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THE ISOLATION AND IDENTIFICATION OF A NAPHTHOQUINONE FROM ELECTRON-TRANSPORT PARTICLES OF *MYCOBACTERIUM PHLEI*

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SUMMARY

1. A naphthoquinone was isolated from electron-transport particles of *Mycobacterium phlei*.

2. The naphthoquinone was identified by chromatography as a vitamin $K_{(45)}$ and by infrared spectroscopy as a vitamin $K_{2(45)}$.

3. This compound was active in restoring DPNH oxidase activity to particles which had been extracted with isooctane. It also reactivated the DPNH oxidase in a crude extract which had been exposed to light at a wavelength of 360 mμ.

4. The inability to restore DPNH oxidase activity in light-exposed particles, with the isolated compound, has been explained by postulating a second light-sensitive component in the electron-transport pathway.

INTRODUCTION

It has been demonstrated that electron transport in *Mycobacterium phlei*, from the level of DPNH through the cytochromes to oxygen, involves the participation of vitamin K (see refs. 1, 2). In an attempt to determine the specificity of naphthoquinones in this reaction, various homologues of vitamin K_1 and K_2 were tested on electron-transport particles[3]. These particles were first exposed to light at 360 mμ or solvent-extracted in order to inactivate or remove the naturally occurring naphthoquinone[1,2]. It was important, therefore, to isolate this naphthoquinone from the particles in order to compare its activity with that of the other K_1 and K_2 homologues in restoring DPNH oxidation to such treated particles.

This naturally occurring naphthoquinone had been isolated from whole cells of *M. phlei* by BRODIE *et al.*[4]. They reported that it appeared to be a new homologue of vitamin K_1 with a maximum molecular weight of 620. JACOBSEN AND DAM[5] similarly isolated a naphthoquinone from *M. phlei* and found that, on the basis of its ultraviolet spectrum, it had a molecular weight of 740.

This report is concerned with the procedure for isolation and purification of the naturally occurring naphthoquinone directly from the electron transport particles, and its identification as a vitamin $K_{2(45)}$. Evidence will also be presented demonstrating the activity of this compound in the terminal electron-transport pathway.

EXPERIMENTAL

Materials

The sodium salt of DPNH was obtained from Sigma Chemical Company. Neotetrazolium chloride was purchased from Sigma Chemical Company and Mann Research Laboratories, Inc. The vitamin K homologues were generously supplied by Dr. O. ISLER, F. Hoffmann LaRoche and Company. A mixture of coenzyme Q_7 and Q_9 from Torula yeast was a gift from Dr. F. CRANE. Spectroquality *n*-hexane was obtained from Matheson, Coleman and Bell Company. A grade of permutit used for Folin ammonia determination was obtained from the Permutit Company. The preparations which gave the most satisfactory results for chromatographic isolation of the naphthoquinone had the following mesh composition: $< 50 = 7.3\,\%$, $50\text{–}80 = 85.4\,\%$, $80\text{–}100 = 7.3\,\%$, and traces of 100 to > 170.

METHODS

Growth of cells and isolation of electron transport particles

M. phlei A.T.C.C. 354, was grown with vigorous aeration in flasks on a New Brunswick Gyrotory Incubator Shaker at 37° for 22 h in a medium described by BRODIE AND GRAY[6]. Cells were harvested in a Servall RC-2 continuous flow apparatus and washed three times with cold deionized water. They were suspended in water (approx. 10 ml/30 g of cells), and the pH was adjusted to 8 with Tris buffer. The cells were disrupted, in 11-ml aliquots, by sonic vibration for 4 min in a 10 kC Raytheon magnetostriction oscillator, which was tuned to a maximum at 1.25 A. The sonic extract was centrifuged at 21000 × *g* at 0° for 30 min to remove cellular debris and the remaining whole cells. The crude supernatant fluid was carefully pipetted off, and recentrifuged two times in order to remove any cellular contamination. This will be referred to as the crude extract.

The particles were obtained by centrifuging the crude extract at 105 000 × *g* for 90 min. The supernatant fluid was decanted and discarded. The particles were then washed with 0.1 M KCl, buffered with Tris at pH 8, and centrifuged at 105 000 × *g* for 60 min. The washing procedure was repeated, and the resultant gelatinous red pellet was stored at −28°. Protein was estimated by the method of LOWRY *et al.*[7].

Isolation of a naphthoquinone from particles of M. phlei

All procedures for the isolation of the naphthoquinone were carried out in subdued light. Approx. 10 g wet weight of particles were suspended in 0.1 M KCl buffered with Tris (pH 8.0). Particles were denatured by repeated freezing and thawing on three successive days. Denaturation was indicated by the rapid settling of particles on standing. If this was not done, a great amount of lipid material present made it difficult to obtain clean hexane extracts suitable for further purification. Ethanol was added to a final concentration of 60 %. The mixture was placed on a New Brunswick Gyrotory Incubator Shaker at 25° for 1 h, and then centrifuged at 4000 × *g* for 15 min. The supernatant fluid containing the water ethanol fraction was decanted into a separatory funnel and extracted with hexane. The residue of denatured particles was extracted repeatedly with hexane. The hexane fraction was yellow in color and extraction was continued until no obvious yellow colored material could be further extracted. The hexane extracts were combined and evaporated to dryness *in vacuo*.

The dried extracts were dissolved in approx. 5 ml of hexane and chromatographed on a column containing permutit (1 × 18 cm) which had previously been washed thoroughly with hexane. The material was adsorbed on to the column and washed with 50 ml of hexane. Elution of the naphthoquinone was obtained with a mixture of anhydrous ether in hexane (5:95, w/v). Approx. 4-ml samples were taken, and the naphthoquinone was found to be in the fourth and fifth tubes. The samples were dried *in vacuo*, dissolved in acetone, and placed at −20° overnight. A white flocculant precipitate formed, composed presumably of triglycerides, which was filtered off at approx. −15°. The yellow acetone solution was placed again at −20° until yellow crystals formed. They were dissolved in acetone at 25° and recrystallized at −20°.

By the method of isolation, the average naphthoquinone concentration, in a series of eighteen experiments, was found to be 1 mg/g wet weight of particles.

Irradiation and solvent extraction of particles

Particles were exposed to light at 360 mμ by methods previously described[1,2]. Isooctane extraction of particles to lower DPNH oxidase activity was performed by a modification of the method of NASON AND LEHMAN[9]. This method presumably removes the naturally occurring naphthoquinone. An equal volume of isooctane was added to the KCl–Tris-suspended particles and mixed in a Vortex Jr. mixer for 2 min at room temperature. They were centrifuged at low speed and the particles recovered by pipetting through a viscous and almost solid lipid layer. The process of extraction was repeated, and after recovery of the particles by centrifugation they were again centrifuged to remove traces of isooctane. As there was a loss of protein during this procedure, the concentration of isooctane-extracted particles was adjusted to that of the untreated particles.

Solubilization of the naphthoquinone

The crystals of naphthoquinone which formed at −20° became an oil at 25°. To test the ability of this compound to restore DPNH oxidase activity to light-exposed or solvent-extracted particles, the oil was emulsified with Tween 80 and brought to volume with water. The final concentration of Tween 80 should be no greater than 0.75 %.

Chromatographic separation of the vitamin K homologues

The method used for separation of the various vitamin K homologues was that of GREEN AND DAM[9] as modified by LESTER AND RAMASARMA[10]. Whatman No. 1 paper was impregnated with 5 % (w/v) Dow Corning Silicone Fluid No. 550 in chloroform. The vitamin K homologues were dissolved in hexane and applied to the paper in a concentration of 0.1 μmole. The solvent system was *n*-propanol–H_2O (4:1). The paper was equilibrated for 30–60 min with the solvent, and the compounds were chromatographed by the ascending technique for approx. 16 h at 25°, at which time the solvent front was approx. 30 cm from the origin. The location of the spots was determined by the reduction of the compounds to the hydronaphthoquinone with potassium borohydride, removal of excess borohydride with 0.1 N HCl, followed by development with 0.25 % neotetrazolium chloride in 0.25 M potassium phosphate buffer (pH 7.0). For coenzyme Q compounds it was necessary to heat the paper to 80–100° for the development of full color intensity[10].

Spectrophotometric procedures

DPNH oxidase activity was measured by a decrease in absorbancy at 340 mμ with a Zeiss PMQ II spectrophotometer. The ultraviolet spectrum of the naphthoquinone was recorded on a Cary Model 11 recording spectrophotometer. Infrared spectra were measured with a Beckman IR-7 double beam recording spectrophotometer with NaCl optics.

RESULTS

Ultraviolet absorption spectrum of the M. phlei naphthoquinone

As can be seen in Fig. 1, the naphthoquinone isolated from *M. phlei* exhibits all the characteristic absorption peaks of the vitamin K_1 and K_2 homologues. The maxima in hexane are 243, 248, 260, 269, 325 and a shoulder at 240 mμ. Although not indicated, exposure of the *M. phlei* compound to light at 360 mμ caused a disappearance of the characteristic absorption spectrum. This was in agreement with results reported by EWING *et al.*[11], for vitamin K_1. A sample calculated to be 95 % pure from infrared data gave an $E^{1\%}_{1\,cm} = 241$ at 248 mμ. Assuming a molecular extinction of 18 900, the molecular weight would be 785.

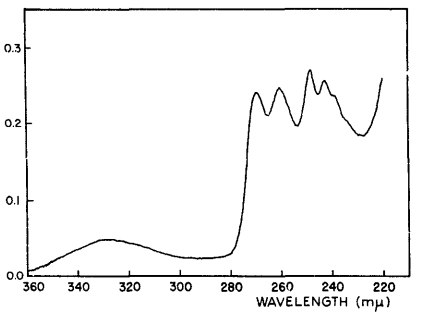

Fig. 1. Absorption spectrum of *M. phlei* naphthoquinone in *n*-hexane.

Chromatographic separation of various naphthoquinones, coenzyme Q_7 and Q_9

The data in Table I indicate that the naphthoquinone isolated from *M. phlei* has the same R_F as authentic vitamin $K_{2(45)}$ and that of *Mycobacterium tuberculosis* naphthoquinone which was isolated by NOLL[12] and identified by NOLL *et al*[13]., as vitamin $K_{2(45)}$. Mixtures of $K_{2(35)}$ and *M. phlei* naphthoquinone gave 2 spots, one identical with $K_{2(35)}$ and the other with $K_{2(45)}$. However, authentic $K_{2(45)}$ mixed with the *M. phlei* compound gave one spot. The two *M. phlei* samples isolated by BRODIE *et al.*[4], gave similar spots identical with our compound. This was the case, although one sample was reported to be active in oxidative phosphorylation whereas the other was not.

Although this chromatographic technique can determine the length of the side chain on the C-3 position of the naphthoquinone ring, it is not possible to differentiate

between a vitamin K_1 or K_2 compound. As can be seen, identical R_F values were obtained with $K_{1(20)}$ and $K_{2(20)}$. Exposure of the *M. phlei* naphthoquinone to light at 360 mμ resulted in a compound which chromatographed as a yellow spot with an R_F of 0.9. This compound did not form an obvious formazan when treated with neotetrazolium after reduction with borohydride.

TABLE I

CHROMATOGRAPHY OF *M. Phlei* NAPHTHOQUINONE, AND KNOWN VITAMIN K AND COENZYME Q HOMOLOGUES

	Compound	*Rp Values**
1	$K_{1(20)}$	0.7
2	$K_{2(20)}$	0.7
3	$K_{2(35)}$	0.48
4	$K_{2(45)}$	0.33
5	*M. phlei* naphthoquinone	0.33
6	$K_{2(45)}$ + *M. phlei* naphthoquinone	0.33
7	$K_{2(35)}$ + *M. phlei* naphthoquinone	0.33, 0.48
8	*M. phlei* naphthoquinone (a)**	0.33
9	*M. phlei* naphthoquinone (b)**	0.33
10	*M. tuberculosis* naphthoquinone ($K_{2(45)}$)	0.33
11	Torula-coenzyme Q_7 and coenzyme Q_9	0.48, 0.6

* Solvent front was 30 cm from the origin.
** Kindly sent by Dr. A. F. BRODIE: a, inactive; b, was labeled biologically active

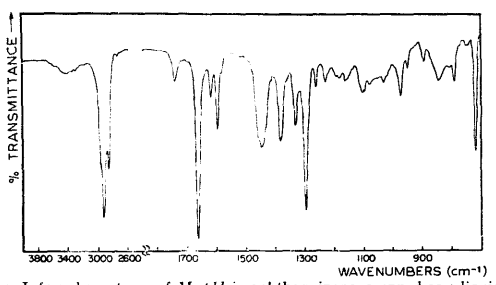

Fig. 2. Infrared spectrum of *M. phlei* naphthoquinone, scanned as a liquid oil

Infrared identification of the naphthoquinone

Conclusive evidence that the vitamin K isolated from *M. phlei* is a vitamin $K_{2(45)}$ was obtained from its infrared spectrum. This spectrum is illustrated in Figs. 2 and 3. Analysis of the spectrum reveals all the qualitative features characteristic for homologues of the K_2 series. The weak ester C=O band near 1740 cm^{-1}, observed in Fig. 2, is due to the presence of a small amount (in the order of about 5 %) of triglyceride impurities which are difficult to separate completely by permutit chromatography[13]. Extensive infrared studies with a large series of synthetic vitamin K homologues by NOLL[14], have shown that K_2 homologues in the non-crystalline state may be differentiated from members belonging to the K_1 series by a number of spectral character-

istics The spectra of K_2 homologues contain two bands, one at 1105 and another at 840 cm^{-1}. These are not present in the spectrum of K_1 homologues. In addition, the position and shape of the CH_2 band near 1450 cm^{-1} was found to be different in the two series. As seen in the expanded scale recordings of the characteristic spectral regions in Fig. 3, these specific K_2 absorptions are clearly recognizable in the spectrum of the *M. phlei* compound when compared with the spectrum of a typical representative of the K_1 series (*e.g.* $K_{1(20)}$). Definite spectral identification of the *M. phlei* compound as $K_{2(45)}$ was made with the aid of an identification scheme based on the observation that each K_2 homologue is characterized by a unique set of relative band intensities[14]. In the present case, the final decision involved comparison of the relative intensities (A) of the bands near 1300 and 2925 cm^{-1} as follows: $K_{2(40)}$, $A_{1300} > A_{2925}$; $K_{2(45)}$, $A_{1300} \leqslant A_{2925}$ and $K_{2(50)}$, $A_{1300} \ll A_{2925}$. From inspection of the spectrum in Fig. 2, it is evident that the side chain in K_2 from *M. phlei* is longer than C_{40} and shorter than C_{50}.

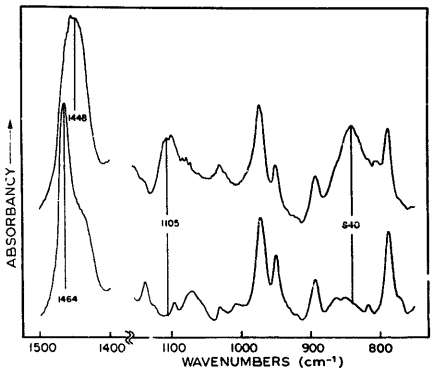

Fig. 3. Expanded scale recordings of the infrared spectral regions, showing the characteristic differences between *M. phlei* naphthoquinone ($K_{2(45)}$) (upper curve) and synthetic DL-vitamin $K_{1(20)}$ (lower curve). Both compounds were scanned as liquid oils.

Reactivation of the DPNH oxidase in solvent-extracted particles

As can be seen in Table II, isooctane extraction of the particles resulted in a 60% reduction in DPNH oxidase activity. This activity was restored with the *M. phlei* naphthoquinone as well as $K_{2(45)}$ and $K_{1(20)}$. This restoration of activity involved the cyanide-sensitive electron-transport pathway. Although not indicated, when the *M. phlei* naphthoquinone was exposed to light at 360 mμ for increasing periods of time, there was a progressive loss in its ability to reactivate the DPNH oxidase.

Reactivation of DPNH oxidase in light-exposed particles and crude extract

The data in Table III show that when crude extracts or particles were exposed to light at 360 mμ, as previously reported[1,2], there was a 90% reduction in DPNH oxidase activity. Addition of the *M. phlei* compound to the light-exposed crude extract

completely restored activity. Vitamins $K_{1(20)}$ and $K_{2(45)}$ presumably in a similar concentration (see DISCUSSION) did not fully reactivate the system. In the light-exposed particles, however, $K_{1(20)}$ more effectively restored activity than either of the other naphthoquinones. As with the isooctane-treated particles, this activity was associated with the cyanide-sensitive pathway.

TABLE II

EFFECT OF VITAMIN K COMPOUNDS ON THE REACTIVATION OF THE DPNH OXIDASE IN ISOOCTANE-EXTRACTED PARTICLES

Reaction mixtures contained 0.5 mg of particle protein, 0.5 ml of 0.1 M potassium phosphate (pH 8) 0.4 μmole of DPNH, and deionized water to a total volume of 3 ml. Where indicated 0.3 μmole of naphthoquinone and 0.02 ml of 95% ethanol were added. Particles were preincubated at 25° for 5 min either alone or with the naphthoquinone and ethanol. The reaction was started with DPNH and the change in absorbancy was recorded from the first 15 sec after mixing.

Material	*Naphthoquinone added*	$-\Delta A_{340}$/min	*Per cent reactivation*	*Per cent inhibition by 10^{-3} M KCN*
Particles	None	0.328	—	79
	$K_{1(20)}$	0.334	—	76
	$K_{2(45)}$	0.280	—	82
	M. phlei	0.338	—	85
Isooctane-extracted particles	None	0.131	—	76
	$K_{1(20)}$	0.330	99	78
	$K_{2(45)}$	0.256	92	82
	M. phlei	0.345	100	84

TABLE III

EFFECT OF VITAMIN K COMPOUNDS ON THE REACTIVATION OF THE DPNH OXIDASE IN LIGHT-EXPOSED PARTICLES AND CRUDE EXTRACT

Reaction mixtures and conditions were the same as indicated in Table II, except that 0.3 mg of particle protein and 3.5 mg of crude extract protein were used.

Material	*Naphthoquinone added*	$-\Delta A_{340}$/min	*Per cent reactivation*	*Per cent inhibition by 10^{-3} M KCN*
Particles	None	0.190	—	76
Light-exposed particles	None	0.015	—	—
	$K_{1(20)}$	0.138	73	75
	$K_{2(45)}$	0.038	20	78
	M. phlei	0.053	23	—
Crude extract	None	0.120	—	78
Light-exposed crude extract	None	0.012	—	—
	$K_{1(20)}$	0.082	68	76
	$K_{2(45)}$	0.063	52	78
	M. phlei	0.144	100	80

DISCUSSION

The electron-transport system of *M. phlei* is contained in particles which contain cytochromes and other factors. In this work it has been demonstrated that one of the factors that contribute to the system is a vitamin $K_{2(45)}$. This compound, extracted

from the particles, was identified by chromatography, and infrared spectroscopy. The compound was isolated as an oil at room temperature rather than in crystalline form characteristic of authentic $K_{2(45)}$. It should be noted, however, that it is difficult to crystallize $K_{2(45)}$ at room temperature when contaminated with triglycerides[13]. When the synthetic crystalline $K_{2(45)}$ was mixed with triolein it crystallized in acetone at −20°, whereas it formed an oil at room temperature similar to the *M. phlei* compound.

The infrared spectrum of crystalline vitamin K_2 homologues show three sharp absorption bands near 876, 796 and 753 cm^{-1}. These are characteristic of the crystalline state and disappear upon melting[12]. Spectra of non-crystalline K_2 preparations, in which these bands are absent appear similar to those of the non-crystalline K_1 compounds. This may have led BRODIE *et al.*[4], to identify the *M. phlei* compound as a vitamin K_1. Similarly, although some of the spectral differences between *M. phlei* naphthoquinone and $K_{1(20)}$ were observed by JACOBSEN AND DAM[5] they were impressed by the great similarity of the two spectra. Nevertheless, they left open the question of whether the differences were due to impurities or to differences in molecular structure.

The reactivation of the DPNH oxidase in isooctane-extracted particles by the compound does not conclusively identify it as a vitamin $K_{2(45)}$. All vitamin K_1 and K_2 homologues with C-3 side chains of 15 and greater will reactivate the cyanide-sensitive DPNH oxidase in such treated particles[3]. DEUL *et al.*[15], CRAWFORD *et al.*[16] and WEBER *et al.*[17,18], have reported that many lipids restore DPNH- and succinate-linked cytochrome *c* reductase activity in heart-muscle preparations extracted with isooctane. Thus the solvent extraction technique, for the removal of naturally occurring electron-transport lipids, is not as specific as might be thought from the results of NASON AND LEHMAN[8]. However, compounds which were found by the above investigators to reactivate their isooctane-extracted preparations such as Tween 80, phytol, or α-tocopherol did not reactivate similarly extracted particles of *M. phlei*[3].

The inability to obtain complete reactivation of the DPNH oxidase with the crystalline synthetic $K_{2(45)}$, in light-exposed crude extract, remains to be explained. Although it is possible that differences in solubility of synthetic and isolated compounds may play a role, *i.e.*, triglyceride impurities in the isolated compound may contribute to its relative solubility, it should be pointed out that the difference in activity of these compounds is reproducible in light-treated crude extracts. An explanation for this effect can be deduced from the data in Table III on light-exposed particles. In this case both the synthetic and isolated $K_{2(45)}$ failed to restore activity to more than 20–30 %. As the particles are devoid of other factors present in the crude extract, it is tempting to suggest the presence of a second light-sensitive component of the electron-transport pathway. In this case the compound as isolated from the particles might contain minute quantities of a catalytically active substance which can interact with the crude extract to restore activity. A more likely possibility is that the high protein content (approx. 70 mg/ml) of the crude extract might protect the postulated second light-sensitive component in the particles from destruction by light. In this case the differences observed between the synthetic and isolated compounds would be explained in differences in their solubilities.

Since complete reactivation could be obtained by the naturally occurring vitamin in isooctane extracted particles, it is obvious that the postulated second light-sensitive component is not extracted.

In contrast to this two component system, vitamin $K_{1(20)}$ can create an artificial by-pass. Vitamin $K_{1(20)}$ would thus accomplish in one step, rather than two, a transfer of electrons to the cytochromes. Work is in progress to test this hypothesis.

While this manuscript was in preparation, BISHOP *et al.*[19], described the isolation of ubiquinone (coenzyme Q) and vitamin K homologues from a wide variety of bacteria. It appears from their results, as well as those of others, that bacteria, when they contain vitamin K, have the K_2 type. This is in contrast to green plants which have been found to contain vitamin K_1.

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REFERENCES

1 M. M. WEBER, AND A. F. BRODIE, *Bacteriol. Proc.*, (1958) 105.
2 M. M. WEBER, A. F. BRODIE AND J. E. MERSELIS, *Science*, 128 (1958) 896.
3 M. M. WEBER AND G. ROSSO, in preparation.
4 A. F. BRODIE, B. R. DAVIS AND L. F. FIESER, *J. Am. Chem. Soc.*, 80 (1958) 6454.
5 B. K. JACOBSEN AND H. DAM, *Biochim. Biophys. Acta*, 40 (1960) 211.
6 A. F. BRODIE AND C. T. GRAY, *J. Biol. Chem.*, 219 (1956) 853.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. C. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 A. NASON AND I. R. LEHMAN, *Science*, 122 (1955) 19.
9 J. P. GREEN AND H. DAM, *Acta Chem. Scand.*, 8 (1954) 1341.
10 R. L. LESTER AND T. RAMASARMA, *J. Biol. Chem.*, 234 (1959) 672.
11 D. T. EWING, F. S. TOMKINS AND O. KAMM, *J. Biol. Chem.*, 147 (1943) 233.
12 H. NOLL, *J. Biol. Chem.*, 232 (1958) 919.
13 H. NOLL, R. RÜEGG, U. GLOOR, G. RYSER AND O. ISLER, *Helv. Chim. Acta*, 43 (1960) 433.
14 H. NOLL, *J. Biol. Chem.*, 235 (1960) 2207.
15 D. DEUL, E. C. SLATER AND L. VELDSTRA, *Biochim. Biophys. Acta*, 27 (1958) 133.
16 R. B. CRAWFORD, M. MORRISON AND E. STOTZ, *Biochim. Biophys. Acta*, 33 (1959) 543.
17 F. WEBER, U. GLOOR AND O. WISS, *Helv. Chim. Acta*, 41 (1958) 1038.
18 F. WEBER, U. GLOOR AND O. WISS, *Helv. Chim. Acta*, 41 (1958) 1046.
19 D. H. L. BISHOP, K. P. PANDYA AND H. K. KING, *Biochim. J.*, 83 (1962) 606.