave at concentrations of 12 $\mu\text{M}$ or higher, reaching a constant value of about 0.25 $\mu\text{A}$. The wave with $E_{pa} = -0.9$ V appeared at this concentration and the wave at $E_{pa} = -0.6$ V became smaller and less well-defined.

In an 8 $M$ urea solution intramolecular hydrogen bonding is destroyed; proteins are denatured, i.e., they lose their rigid three dimensional structure and attain a loose helical one. The C.V. for a 0.1 mM insulin solution, pH 7.4 made 8 $M$ in urea was very similar to that of the undenatured insulin. The cathodic adsorption peak at $-0.63$ V was still present and its magnitude and scan rate dependence were the same as those in a urea-free solution. In the presence of urea the background process occurred at less negative potentials so that the wave at $-1.3$ V merged with the final current rise, and the oxidation peak with $E_{pa} = -0.9$ V was not observed.

**Coulometry of insulin at mercury pool**

Coulometric reduction of insulin at a large area (7.6 cm$^2$) stirred mercury pool electrode was carried out at several different potentials; typical results are shown in Table 2. For reduction at potentials following the first wave $n_{app}$ (electrons per molecule of insulin) values of 3.8 to 4.7 were found. The current decay in these insulin coulometry experiments was much slower than for the bulk reduction of a small soluble species in the same cell under similar conditions. Thus coulometric trials extended for three or more hours before the current level was less than 5% of its initial value. Under these conditions the correction for background processes becomes rather large and this introduced some uncertainty in the reported $n_{app}$ values. The product of the reduction was a colloidal suspension. A C.V. of this solution obtained at a fresh drop of a HMDE located above the mercury pool is shown in Fig. 5; a C.V. on the pool itself had a very similar shape. The scan, initiated in a positive direction from $-0.75$ V showed the pair of waves at $-0.6$ V, but the second reduction wave at $-1.2$ V and the reversal anodic wave at $-0.9$ V were absent. The peak currents for these waves were slightly larger than those found for the original unreduced insulin solution and plots of both $i_{pa}$ and $i_{pa}$ with $v$ were linear and intersected the origin. The integrated areas of the peaks were $Q_c = 13.5$ $\mu\text{C cm}^{-2}$ and $Q_a = 11$ $\mu\text{C cm}^{-2}$ after attempted correction for the charging and background current. Oxidation of the reduced insulin colloidal suspension yielded $n_{app}$ values of 1.4 to 2.5. A spot test for mercuric ion in solution [16] was positive following oxidation (but not before oxidation or for the original solution stirred in contact with the mercury pool). A C.V. at the HMDE following reoxi-
<table>
<thead>
<tr>
<th>$10^6$ Insulin/mol</th>
<th>Solution volume/ml</th>
<th>$10^4$ Insulin concentration/M</th>
<th>First reduction</th>
<th>Reoxidation</th>
<th>Second reduction</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduction potential/V</td>
<td>$n_{app}$</td>
<td>Oxidation potential/V</td>
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<tr>
<td>1.66</td>
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<td>1.1</td>
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<td>1.14</td>
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<td>1.79</td>
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<td>-1.26</td>
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<tr>
<td>2.1 $^b$</td>
<td>18</td>
<td>1.16</td>
<td>-1.0</td>
<td>4.8</td>
<td>Irreversible</td>
</tr>
</tbody>
</table>

$^a$ Solution was a pH 7.4 buffer and pool area 7.6 cm².

$^b$ 8 M urea.
Fig. 5. Cyclic voltammogram of 0.1 mM insulin reduced at Hg pool at \(-0.70\) V vs. SCE (a) First scan; (b) second scan. HMDE; scan rate, 200 mV s\(^{-1}\).

Fig. 6. Cyclic voltammogram of 0.1 mM insulin reoxidized at \(-0.35\) V vs. SCE (following reduction at \(-0.75\) V). HMDE, scan rate, 200 mV s\(^{-1}\).

tation is shown in Fig. 6. The adsorption wave pair at \(-0.6\) V is present again and is very well-defined compared to previous voltammograms. The wave at \(-1.2\) V reappeared. This wave increased in height and took on a “polarogram-like” appearance when the solution was stirred during the scan, so that this wave can be attributed, at least partially, to reduction of a diffusing species. Reduction of this oxidized solution at \(-0.75\) V gave \(n_{\text{app}}\) values of 2.1 to 3.1.

Coulometric reduction at potentials at the second wave (\(-1.3\) V) showed \(n_{\text{app}}\) values of 4.7 to 5.2. The reduction products precipitated out of solution and could not be reoxidized. During the course of reduction however the C.V. of the solution at a HMDE resembled those in Fig. 5. For a coulometric reduction of insulin denatured in 8 \(M\) urea, an \(n_{\text{app}}\) of 4.8 was obtained. The reduced product was completely soluble but could not be reoxidized at the mercury pool.

DISCUSSION

A mechanism for the electrochemical behavior of insulin which is generally consistent with our experimental findings and past results can be proposed. Insulin will be represented by \(\text{In(SS)}_3\), where (SS) represents one of the three disulfide bonds in the molecule. The first reduction wave (at \(-0.6\) V) is clearly an adsorption wave by the scan rate and concentration dependence. The inte-
grated area, $Q_c$, for the first scans for maximum coverage was 10 $\mu$C cm$^{-2}$. Assuming that this represents a monolayer and taking the crystallographic area ($6.9 \times 10^{-14}$ cm$^2$) as representing the area occupied by the molecule on the electrode surface, one calculates $n = 4.3$. This number is perhaps smaller than the true $n$ value, since the molecular conformation would change on adsorption with a larger area probably occupied by the molecule compared to its crystallographic area. This number is in good agreement with that determined by coulometry. Thus we first wave reduction process if probably a 4-electron reaction involving breakage of two disulfide bonds to form four sulphydryl groups and represented by

$$[\text{In(SS)}_3]_{\text{ads}} + 4e \rightarrow [\text{In(SS)}(\text{SS}^2-)_{2}]_{\text{ads}}$$  \hspace{1cm} (1)

While it is possible that some reaction of the adsorbed insulin directly with the mercury occurs to produce a form such as $\text{In(SS)}_3(\text{SS}^2-)\text{Hg}^{2+}$ which could then be reduced, the previous results with the model compound cystine [7] make this appear less likely. At low concentrations and higher scan rates the integrated area of the first reversal scan, $Q_a$, is only slightly smaller than $Q_c$. At the higher concentrations, $Q_a$ is about one-half of $Q_c$ and the anodic wave breaks up into two separate peaks or a broad, less distinct, wave. This behavior is consistent with the reoxidation of the reduced species to parent at short times and low concentrations, but reoxidation of only one disulfide group at longer times. Thus the rearrangement represented by (2) can

$$[\text{In(SS)}(\text{SS}^2-)_{2}]_{\text{ads}} \rightarrow [\text{In(SS)}(\text{S}^-\text{S}^-)(\text{SS}^2-)]_{\text{ads}}$$  \hspace{1cm} (2)

occur, where $(\text{S}^-\text{S}^-)$ represents reduced sulfurs or sulphydryls which have changed their orientation with respect to one another so that the disulfide bond can no longer form on reoxidation. The oxidation reaction then can be represented as

$$[\text{In(SS)}(\text{S}^-\text{S}^-)(\text{SS}^2-)]_{\text{ads}} \rightarrow [\text{In(SS)}(\text{S}^-\text{S}^-)(\text{SS})]_{\text{ads}} + 2e$$  \hspace{1cm} (3)

This result is also consistent with the coulometric results where $n_{\text{app}}$ for the reoxidation is about two. The form $\text{In(SS)}(\text{S}^-\text{S}^-)(\text{SS}^2-)$ must be somewhat soluble, because in the coulometric experiment it desorbs and is replaced by parent compound which is reduced, and because an oxidation wave corresponding to this reduced species is found at the HMDE above the mercury pool (Fig. 5). Moreover continuous cycling over the wave system at $-0.6$ V shows that the cathodic wave decreases to about one-half of its initial value (i.e. $Q_{c}\text{as}/Q_c \approx 0.5$). The steady state then represents conversion to the $\text{In(SS)}(\text{S}^-\text{S}^-)(\text{SS})$ form and the steady state reduction wave involves reduction of this species (i.e. the reverse of reaction 3). Since the time to attain steady state was of the order of 100 s, the rate constant for the rearrangement reaction is about $10^{-2}$ s$^{-1}$. It is difficult within this mechanism to account for the ratio $(Q_{c}\text{as})/(Q_c)$ being less than one at the high concentrations. One possibility is that the true integrated area of the anodic wave is actually larger than that reported because of the difficulty in correcting for background. Another is that adsorbed reduced species is displaced by parent insulin so that $(Q_a)$ is smaller than that for a monolayer while $(Q_{c}\text{as})$ represents actual monolayer coverage.

Another possibility exists for the oxidation reaction rather than (3). By
analogy to cysteine [7], the oxidation could involve Hg, as shown in (4), with the next reduction scan then being the reverse reaction. Indeed following
\[ \text{[In(SS)(S}^-\text{S}^-\text{)(S}^2^-\text{)]}_{\text{ads}} + \text{Hg} \rightarrow \text{[In(SS)(S}^-\text{S}^-\text{)(S}^2^-\text{)Hg}^{2+}\text{]}_{\text{ads}} + 2e \] (4)
the coulometric oxidation a positive spot test for Hg\textsuperscript{2+} was obtained. However, we are persuaded that the bond reformation shown in (3) occurs, at least to some extent, by the following findings. While for cystine the second cathodic scan (i.e. following reduction of RSSR to 2RS\textsuperscript{-} and oxidation of 2RS\textsuperscript{-} to Hg\textsuperscript{2+} (RS\textsuperscript{-})\textsubscript{2}) is quite different from the first, for insulin the form and location of all cathodic peaks are quite similar. Moreover after coulometric reduction and oxidation the diffusion wave at \(-1.2\) V reappeared (Fig. 6), while this peak is absent following the reduction.

The nature of the reactions occurring at the reduction wave at \(-1.2\) V is unclear. The cyclic voltammetric scan rate and concentration dependence suggest that it involves a solution species rather than an adsorbed one. This could represent reduction of dissolved insulin; dissolved cystine reduction was shown to occur at potentials more negative than that for the adsorbed form [7]. The reduction at this wave on a coulometric time scale leads to insoluble products, but the \(n_{\text{app}}\) value is not large enough to account for total reduction of all three disulfide bonds. The monomer form of insulin is in equilibrium with dimeric and hexameric forms, and the association constants reported for insulin at pH 7 [17] suggest that at concentrations above 0.03 mM appreciable amounts of the dimer exist. This form could be involved in the second wave reactions but further work is required to elucidate the nature of this wave. We might add that zinc-containing forms of insulin are also known [10]. However, analysis of a 3 \times 10\textsuperscript{-5} M insulin solution by flameless atomic absorption showed that the zinc concentration was at most 3 \times 10\textsuperscript{-7} M, so that these forms can probably be neglected.

In general the mechanism presented here agrees with the previous bulk electrolysis results [4–6] where the rapid breaking of two disulfide bonds, presumably the A7B7 and A20B19, with only slow reduction of the third was reported. The results here suggest a mechanism in which the molecule shows strong adsorption to a mercury electrode because of the large mercury-sulfur interaction (as well as the general non-polar nature of the protein). These disulfide bonds are the reducible moieties in proteins and the results shown here suggest that at short times they can be reformed on oxidation, presumably because the molecular conformation is partially maintained at the electrode surface by the Hg–S interaction and intramolecular hydrogen bonding.

ACKNOWLEDGMENTS

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REFERENCES

3  S.J. Leach, A. Neschers and O.A. Swanepoel, Biochemistry, 4 (1965) 23.
17 A. Pekar and B. Frank, Biochemistry, 11 (1972) 4103.