pH-DEPENDENT CHANGES IN THE SPIN STATE OF CYTOCHROME *P*450 FROM ADRENAL MITOCHONDRIA*

C.R. JEFCOATE and G.S. BOYD

Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland

Received 16 December 1970

1. Introduction

Cytochrome *P*450 in the adrenal cortex participates as a mixed function oxidase in several steps in the biosynthesis of corticosterone, of which side chain cleavage (SCC) of cholesterol [1] and 11β-hydroxylation of 11-deoxycorticosterone (11-DOC) occur in the mitochondria [2]. These two oxidations are specifically inhibited by aminoglutethimide and metapyrone [3] respectively, suggesting that different enzymes are involved in these oxidations. Recently the cytochrome *P*450 of adrenal mitochondria has been separated into two fractions which, with the same reducing system, specifically carry out 11β-hydroxylation of 11-deoxycorticosterone of SCC of cholesterol [4].

EPR spectra of subparticles from adrenal mitochondria have indicated that the haems of the constituent cytochrome *P*450 exist in one of two spin states; high spin (s_m = 7.49) and low spin (s_m = 2.45, g = 2.26, s_m = 1.91) [5]. In addition, steroid-induced changes in the above EPR spectra and in the absorption spectra of the separated cytochrome *P*450 fractions [4] indicate that low spin 11β-hydroxylase cytochrome *P*450 changes to a high spin state binding 11-DOC, while high spin SCC-*P*450 is converted to a low spin state by pregnenolone. In this paper we show that the spin state and ligand binding properties of the SCC-cytochrome *P*450, but not 11β-hydroxylase cytochrome *P*450, are very sensitive to pH.

* Presented as a communication at the 8th International Congress of Biochemistry, Interlaken, September 1970.

2. Methods

Sonicated subparticles and the separated SCC-cytochrome *P*450 fraction were prepared from bovine adrenal mitochondria as previously described [4]. After the SCC-cytochrome *P*450 fraction had been dialysed for 12 hr against distilled water to remove sodium cholate, the absorption spectrum of the preparation at pH 6.5 (10 mM potassium phosphate buffer) indicated variable amounts of a haemoprotein contaminant (λ_{max} 415 nm). Centrifugation (105,000 *g*, 45 min) removed most of the haem contaminant into the pellet while about half of the cytochrome *P*450 remained in the supernatant fraction. This optically clear supernatant was used for all absolute spectra. The SCC activities (pregnenolone formation) of both fractions were similar as assayed by the ^{14}C-cholesterol method [1, 4] and activities of 10 nmoles pregnenolone/nmole cytochrome *P*450/min were typically found for a cholesterol concentration of 100 μg/ml. The NADPH-cytochrome *P*450 reductase was prepared by the method of Omura et al. [6]. Careful fractionation on Sephadex G 100 was necessary to separate the reductase from small amounts of cytochrome *P*450 in the preceding fractions, which had very high SCC activity (~ 30 nmoles pregnenolone/nmole cytochrome *P*450/min). The cholesterol content of preparations was measured by gas liquid chromatography using a Pye 104 instrument (S.E. 30/240°) and protein estimations were performed by the Biuret method [7].

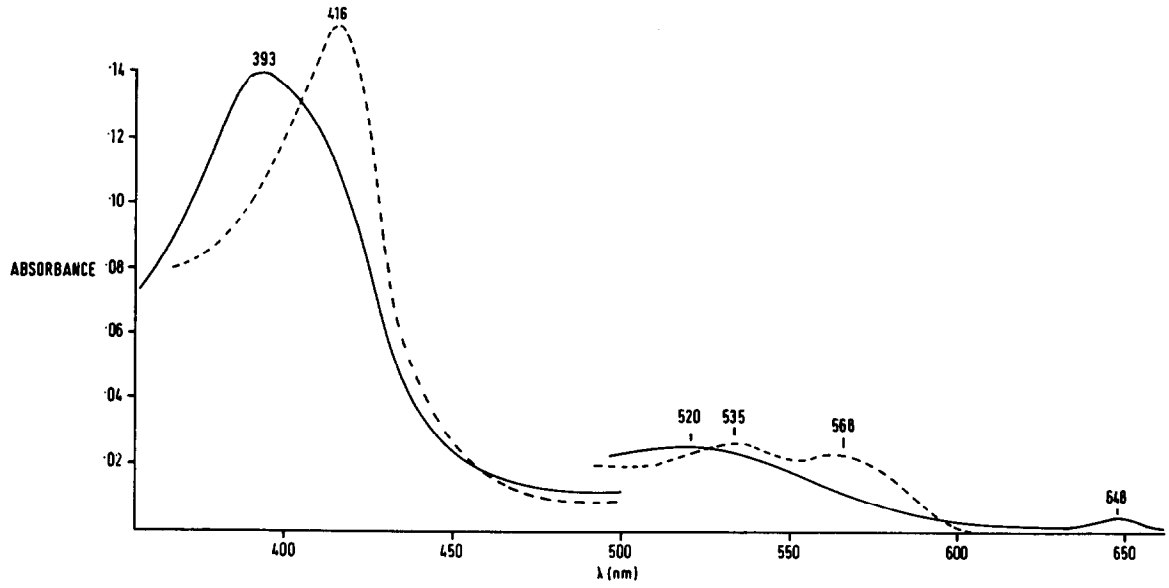

Fig. 1. Spectra of oxidised SCC-cytochrome *P*450 (——) and the pregnenolone complex (- - -) in 10 mM potassium phosphate buffer at pH 6.8. Protein = 0.9 mg/ml; [pregnenolone] = 10 μM.

3. Results

The absolute absorption spectra of oxidised SCC-cytochrome *P*450 and of the pregnenolone complex at pH 6.5 are shown in fig. 1. The spectrum of the oxidised cytochrome *P*450 (λ_{max} 393, 520, 648 nm) was almost identical with that of the high spin camphor complex of *Pseudomonas putida* cytochrome *P*450 [8], while that of the pregnelonone complex (λ_{max} 416, 535, 568 nm) compared closely with the free low spin *Pseudomonas* cytochrome *P*450. The distortion of the oxidised Soret band was partly due to the contaminant haemoprotein previously mentioned which was rapidly reduced by dithionite to produce a *b*-type oxidised reduced difference spectrum (fig. 2A, 1 min). The contribution to the spectrum from reduction of SCC-cytochrome *P*450 appeared very slowly (fig. 2A, 30 min), unless adrenoxoxin was added to the preparation. Although 11β-hydroxylase cytochrome *P*450 was not spectrally detectable [4], there remains the possibility that the low pH form of SCC-cytochrome *P*450 still remains a low spin contribution. EPR spectra of the preparation should resolve this question. The absorption spectra in the Soret regions of the reduced cytochrome *P*450 (λ_{max} 414 nm) and the CO complex

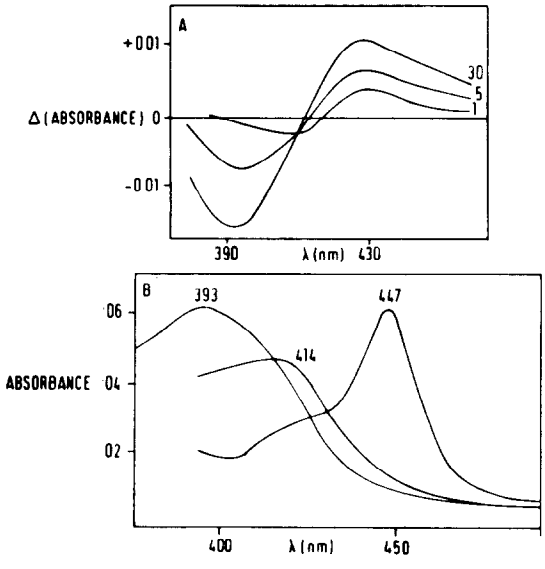

Fig. 2. A) Reduced-oxidised difference spectra of the SCC-cytochrome *P*450 preparation (10 mM K phosphate, pH 6.8, protein = 0.45 mg/ml) obtained with sodium dithionite. Spectra were commenced after 1, 5 and 30 min. B) Spectra of oxidised (max 393 nm), reduced (max 414 nm) and reduced CO (max 447 nm) SCC-cytochrome *P*450. Conditions were the same as for 2A.

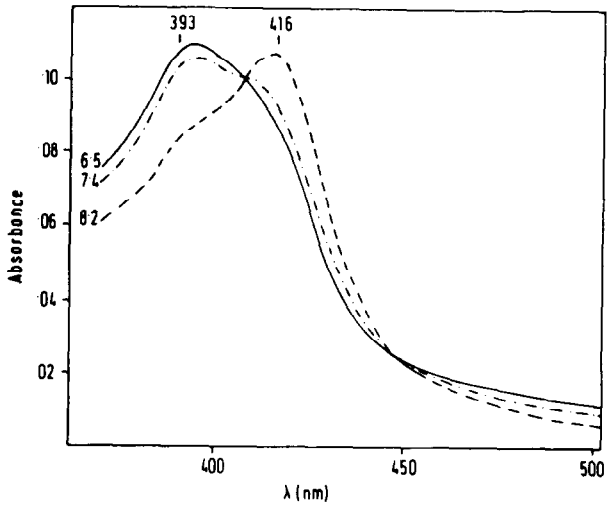

Fig. 3. Spectra of SCC-cytochrome *P*450 in buffers (50 mM) at different pHs: (——), pH 6.5; (----), pH 7.4; (- - -) pH 8.2. Protein = 0.9 mg/ml. Potassium phosphate buffers were used at pH 6.5 and 7.4, tris-HCl at pH 8.2.

(λ_{max}447 nm) are shown in fig. 2B. Since the reduced spectrum contains a contribution from a *b*-type haem with a peak at over 420 nm, the true Soret maximum for reduced SCC-cytochrome *P*450 is probably close to 410 nm.

An increase in the pH above caused a decrease in the absorbance of oxidised SCC-cytochrome *P*450 at 393 nm, with a concomitant increase at 416, 535 and 568 nm. The spectra shown in fig. 3 represent equilibrium positions at each pH while lowering the pH from 8.2 produced a complete reversal of the above spectral changes.

Since an increase in pH or addition of pregnenolone resulted in very similar difference spectra, it was assumed that the absolute spectra of the respective low spin cytochrome *P*450 species were the same. Thus the percentage conversion of high spin cytochrome *P*450 (λ_{max} 393 nm) to low spin cytochrome *P*450 (λ_{max} 416 nm) as a function of pH (fig. 4A) was calculated by comparison with the spectrum of the low spin pregnenolone complex (fig. 1).

As a consequence of the pH-induced spin change in SCC-cytochrome *P*450, the maximal absorbance change (ΔA_{max})* which could be induced by pregnenolone, decreased with increase of pH (pH 6.8). The pH

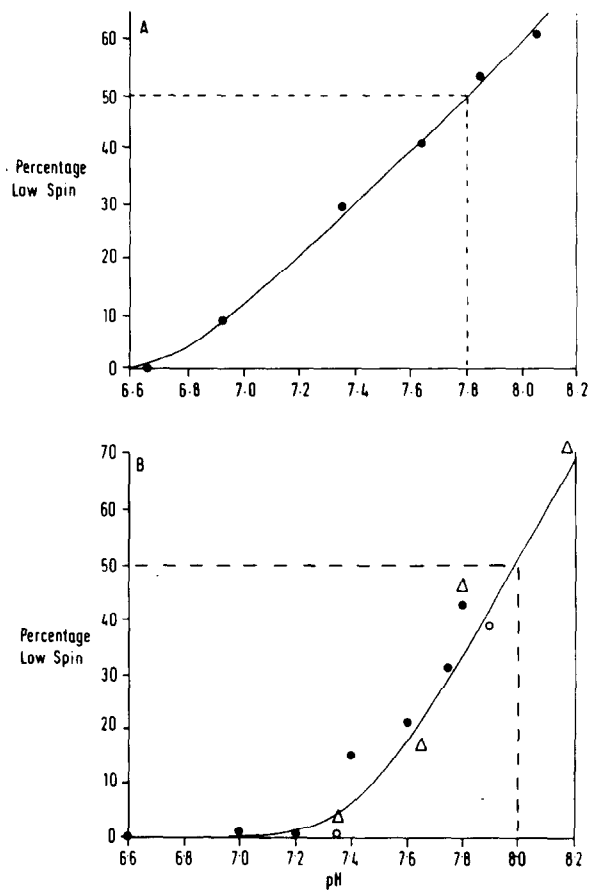

Fig. 4. Changes in the spin state of SCC-cytochrome *P*450 induced by alteration of the pH (0.1 M K phosphate). A) Soluble preparation. B) Sonicated mitochondrial subparticles: pregnenolone method (•), (○) 2 different preparations; aminoglutethimide method (△).

change in SCC-cytochrome *P*450 of sonicated mitochondria was investigated by determining the ΔA_{max} produced by pregnenolone at different pH values. The decrease in ΔA_{max} which was found between pH 7.4 and 8.0 was a clear indication of a pH-induced interconversion of the spin states of SCC-cytochrome *P*450 (fig. 4B). By contrast, there was no change in the 11-DOC binding of the 11β-hydroxylase cytochrome *P*450 of sonicated mitochondria (ΔA_{max} or K_S) in the pH range 6–8. In addition there was no appreciable con-

* Determined from a plot of $1/C$ versus $1/A$

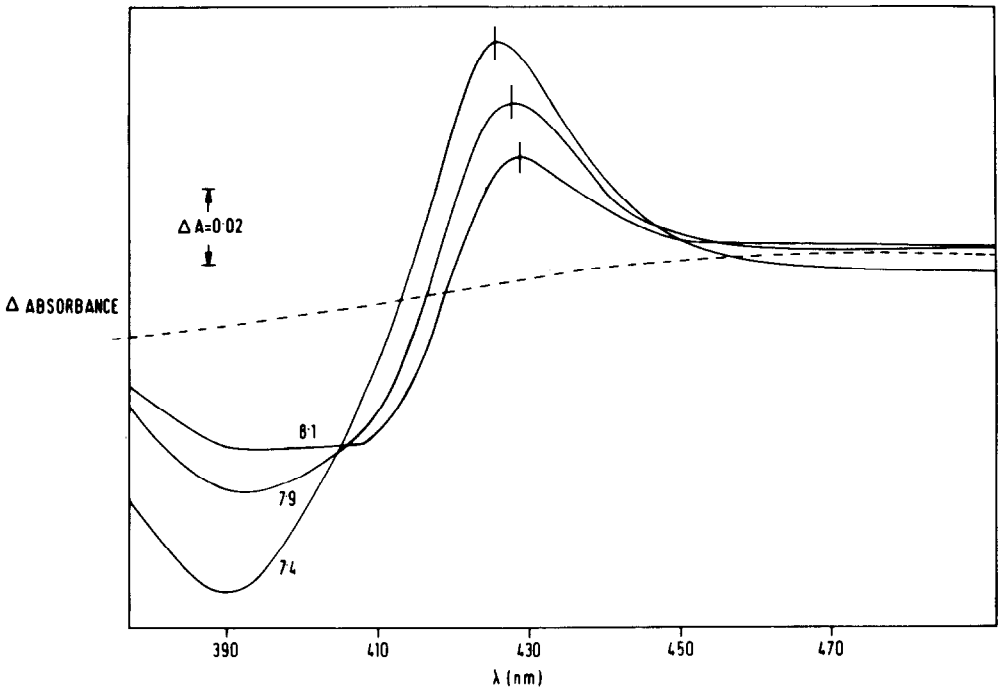

Fig. 5. Difference spectra due to the addition of a saturating amount of aminoglutethimide (0.5 mM) to sonicated mitochondrial subparticles suspended in buffers of different pH. (pH 7.4, 7.9, K phosphate; pH 8.1 tris-HCl). Protein = 5.0 mg/ml.

version of cytochrome *P*450 to cytochrome *P*420 in the range 6–8.

Difference spectra induced by amine binding have been used to determine the proportions of high and low spin cytochrome *P*450 in adrenal mitochondria [9]. Aminoglutethimide bound strongly to both cytochrome *P*450s in adrenal mitochondria [11] producing difference spectra similar to *n*-octylamine [9]. The difference spectra obtained from sonicated mitochondria, upon the addition of saturating amounts of aminoglutethimide, changed dramatically as the pH increased from 7.4 to 8.2. The difference minimum increased at 410 nm and decreased at 390 nm, while the maximum shifted to longer wavelength (fig. 5). The ratios of ΔA_{410} to ΔA_{390} are shown in table 1, together with the proportions of high and low spin cytochrome *P*450, which were calculated from the formula described by Jefcoate and Gaylor [9]. The low spin contribution which was found throughout the pH range 6.0–7.0 was attributed to 11β-hydroxylase cytochrome *P*450. This proportion was consistent with that estimated from the difference spectra induced by 11-DOC, and was associated with a lower binding constant for aminoglutethimide in this pH range. The change in the spin state of SCC-cytochrome *P*450 determined with aminoglutethimide (fig. 4B) was substantially the same as that obtained using pregnenolone and in sonicated mitochondria showed an apparent p*K* of 8.0. The changes observed using the soluble preparation were spread over a wider pH range (fig. 4A), probably due to the presence of cytochrome *P*450 in different particulate states, each with distinct p*K*s. The supernatant fraction after centrifugation at 105,000 *g* for 45 min clearly had a p*K* which was substantially lower than 8.0 (fig. 3). Thus, solubilisation greatly facilitated the conversion of high spin to low spin SCC-cytochrome *P*450. Increase in ionic strength by addition of KCl (10 mM K phosphate, 0.25 M KCl) produced only a slight increase in low spin cytochrome *P*450.

The binding constants for the interactions of pregnenolone and aminoglutethimide with SCC-cytochrome *P*450 in sonicated mitochondria have also been determined as a function of pH (fig. 6). Al-

Table 1
Ratio of spin types from aminoglutethimide spectra[a].

pH	Spectral ratio $\Delta A_{410}/\Delta A_{390}$	% high spin[b]	% conversion of SCC-cytochrome *P*450 to low spin state
7.05	0.195	60[c]	0
7.35	0.23	58	3.5
7.65	0.35	50	17
7.89	0.67	32	46.5
8.2	1.14	17	71.5

a Spectra shown in fig. 5
b Determined according to the relationship for octylamine spectra described in [9].
c Residual low spin *P*450 at pH 7.0 is taken as 11β-hydroxylase *P*450

though aminoglutethimide is fully protonated in this pH range, the pH-dependence of binding of steroid and amine were surprisingly similar. Since pregnenolone cannot lose a proton upon binding, this similarity suggests that aminoglutethimide also binds without losing a proton to the solution, although probably occupying a different site. One possibility for the first step in the binding of aminoglutethimide would be transfer of the amine proton to a protein ligand displaced from the haem:

$$\begin{array}{l} Y\text{–}Fe\text{–}X + R\text{–}NH_3^+ \rightleftharpoons Y\text{–}Fe\text{–}NH_2\text{–}R \\ \quad\lfloor\text{–}Pr\text{–}\rfloor \qquad\qquad\quad \lfloor\text{–}Pr\text{–}XH^+ \end{array}$$

The pH-dependence of binding in each case thus probably arises from a pH-induced change in SCC-cytochrome *P*450. The spin state equilibrium provides a *possible* explanation if both ligands bind preferentially to the high pH, low spin form of SCC-cytochrome *P*450. The curves shown in fig. 6 were calculated on this basis with binding constants of 3 and 100 μM for the high and low pH forms respectively. Since the binding of 11-DOC to 11β-hydroxylase cytochrome *P*450 was constant in the range 6.0–8.0, the pH-dependence was not an inherent property of a spin state change in cytochrome *P*450, but rather a specific

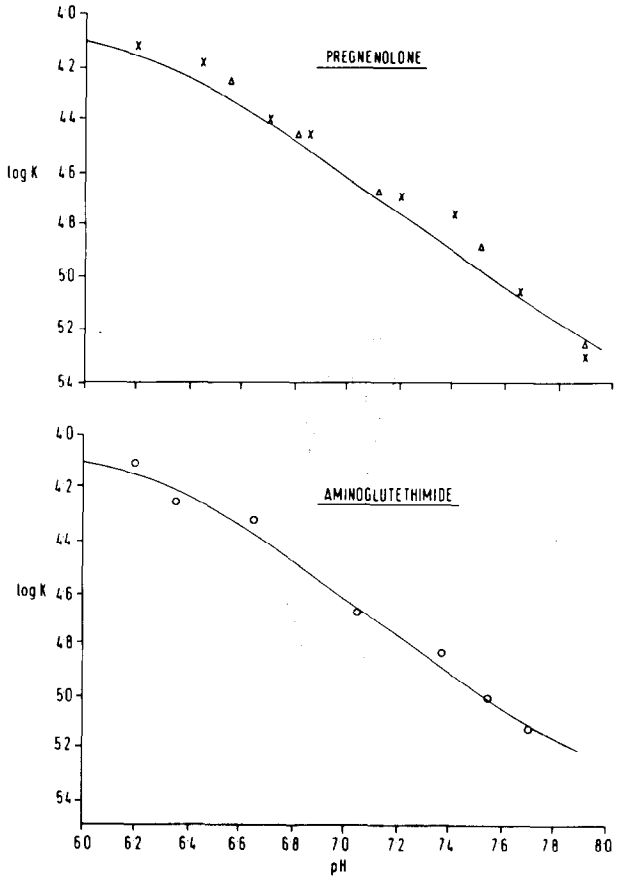

Fig. 6. pH-dependence for the binding of pregnenolone and aminoglutethimide to sonicated mitochondrial subparticles (protein = 2.8 mg/ml). (x, △) indicate separate adrenal preparations. The curve was calculated on the basis of following possible scheme:

$$\begin{array}{ccc} P450\,(H) & \underset{K_1}{\overset{pK\ 8.0}{\rightleftharpoons}} & P450\,(L) + H^+ \\ K_3 \uparrow\downarrow B & & K_2 \uparrow\downarrow B \\ P450\text{–}B(L) & \overset{H^+}{\rightleftharpoons} & P450\text{–}B(L) \end{array}$$

B = aminoglutethimide or pregnenolone, (H) = high spin, (L) = low spin, K_2 = 3 μM, K_3 = 100 μM.

property of SCC-cytochrome *P*450.

The addition of cholesterol (100 μg/ml) to these preparations caused only a slight *increase* in the proportion of low spin cytochrome *P*450, possibly by weak binding at the pregnenolone site. Thus the simple explanation of the high spin state of SCC-cytochrome *P*450 as a complex with endogenous

cholesterol (2.5 nmoles/nmole cytochrome *P*450 in the soluble preparation) requires further investigation. Either cholesterol is not displaced in the high pH form, or the displaced cholesterol is structually bound and does not exchange with exogenous cholesterol. However, for a different preparation cholesterol does appear to increase the proportion of high spin cytochrome *P*450 [10].

The pH-dependence of SCC-cytochrome *P*450 has been observed in tightly coupled mitochondria [11]. Thus the activity of SCC-cytochrome *P*450 in adrenal mitochondria may be very sensitive to pH changes localised within the mitochondria, (cf. the Bohr effect shown by haemoglobin [12]). This sensentivity may arise both from pH-dependent changes in both the spin state of SCC-cytochrome *P*450 and in the binding of pregnenolone. It may be significant that this pH sensitivity appears to be confined to SCC-cytochrome *P*450 which is likely to be the slow step of adrenal steroidogenesis. In addition, since the pH-dependent properties of SCC-cytochrome *P*450 were considerably affected by solubilisation, the state of the surrounding membrane must be a factor in determining the properties of the cytochrome.

References

[1] E.R. Simpson and G.S. Boyd, European J. Biochem. 2 (1967) 275.
[2] L.D. Wilson, D.H. Nelson and B.W. Harding, Biochem. Biophys. Acta 99 (1965) 391.
[3] B.W. Harding, J.J. Bell, L.D. Wilson and J.J. Whysner, in: Advances in Enzyme Regulation, ed. G. Weber Vol. 7(Pergamon Press, London, 1969) p. 237.
[4] C.R. Jefcoate, R. Hume and G.S. Boyd, FEBS Letters 9 (1970) 41.
[5] J.A. Whysner, J. Ramseyer and B.W. Harding, J. Biol. Chem. 245 (1970) 5441.
[6] T. Omura, E. Sanders, R.W. Estabrook, D.Y. Cooper and D. Rosenthal, Arch. Biochem. Biophys. 117 (1966) 660.
[7] A.G. Gornall, G.J. Bardawill and M.M. David, J. Biol. Chem. 177 (1949) 731.
[8] R.L. Tsai, C.A. Yu, I.C. Gunsalus, J. Peisach, W. Blumberg, W.H. Orme-Johnson and H. Beinert, Proc. Natl. Acad. Sci. U.S. 66 (1970) 1157.
[9] C.R. Jefcoate and J.L. Gaylor, Biochemistry 9 (1970) 3816.
[10] F. Mitani and S. Horie, J. Biochem. (Tokyo) 65 (1969) 269.
[11] E.R. Simpson, C.R. Jefcoate and G.S. Boyd, in preparation.
[12] J. Wyman, Advan. Protein Chem. 4 (1948) 407.