Inhomogeneity of Vitamin K_2 in *Mycobacterium phlei**

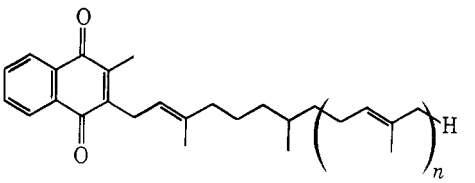
Iain M. Campbell and Ronald Bentley

ABSTRACT: The use of gel filtration in the isolation of pure menaquinones from bacterial nonpolar lipids has been developed. During this procedure, fractionation of any naturally occurring menaquinones with side-chain variation takes place. Thus, in addition to the previously known dihydromenaquinone-9, *Mycobacterium phlei* has been shown to produce a dihydromenaquinone-8 and probably a dihydromenaquinone-10. The dihydromenaquinone-8 has been characterized, the position of the saturated double bond being determined mass spectrometrically.

Some observations on the natural existence of geometrical isomerism in menaquinones and biosynthesis of dihydromenaquinones are made.

During investigations of the biosynthesis of bacterial menaquinones (Campbell *et al.*, 1967), considerable difficulty has been encountered in obtaining rigorously pure samples of these quinones by standard methods. Repeated adsorption or partition chromatography of the quinones or of their dihydrodiacetates fails to remove completely such persistent contaminants as fatty acid esters, aromatic esters (Guérin *et al.*, 1968), and long-chain alcohols. Needless to say, the consequence of such contamination on the interpretation of the results of tracer experiments is sometimes profound.

In an attempt to resolve this unfortunate situation, we have investigated gel filtration in organic solvents. The use of methylated Sephadex in the fractionation of menaquinones[1] was first studied by Nyström and Sjövall (1966) using small samples of material. By application of their methods on a preparative scale with commercial Sephadex LH-20, we have been able to solve the purification problem. At the same time, the technique has revealed that the menaquinone composition of *Mycobacterium phlei* is quite complex. The previously known menaquinone of this organism was not one of the prenylogs investigated by Nyström and Sjövall. Instead, it was a 2-methyl-3-nonaprenyl-1,4-naphthoquinone in which the double bond in the second isoprenyl unit, counting from the aromatic ring, had been hydrogenated: Ia (Azerad *et al.*, 1967). We have now observed that this material, $MK_9(II\text{-}H_2)$, is accompanied by a related dihydro compound possessing one less prenyl unit, $MK_8(II\text{-}H_2)$, Ib, and probably by the compound with one more prenyl unit, $MK_{10}(II\text{-}H_2)$, Ic. This paper reports



Ia, $n = 7$
b, $n = 6$
c, $n = 8$

the details of the purification process and summarizes the evidence for the quoted structures.

Materials and Methods

General. All solvents, etc., were reagent grade. The silica gel used in layer chromatography was obtained from Brinkmann Inc. (Macherey and Nagel, G-HR) and contained a calcium sulfate binder. Ultraviolet spectrometry was performed on a Zeiss PMQII spectrophotometer in the specified solvents. Mass spectra were obtained by the direct probe method using an LKB 9000 spectrometer working at the indicated electron voltages with an electron current of 60 μA and an accelerating voltage of 3.5 kV.

Growth and Harvest of the Organism. M. phlei, the generous gift of Professor M. Weber, was maintained on nutrient agar slopes at 37°. Liquid innocula were prepared by growth for 24 hr at 37° as shake cultures in 250-ml Erlenmeyer flasks containing 25 ml of the following medium: Casamino Acids (Difco), 1.5%; dipotassium hydrogen phosphate, 0.12%; fumaric acid (neutralized with aqueous potassium hydroxide), 0.075%; Tween 80, 0.2%; magnesium sulfate·$2H_2O$, 7.5×10^{-3}%; ferrous sulfate·$7H_2O$, 6.6×10^{-4}%. Prior to sterilization, the medium was adjusted to pH 7.5 with concentrated aqueous potassium hydroxide.

The liquid innocula so produced were used to seed 2.8-l. Fernbach flasks containing 700 ml of the above culture medium. Growth as shake cultures continued at 37° for a further 24-hr period. Thereafter the cells were harvested by continuous centrifugation and the paste

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[1] In this paper, vitamins K_2 will be referred to as menaquinones following the recommended nomenclature (Folkers *et al.*, 1965). The abbreviations which are used are as follows: menaquinone with a 3 substituent of n prenyl units, MK_n; a menaquinone with a reduced double bond in the second prenyl unit, counting from the nucleus, $MK_n(II\text{-}H_2)$.

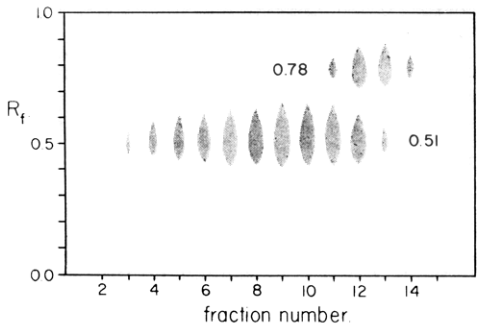


FIGURE 1: Thin-layer chromatogram of menaquinone-containing fractions from Sephadex LH-20 column on silica gel containing 0.02% Rhodamine 6G and 1.5% paraffin oil; solvent, acetone–water (19:1). Visualization was by means of ultraviolet light (λ 254 mμ) and 5% ethanolic phosphomolybdic acid.

was washed twice with distilled water. The yield of wet paste was approximately 10 g/flask.

Isolation of Nonpolar Lipids. Initially the isooctane–isopropyl alcohol method described by Brodie (1963) was employed. This, however, led to excessive and persistent contamination of the menaquinone with, *inter alia*, isopropyl fatty esters (I. M. Campbell and R. Bentley, unpublished data).

A slight modification of the acetone homogenization technique recently reported by Dunphy *et al.* (1968) gave a cleaner preparation. Thus, the wet paste was homogenized in a Waring Blendor (ten bursts of 10 sec each) with three to four volumes of acetone. The solid material was filtered and recycled twice. The combined acetone extracts were reduced in volume to 10% and were then partitioned between ether and water, about ten volumes each. The yield of nonpolar lipid, obtained by separation, drying, and evaporation of the ether layer, was in the range 50–70 mg/100 g wet wt of paste.

It must be emphasized that the above operation, and all those that follow, must be conducted in virtual darkness if extensive *trans–cis* isomerism and decomposition of the menaquinones are to be avoided.

Isolation of the Menaquinones. In a typical run, the nonpolar lipids (179.2 mg) were fractionated on a column of Sephadex LH-20 (130 g; 1.8 × 192 cm; flow rate, 34 ml/hr; swelling time, 22 hr) established in the solvent system isooctane–methanol–chloroform (2:1:1). After development of the column with 100 ml of the solvent, 3.5-ml fractions were collected. Ultraviolet spectrometry revealed that the characteristic chromophore of a 2,3-disubstituted naphthoquinone was restricted to fractions 3–14, with fractions 3 and 14 containing only trace amounts; yields: fractions 3–10 (MK_9-(II-H_2) and MK_{10}(II-H_2)), 91.1 mg; fractions 11–14 (MK_9(II-H_2) and MK_8(II-H_2)), 63.4 mg. For chromatographic and mass spectral assay of these fractions, see Figures 1 and 2, respectively.

Isolation and Characterization of MK_8(II-H_2). The combined fractions 11–14 (63.4 mg) from the above column were rechromatographed on Sephadex LH-20 (130 g; 1.8 × 192 cm; flow rate, 14 ml/hr; swelling time, 24 hr) using the previously described solvent system. After development with 109 ml of solvent, 3.5-ml fractions were taken. Ultraviolet spectrometry located menaqui-

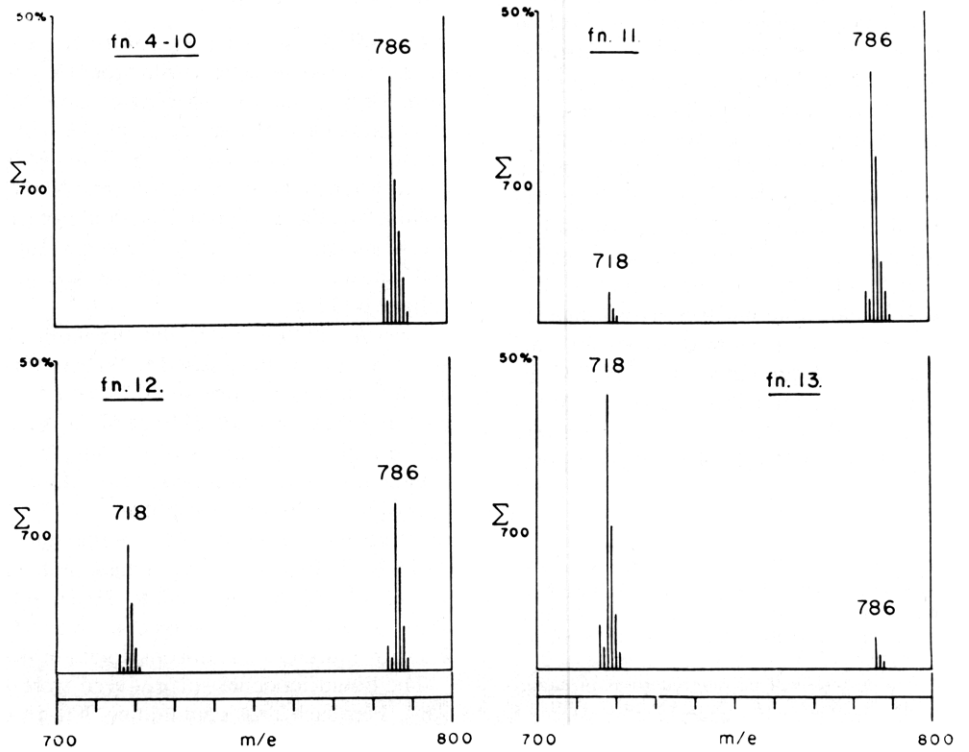


FIGURE 2: Mass spectrometric assay of menaquinone-containing fractions from Sephadex LH-20 column, recorded at 15 eV. Molecular parent ion at *m/e* 786 is MK_9(II-H_2), at *m/e* 718 is MK_8(II-H_2).

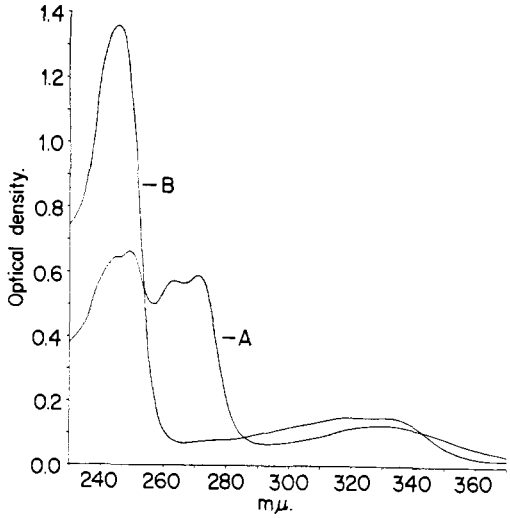


FIGURE 3: Ultraviolet spectra of $MK_8(II\text{-}H_2)$ (A) and the corresponding hydroquinone, (B) taken in ethanol containing 1% aqueous molar ammonium acetate. The hydroquinone was obtained by *in situ* reduction of the quinone with sodium borohydride.

none in fractions 1–5. Mass spectrometry indicated that fractions 4 and 5 (18.8 mg) were rich in $MK_8(II\text{-}H_2)$.

Final purification was achieved by repeated reversed-phase chromatography on layers of silica gel (250 μ) prepared with 0.02% Rhodamine 6G and impregnated with 1.5% paraffin oil. The developing solvent was acetone–water (19:1). The quinone was visualized by *very* brief exposure to ultraviolet light of wavelength 254 mμ and was eluted from the silica gel with ethyl acetate. The paraffin oil contaminating the eluted quinone was removed by thin-layer chromatography on silica gel (250 μ) using cyclohexane–benzene (2:1) as solvent. Pure $MK_8(II\text{-}H_2)$ was obtained as an oil (*ca.* 1.5 mg): λ_{max} (isooctane) 239 (s), 243, 249, 261, 270, and 327 mμ; of authentic $MK_9(II\text{-}H_2)$, 239 (s) 243, 261, 270, and 327 mμ (*cf.* Figure 4); λ_{max} (ethanol–sodium acetate), 245, 249, 263, 271, and 330 mμ; on treatment with sodium borohydride, λ_{max} 247, 320, and 332.5 mμ (*cf.* Figure 3); parent molecular ion *m/e* 718 and base peak *m/e* 225 (*cf.* Figure 5).

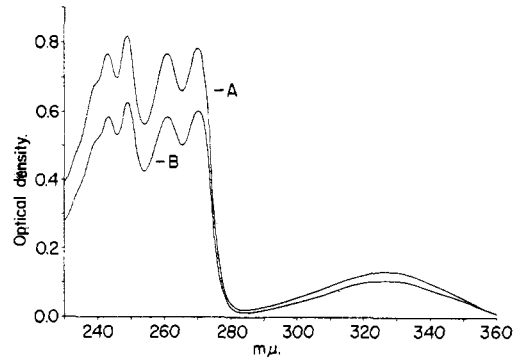


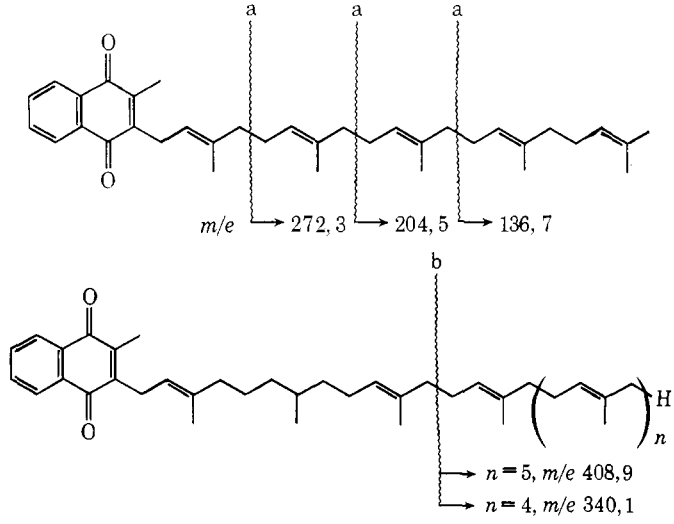
FIGURE 4: Comparison of ultraviolet spectra of $MK_8(II\text{-}H_2)$ (A) with that of authentic $MK_9(II\text{-}H_2)$ (B), solvent, isooctane.

Results

Chromatography of the neutral lipids of *M. phlei* on Sephadex LH-20 in a modification of the solvent system of Nyström and Sjövall (1966) leads to elution of the menaquinone fraction as a single band. The composition of this band, however, is not homogeneous. If consecutive fractions are subject to reversed-phase chromatography on layers of silica gel impregnated with paraffin oil, resolution into two distinct areas is clearly seen (Figure 1). Mass spectrometry demonstrates this multiplicity even more forcefully. In Figure 2 are presented the higher mass regions of the mass spectra of the contents of the ten fractions concerned. The spectra were determined at 15 eV at which voltage the contribution of the M − 68 and M − 69 ions to the total ion current is negligible. While fractions 4–10 can be seen to contain solely a substance of parent molecular ion *m/e* 786, fractions 11–13 possess, in addition, a parent ion at *m/e* 718.

Standard methods of analysis confirmed that the dihydromenaquinone, $MK_9(II\text{-}H_2)$, Ia, was responsible for the *m/e* 786 parent ion and the chromatographic spot of R_F 0.51.

The material of mol wt 718 and R_F 0.78 was isolated free from $MK_9(II\text{-}H_2)$ by further Sephadex LH-20 and reversed-phase chromatography. Its identity as a 2,3-disubstituted naphthoquinone was established by



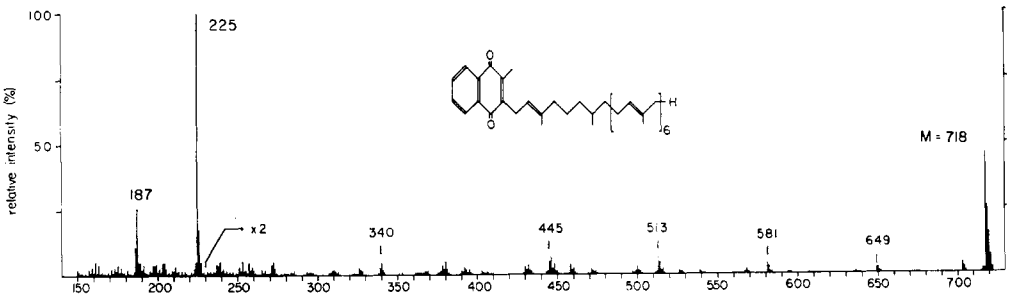


FIGURE 5: Mass spectrum of $MK_8(II\text{-}H_2)$ taken at 70 eV.

the alteration of its ultraviolet chromophore on treatment in buffered ethanol with borohydride (Figure 3, Dunphy *et al.*, 1968) and by the superimposability of its ultraviolet spectrum with that of authentic $MK_9(II\text{-}H_2)$ (Figure 4). Comparison of its 70-eV mass spectrum (Figure 5) with that of $MK_9(II\text{-}H_2)$ indicated that the new material was a dihydro-2-methyl-3-octaprenyl-1,4-naphthoquinone and suggested that its structure might be Ib.

Since insufficient material was available for a chemical degradation to confirm the location of the saturated double bond, a mass spectrometric method had to be used for this purpose. It had been noted (I. M. Campbell, unpublished data) that the 15-eV mass spectra of menaquinones MK_5, MK_7, MK_8, and MK_9 contained sizeable ions at *m/e* 135, 136, and 137, and then at positions corresponding to *m/e* 136 + *n*68 and 137 + *n*68. Since these ions also occurred in the 15-eV spectrum of 2-demethyl-MK_8, it was deduced that they represented side-chain fragments $(C_5H_8)_n$ and $(C_5H_8)_nH$, produced by fission through the diallylic bonds a, the process occurring with or without hydrogen transfer. The validity of this conclusion was ascertained by mass measurement. It was further noted (Figure 6) that in the spectrum of $MK_9(II\text{-}H_2)$, the $(C_5H_8)_n$:$(C_5H_8)_nH$ ion sequence was significantly interrupted after *m/e* 408, *i.e.*, at that point b, between isoprene units 3 and 4, where the last diallylic fission was possible. Since the corresponding interruption in the sequence in the spectrum of the new $MK_8(H_2)$ (Figure 6) occurred after the *m/e* 340 level, it was deduced that in this compound the last diallylic bond also occurred between isoprene units 3 and 4. Hence, the saturated double bond must be located in the second isoprene unit, counting out from the aromatic ring, and structure Ib must indeed be that of the newly isolated quinone.

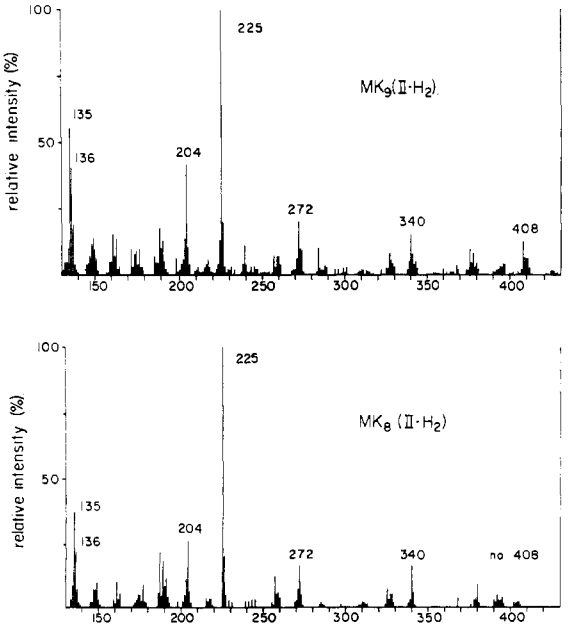


FIGURE 6: Central regions of the 15-eV mass spectra of $MK_9(II\text{-}H_2)$ and $MK_8(II\text{-}H_2)$. Note absence of *m/e* 408 in spectrum of $MK_8(II\text{-}H_2)$.

The detection of one addition prenylog of $MK_9(II\text{-}H_2)$ in *M. phlei* suggested that there might be others. A search for prenylogs of lower molecular weight than $MK_8(II\text{-}H_2)$ has so far been unfruitful. However, when relatively large samples of fractions 4–10 were used for 15-eV mass spectrometry, the presence of a material of parent molecular ion *m/e* 854 was routinely detected. This is almost certainly due to an $MK_{10}(H_2)$, possibly $MK_{10}(II\text{-}H_2)$, Ic. It is hoped that a lipophilic gel of higher exclusion limit than LH-20 will permit its separation from the lower molecular weight prenylogs.

Since major differences in the relative volatility of related compounds in the molecular weight region 700–900 might not be expected to exist, a first approximation to the composition of the menaquinone fraction of *M. phlei* may be obtained by measuring the relative abundances of the parent molecular ions in the 15-eV mass spectrum of the unfractionated mixture. The figures were determined to be: $MK_8(II\text{-}H_2)$, 7.6%; $MK_9(II\text{-}H_2)$, 92.3%; and $MK_{10}(H_2)$, 0.1%.

It is of interest to note one further result. In view of the recent report of the occurrence of a *cis* geometrical isomer of $MK_9(II\text{-}H_2)$ in *M. phlei* (Dunphy *et al.*, 1968), we have examined our samples by the prescribed chromatographic methods for the detection of such isomerism. *cis* isomers were found only when ultraviolet light had been used in the visualization of quinones on thin-layer plates, or when the isolation, etc., was not conducted in virtual darkness. The implication of this finding will be discussed elsewhere.

Discussion

The isolation of dihydro-2-methyl-3-octaprenyl-1,4-naphthoquinones from several sources has already been

reported (Scholes and King, 1965; Jeffries *et al.*, 1967). However, this is the first instance where such a compound has been characterized.

Inhomogeneity in the composition of bacterial polyprenylated metabolites appears to be quite common. Thus, in addition to this instance in *M. phlei*, a similar situation arises in the menaquinones of aerobic *Micrococcacea* (Jeffries *et al.*, 1967), in the menaquinones (I. M. Campbell and R. Bentley, unpublished data) and ubiquinones (Daves *et al.*, 1967) of *E. coli*, and in the ubiquinones of *Rhodospirillum rubrum* (Daves *et al.*, 1967). Inhomogeneity in fungal (Lavate and Bentley, 1964), yeast (Daves *et al.*, 1967), and animal (Matschiner and Amelotti, 1968) polyprenylated metabolites has also been demonstrated. As already pointed out (Daves *et al.*, 1967), this phenomenon appears to indicate that the polyprenyl synthases and transferases involved in the synthesis of such materials are less than absolutely specific.

With particular reference to the biosynthesis of the menaquinones of *M. phlei*, the existence of a series of polyprenyl derivatives in which the double bond of the penultimate isoprene unit is consistently reduced may indicate that hydrogenation takes place after coupling of the polyprenyl chain with the naphthoquinone ring system, rather than before it.

Acknowledgment

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References

Azerad, R., Cyrot, M., and Lederer, E. (1967), *Biochem. Biophys. Res. Commun. 27*, 249.

Brodie, A. F. (1963), *Methods Enzymol. 6*, 295.

Campbell, I. M., Coscia, C. H., Kelsey, M., and Bentley, R. (1967), *Biochem. Biophys. Res. Commun. 28*, 25.

Daves, G. D., Muraca, R. F., Whittick, J. S., Friis, P., and Folkers, K. (1967), *Biochemistry 6*, 2861.

Dunphy, P. J., Gutnick, D. L., Phillips, P. G., and Brodie, A. F. (1968), *J. Biol. Chem. 243*, 398.

Folkers, K., Green, D. E., Isler, O., Martius, C., Morton, R. A., and Slater, E. C. (1965), *Biochim. Biophys. Acta 107*, 5.

Guérin, M., Azerad, R., and Lederer, E. (1968), *Bull. Soc. Chim. Biol. 50*, 187.

Jeffries, L., Cawthorne, M. A., Harris, M., Diplock, A. T., Green, J., and Price, S. A. (1967), *Nature 215*, 257.

Lavate, W. V., and Bentley, R. (1964) *Arch. Biochem. Biophys. 108*, 287.

Matschiner, J. T., and Amelotti, J. M. (1968), *J. Lipid Res. 9*, 176.

Nyström, E., and Sjövall, J. (1966), *J. Chromatog. 24*, 212.

Scholes, P. B., and King, H. K. (1965), *Biochem. J. 97*, 766.