It was very soluble in ether and benzene and slightly soluble in alcohol and glacial acetic acid. *Anal.* Calcd. for $C_{26}H_{17}N$: C, 90.93; H, 4.99; N, 4.08; mol. wt., 343; m. p. (lit.[10]) 144°. Found: C, 90.26, 90.55; H, 4.97, 5.01; N, 4.20, 4.27; mol. wt., 350, 354, 335, 337 (average 344); m. p. 143.5–144.5°.

7-Phenyl-dibenzo[*c,g*]carbazole Dipicrate.—Conversion of a sample of the carbazole to the picrate by the procedure described above yielded the red crystalline product. *Anal.* Calcd. for $C_{26}H_{17}N \cdot 2C_6H_2OH(NO_2)_3$: N, 12.23; m. p. (lit.[10]) 169°. Found: N, 12.10, 12.05; m. p. 168.5–169.5°.

Lead Dioxide Oxidation of N-Phenyl-2-naphthylamine.—Two hundred fifty grams of the amine was dried *in vacuo* at 70°, dissolved in 5 liters of dry benzene, and stirred with 250 g. of lead dioxide for 48 hours at room temperature. The solution was filtered and evaporated to yield a dark tar. When the tar was taken up in about 1 liter of ether, about 75 g. of a crystalline residue remained. A portion of the residue was recovered from ether and was identified as unreacted amine, m. p. 108°; mixed m. p. unchanged. On evaporation of the ether extract 157 g. of a dark tar (C) was obtained. Seventy-five grams of C was distilled under reduced pressure to give three fractions: (1) Forty-seven and five-tenths grams of a white crystalline product, b. p. 235–270° (20 mm.), which was recrystallized twice from a cyclohexane–acetone mixture and identified as additional unreacted N-phenyl-2-naphthylamine. (2) Five grams of a red viscous tar, b. p. 275–285° (20 mm.), which could not be crystallized, but after reaction with 5.8 g. of picric acid in a small amount of hot benzene, a red crystalline picrate separated immediately. This was recrystallized twice from benzene and decomposed with hot dilute ammonium hydroxide to yield 2 g. of a dark tar which underwent an almost complete transition to yellowish crystals on standing at 10° for several days. Recrystallization twice from a benzene–alcohol–acetone mixture yielded yellow crystals, m. p. 190° (dec.). *Anal.* Calcd. for $C_{27}H_{20}N_3O_2$: C, 77.49; H, 4.82; N, 10.04; mol. wt., 418. Found: C, 76.86; H, 4.79; N, 10.84; ash, 0.0; mol. wt. (in camphor), 412. The dipicrate melted at 156° (dec.). *Anal.* Calcd. for $C_{27}H_{20}N_3O_2 \cdot 2C_6H_2OH(NO_2)_3$: C, 53.43; H, 2.99; N, 14.38. Found: C, 57.24; H, 3.05; N, 12.33. (3) Twelve grams of a very viscous red tar, b. p. 320–350° (20 mm.), which reacted with 19.9 g. of picric acid in hot benzene to yield a red crystalline picrate. Recrystallization twice from hot benzene and decomposition with hot dilute ammonium hydroxide gave 3.9 g. of dirty yellow crystals. These were recrystallized 3 times from benzene–alcohol mixtures to yield pale green crystals, m. p. 137–140°, which gave the same color tests, fluorescence, and solubility properties as were described above for the 7-phenyl-dibenzo[*c,g*]carbazole isolated from the permanganate oxidation products; mixed m. p. with the latter, 140–142°. Conversion to the picrate gave a product with m. p. 165–168°; mixed m. p. with the corresponding picrate obtained from the permanganate oxidation product, 165–168.5°.

Absorption Spectra.—The measurements were made with a Beckman quartz prism spectrophotometer, a hydrogen discharge tube serving as the light source. Readings were taken at 1 mμ intervals in the vicinity of maxima and minima, and at 5 mμ intervals elsewhere. Solute concentrations of about 0.01 g./l. were employed. Selected lots of the purified solvents were used, and suitable corrections were made for background absorption.

Acknowledgment.—The authors wish to thank Mr. Julius V. Sommer for some of the analyses reported in this work, and Miss Jean Hund for technical assistance in the spectrophotometric measurements.

Summary

1. The oxidation of N-phenyl-2-naphthylamine by potassium permanganate in acetone and by lead dioxide in benzene has been carried out.

2. From the permanganate oxidation products there have been isolated N-(2-naphthyl)-N,N'-diphenyl-1,2-naphthylenediamine, and 7-phenyl-dibenzo[*c,g*]carbazole.

3. From the lead dioxide oxidation products there have been isolated 7-phenyl-dibenzo[*c,g*]-carbazole and an unidentified product having the composition $C_{27}H_{20}N_3O_2$.

4. Some derivatives of N-(2-naphthyl)-N,N'-diphenyl-1,2-naphthylenediamine and of $C_{27}H_{20}N_3O_2$ have been synthesized and described.

5. The ultraviolet absorption spectra, in isooctane solution, of N-phenyl-2-naphthylamine and some of its oxidation products have been compared and the spectral characteristics have been recorded.

ELIZABETH, NEW JERSEY RECEIVED DECEMBER 29, 1944

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF WINTHROP CHEMICAL COMPANY, INC.]

The Absorption Spectra of the Vitamins and Provitamins D[1]

BY WOLFGANG HUBER, GALEN W. EWING AND JACOB KRIGER

Observations on the ultraviolet absorption spectra of the various D vitamins[2,3,4,5] and provitamins[6] have been reported in the literature by various authors. There also have been reported spectra of several of the esters of these compounds, particularly of the nitro substituted benzoyl esters.[5,7]

It is the purpose of this paper to describe the spectra of the D vitamins, provitamins and several of their esters, including those mentioned by Huber and Barlow,[5] with a view to characterization and possible assay of vitamin preparations and intermediates by absorption spectrophotometry. We have examined particularly the 3,5-dinitrobenzoates, the 3,5-dinitro-4-methylbenzoates, the 3-nitro-4-methylbenzoates, and the 4-nitrobenzoates of vitamins D_2 and D_3, ergosterol and 7-dehydrocholesterol. These have been studied variously in alcohol, hexane, and chloroform solutions.

The D provitamins, such as ergosterol (I), 7-

(1) Presented in part before the Division of Biological Chemistry of the American Chemical Society, Pittsburgh Meeting, September, 1943.

(2) Brockmann and Busse, *Z. physiol. Chem.*, **256**, 252 (1938).

(3) Anderson, Bacharach and Smith, *Analyst*, **62**, 430 (1937).

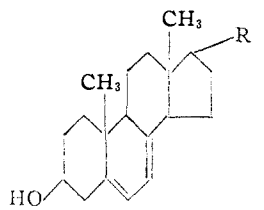
(4) Schenck, *Naturwiss.*, **25**, 159 (1937).

(5) Huber and Barlow, *J. Biol. Chem.*, **149**, 125 (1943).

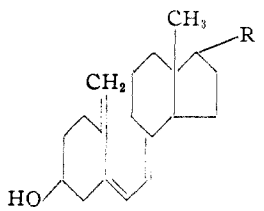
(6) Hogness, Sidwell and Zscheile, *J. Biol. Chem.*, **120**, 239 (1937).

(7) Windaus and Rygh, *Nachr. Ges. Wiss. Göttingen*, Math.-physik. Klasse, **25**, 202 (1928).

dehydrocholesterol (II), and several others, all show the ultraviolet absorption characteristic of conjugated dienes in which two double bonds occur in the same ring. In vitamins D_2 (III) and D_3 (IV), however, the absorption spectra are typical of conjugated trienes in which the double bonds are not co-cyclic.



(I). (II)



(III), (IV)

(I) and (III), R = $-CH(CH_3)-CH=CH-CH(CH_3)-CH(CH_3)_2$; (II) and (IV), R = $-CH(CH_3)-(CH_2)_3-CH(CH_3)_2$

Ergosterol.—Since ergosterol is the most readily obtainable of the various D provitamins, more numerous and extensive investigations have been made of its properties than of any of the others. The ergosterol spectrum is well known to show intense maxima at wave lengths approximately 271 and 281 mμ and a third, less intense, at 293 mμ. The most recent data in the literature are those given by Hogness, Sidwell and Zscheile.[6] These authors used commercial ergosterol[8] which they recrystallized once from alcohol, once from benzene and twice from iso-octane, successively. We have performed similar experiments, also using commercial ergosterol,[9] and find after repeated recrystallizations from benzene–ethanol, extinction data almost identical with Hogness' values (Fig. 1). It is found, however, that if spectral determinations are made after each recrystallization, the extinction values become successively lower, not higher as might be expected if the recrystallizations increase the purity of the sample. The highest values are obtained by recrystallizing once only the commercially available material. It is well known that the provitamins D are rather unstable, and hence it is not surprising that repeated manipulation may tend to decrease the purity on account of decomposition, rather than to increase it.

We have used benzene and alcohol (containing 5% water) for our recrystallization of ergosterol, as it is the most satisfactory solvent mixture for use on a large scale where reproducible purity is important. It is recognized that this procedure gives crystals of ergosterol which contain one mole of water of crystallization, whereas Hogness' ergosterol is probably anhydrous.[10]

Our curve for ergosterol, obtained as above described, is given in Fig. 1, together with the maxima and minima from Hogness' paper for comparison. The extinction data are presented in Table I, along with a notation of the width of the spectral band isolated in each case.[11] A slight discrepancy between the data of Hogness and of the present research lies in the neighborhood of 262 to 263 mμ. Hogness finds a maximum at 262 with an accompanying minimum at 263 mμ, whereas we find only an inflection in the curve at this wave length.[12]

The solvent used in the spectral determinations of ergosterol has a considerable effect on the absorption. Alcohol and hexane solutions give nearly identical curves, while a chloroform solution shows extinction coefficients reduced by about 10% and shifted 3 mμ toward the longer wave lengths (Fig. 1). Chloroform for this use should be tested for traces of hydrochloric acid,

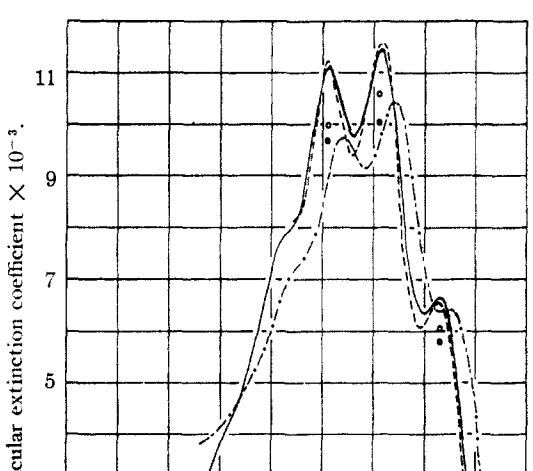


Fig. 1.—Absorption spectra of ergosterol, (a) in alcohol ——, (b) in hexane — — — —, (c) in chloroform — · — · —. The maxima reported by Hogness[6] are indicated by (○) for alcohol and (●) for iso-octane.

(8) Obtained from Mead, Johnson and Co.

(9) Obtained from Montrose Chemical Co.

(10) Lettré and Inhoffen, "Über Sterine, Gallensäuren und verwandte Naturstoffe," Ferd. Enke, Stuttgart, 1936, p. 121.

(11) For the significance of this, see Hogness, Zscheile and Sidwell, *J. Phys. Chem.*, 41, 379–415 (1937).

(12) Hogness reports maxima in the ergosterol and 7-dehydrocholesterol curves in the region between 320 and 340 mμ. We have made no attempt to verify these observations, since the maxima are so weak that they are not likely to be of much value is characterizing or assaying the sterols. Similar remarks apply to the inflections at 262–263 and at 252 mμ, which we have accordingly not included in Table I.

TABLE I

Compound	Solvent	$\lambda_{max.}$	Our values ϵ[a]	Our values Band width	Hogness' values[6] ϵ[a]	Hogness' values[6] Band width
Ergosterol	Alc	271.0 ± 0.5	11090 ± 200	1.46	10000 ± 100	0.52
		281.0 ± 0.5	11500 ± 200	1.04	10600 ± 100	.61
		293.0 ± 1.0	6540 ± 100	1.52	6060 ± 50	.71
	Hex	271.0 ± 0.5	11260 ± 200	2.18	9700 ± 70[b]	.73
		281.0 ± 0.5	11570 ± 200	2.49	10050 ± 70	.84
		293.0 ± 1.0	6660 ± 100	2.26	5800 ± 50	.86
	Chl	274.0 ± 0.5	9760 ± 200	2.42		
		284.5 ± 0.5	10440 ± 200	2.71		
		295.0 ± 1.0	6390 ± 100	2.89		
7-Dehydrocholesterol	Alc	271.0 ± 0.5	10470 ± 200	1.75	10400 ± 100	.94
		281.5 ± 0.5	10920 ± 200	1.98	10750 ± 100	1.10
		293.0 ± 0.5	6300 ± 100	2.26	6150 ± 150	1.14
	Hex	271.0 ± 0.5	10550 ± 200	1.46		
		281.5 ± 0.5	11020 ± 200	1.50		
		293.0 ± 0.5	6330 ± 100	1.70		
	Chl	274.0 ± 0.5	9250 ± 200	1.52		
		284.5 ± 0.5	9950 ± 200	1.70		
		295.0 ± 0.5	6080 ± 100	1.93		
Vitamin D_2	Alc	264.5 ± 0.5	18200 ± 300	2.66		
	Hex	264.5 ± 0.5	18200 ± 300	2.66		
Vitamin D_3	Alc	264.5 ± 0.5	18200 ± 300	2.92		
	Hex	264.5 ± 0.5	18200 ± 300	2.00		

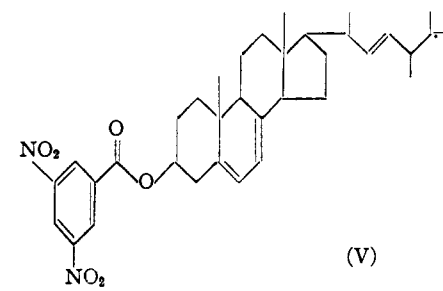
[a] Molecular extinction coefficient, defined by the equation, $\epsilon = (M/ct) \log_{10}(I_0/I)$, where M is the molecular weight of the solute, c its concentration expressed in grams per liter of solution, t the thickness of the absorption cell in cm., I_0 the intensity of the incident light, and I that of the light transmitted by the solution. Note that Hogness' α is identical with ϵ as here defined. [b] Hogness used iso-octane rather than hexane.

since its presence may result in partial isomerization of the sterol.

7-Dehydrocholesterol.—Since the chromophoric group of 7-dehydrocholesterol is the same as that of ergosterol, it is to be expected that their spectra will be similar, and such is found to be the case. Our values for the extinction coefficients agree with those of Hogness,[6] within the stated limits of error, as shown in Table I. Hogness states that his sample was prepared by the method of Windaus, Lettré and Schenck,[13] which was essentially the method followed in the present work. Hogness' product was recrystallized from anhydrous solvents, and so may have been anhydrous, whereas our material is known to be a monohydrate. Just as with ergosterol, we find that 7-dehydrocholesterol should not be recrystallized after it is separated from the saponification mixture. Our curve, as well as the maxima of Hogness, is given in Fig. 2.

Provitamin Esters.—As mentioned above, the provitamins ergosterol and 7-dehydrocholesterol are difficult to obtain and maintain in a condition of high purity, and hence it is difficult to establish a true criterion of purity by means of spectroscopic examination. However, the esters of these provitamins with the common nitrobenzoic acids do not share this instability. Thus it is comparatively a simple matter to determine reproducibly the extinction curves of these esters. It should then be possible to subtract the absorption due to the acid residue from the absorption of the ester to obtain an extinction curve for the provitamin itself, unaffected by impurity due to decomposition.

The validity of this procedure is based on the independence of two chromophore groups separated in the molecule by one or more saturated linkages. In the case of the esters under consideration, such for example as ergosteryl 3,5-dinitrobenzoate (V), the benzenoid chromophore



(V)

of the acid is separated from the diene chromophore of the sterol not only by the oxygen link of the ester but also by a whole cyclohexane ring. Hence it is to be expected that the spectrum of the ester should be very nearly equal to the sum of the spectra of the sterol and acid components. It is also assumed that all three substances (acid, sterol, and ester) obey Beer's law. This assumption has been found to be valid for the esters and sterols, but may not hold for the free acids, as they

(13) Windaus, Lettré and Schenck, *Ann.*, **520**, 98 (1936).

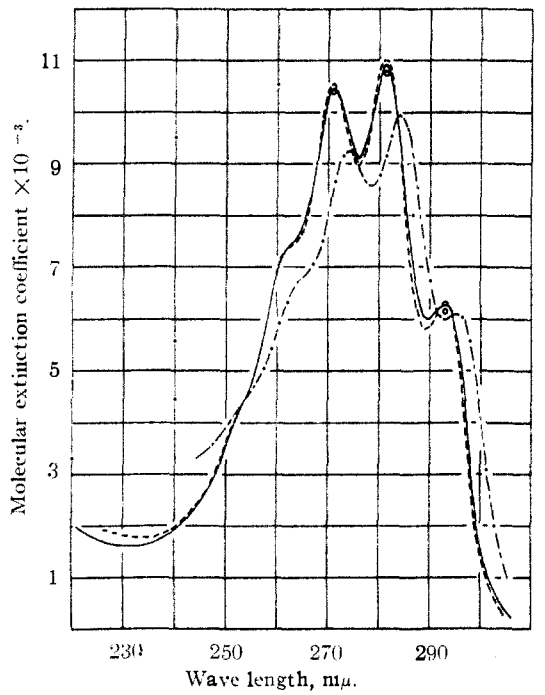


Fig. 2.—Absorption spectra of 7-dehydrocholesterol, (a) in alcohol ——. (b) in hexane — — — —, (c) in chloroform — · — · —. The maxima reported by Hogness[6] for alcohol are indicated by (O).

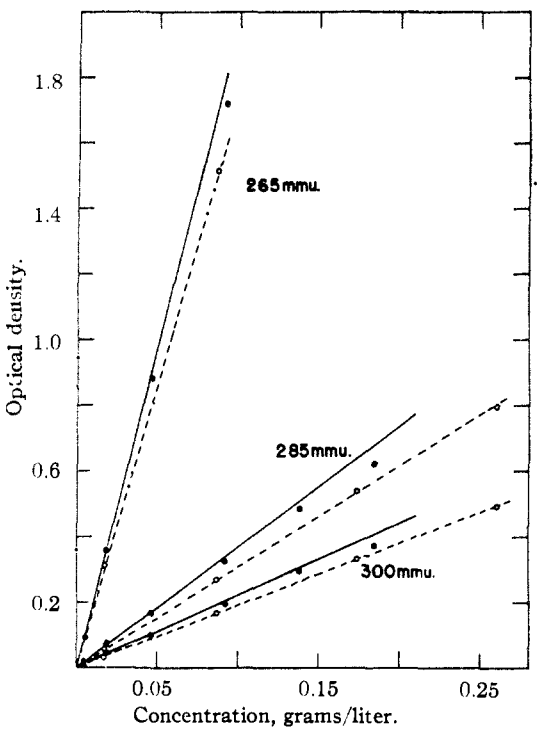


Fig. 3.—Beer's law diagram for 3,5-dinitrobenzoic acid. —●—●—. and cyclohexyl 3,5-dinitrobenzoate — — — ⊙ — — — ⊙ — — —, both in alcohol. The observed densities for the acid have been multiplied by the ratio of molecular weights of acid to ester (212/293) to render them comparable.

tend to ionize appreciably at the low concentrations employed, particularly in such a polar solvent as alcohol. Figure 3 shows a plot of optical density against concentration for 3,5-dinitrobenzoic acid in alcohol, as determined at three different wave lengths. Their deviation from straight lines is a measure of the failure of Beer's law. In the same figure are shown similar curves for the cyclohexyl ester of the same acid, and these are seen to obey Beer's law within the limits of experimental error. The cyclohexyl esters of the other acids considered were also prepared and their spectra determined for use in place of those of the free acids.

In connection with the validity of the additivity relations, the following observations are of interest. H. Dannenberg[14] includes in his extensive list of spectra of sterols, six sterols for which the spectra of the acetates have also been determined. In four of these the hydroxyl group is attached directly to a completely saturated ring (lumisterol, cholesterol B_3, $\Delta^{5,16}$-pregnadienol-3-one-20, and $\Delta^{3,5}$-androstadienol-17-one-7). The acetates of these sterols have absorption maxima at exactly the same wave lengths as the respective free alcohols. This has also been found to be true for ergosterol and its acetate.[15] In another of Dannenberg's entries, Δ^4-cholestenol-3-one-6, the hydroxyl group is attached to a ring which contains a double bond in the position α,β to the hydroxyl group; its acetate shows a maximum about 3 mμ lower than the free alcohol. In his other instance, tetradehydro-*neo*-ergosterol, the hydroxyl is connected to an aromatic ring system. This sterol shows two maxima, at wave lengths 250 and 280 mμ. In the spectrum of its acetate, the lower of these has been shifted to 240, while the 280 maximum is unchanged. Thus it is seen that esterification of a sterol in which the hydroxyl is attached to a saturated ring does not change the spectrum of the sterol, as long as the esterifying acid is itself non-absorbing.

Baxter and his co-workers[16] have reported wave-length maxima of the tocopherols and their esters with saturated acids. These esters, in which the acid residue contains no chromophore group at all, show maxima at 6 to 14 mμ shorter wave lengths than those of the tocopherols themselves, and with lower extinction values. At first sight this would seem to be in disagreement with the present work. However, it should be pointed out that the tocopherols are substituted phenols, rather than substituted alicyclic alcohols as are the provitamins and vitamins D. A similar shift to shorter wave lengths and lower extinctions is shown upon esterification of phenol itself (phenol, $\lambda_{max.} = 271$ mμ,

(14) Dannenberg, *Abhandl. Preuss. Akad. Wiss.*, Math.-naturwiss. Kl., No. 21, pp. 1–68 (1939, published 1940).

(15) Mazur, THIS JOURNAL, **63**, 2442 (1941).

(16) Baxter, Robeson, Taylor and Lehman, *ibid.*, **65**, 918 (1943).

ϵ = 2750; phenyl acetate, $\lambda_{max.}$ = 261 mμ, ϵ = 229).[17]

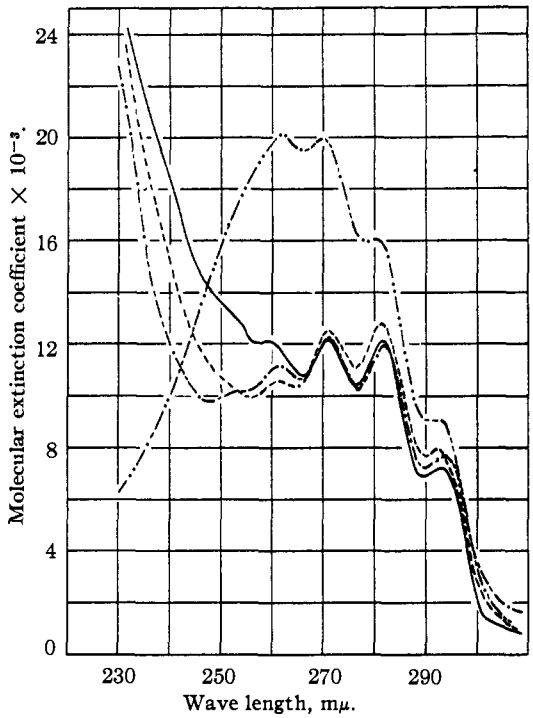


Fig. 4.—Absorption spectra of ergosterol esters in hexane, (a) 3,5-dinitrobenzoate ——, (b) 3,5-dinitro-4-methylbenzoate — — — —, (c) 3-nitro-4-methylbenzoate — • — • —, (d) 4-nitrobenzoate — •• — •• —.

To test these assumptions, spectra have been determined for the various esters of ergosterol (Fig. 4) and 7-dehydrocholesterol in alcohol, hexane and chloroform solutions. A subtraction plot was made for each ester in each solvent (Figs. 5 and 6 are representative). The extinction values for the provitamin maxima thus obtained are given in Table II in comparison with those obtained from the pure crystalline sterols.

Vitamins D_2 and D_3.—There is no report in the literature giving absorption data on these vitamins with precision comparable to that of Hogness and co-workers for the provitamins. The curve given by Brockmann and Busse[2] is representative, and shows a single maximum at wave length 265 mμ, with a molecular extinction coefficient of 18,200. All the literature reports agree on the wave length, and within limits of several per cent. on the extinction as well. Our own determinations are summarized in Table I and Fig. 7.

The vitamins are even more difficult to maintain in a state of purity than the provitamins, but in this case also the nitrobenzoic esters are quite stable, as shown by Huber and Barlow.[5] The spectra of the several esters of both vitamins were determined in alcohol and in hexane, as shown in Fig. 8 for the case of D_2 in alcohol. The vitamins were not run in chloroform since they are extremely sensitive to minute traces of hydrochloric acid, such as may be liberated by action of the

(17) Dimroth, *Z. angew. Chem.*, **52**, 545–556 (1939).

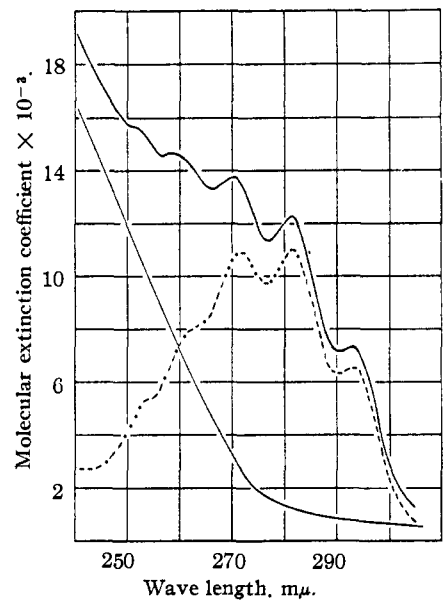


Fig. 5.—Absorption spectra, in alcohol, of (a) ergosteryl 3,5-dinitrobenzoate, (b) cyclohexyl 3,5-dinitrobenzoate, and (c) (— — — —) the curve for ergosterol obtained by subtracting graphically (b) from (a).

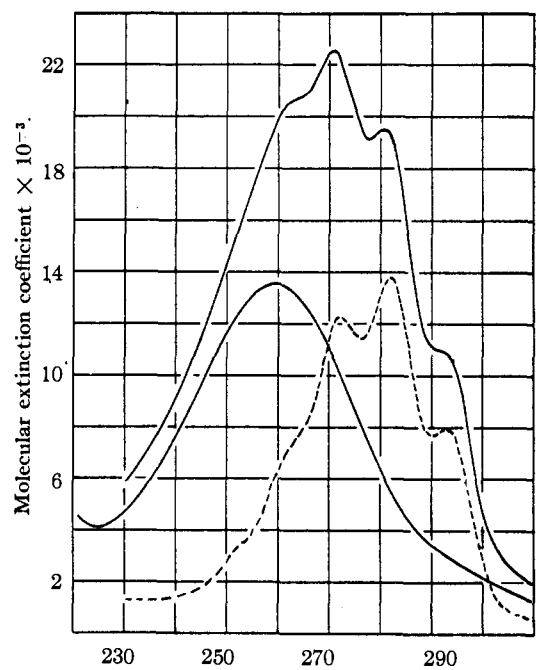


Fig. 6.—Absorption spectra, in alcohol, of (a) 7-dehydrocholesteryl 4-nitrobenzoate, (b) cyclohexyl 4-nitrobenzoate, and (c) (— — — —) the curve for 7-dehydrocholesterol obtained by subtracting (b) from (a).

TABLE II

MOLECULAR EXTINCTION COEFFICIENTS OF THE ABSORPTION MAXIMA OF THE PROVITAMINS D_2 AND D_3, OBTAINED DIRECTLY AND FROM THE SUBTRACTION CURVES OF THEIR ESTERS

	Alcohol			Hexane			Chloroform		
	271–2	281–2	292–3	271–2	281–2	292–3	274–5	284–5	295–6
Ergosterol:									
Pure crystals	11090	11500	6540	11260	11570	6660	9760	10440	6390
Benzoates:									
3,5-dinitro-	10900	11150	6550	11000	11400	6700	9350	10070	6150
3,5-dinitro-4-methyl-	...	...	..	11420	11850	6950	10150	10800	6600
3-nitro-4-methyl-	9700	10500	6300	10450	10850	6600	9730	10350	6500
4-nitro-	11000	11900	6550	11300	12050	6800	9900	11200	6900
7-Dehydrocholesterol									
Pure crystals	10470	10920	6300	10550	11020	6330	9250	9950	6080
Benzoates:									
3,5-dinitro-	...	...	..	12000	12500	7250	10700	11450	7000
3,5-dinitro-4-methyl-	11550	11800	7000	11300	11700	6900	10000	10600	6750
3-nitro-4-methyl-	12500	12900	7400	12700	13100	7500	11100	11800	7250
4-nitro-	12300	13800	7900	12400	13500	7800	11000	12700	7800

ultraviolet radiation during the spectrophotometric determinations.

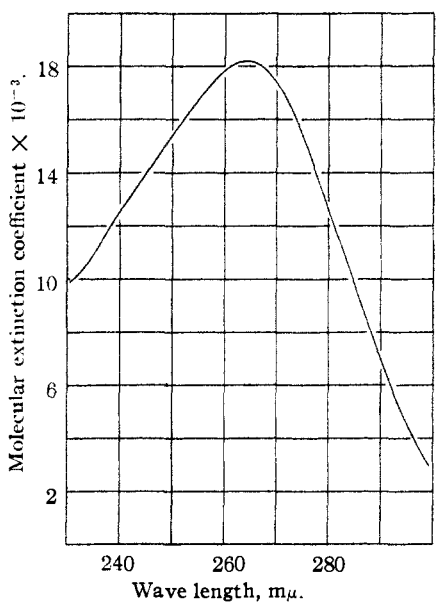


Fig. 7.—Absorption spectrum of vitamin D_2 in alcohol. The spectra of vitamins D_2 and D_3 in both alcohol and hexane coincide within the limits of experimental precision.

Subtraction plots were made for each ester in each solvent and the extinction values for the vitamins so obtained listed in Table III for comparison with the values obtained from the pure crystalline vitamins. Figure 9 shows a representative subtraction curve.

Discussion

It is seen from an examination of the data that the absorption maxima of the provitamins and vitamins, respectively, as determined by the subtraction method, agree within the limit of error as far as the wave length location of the maxima is concerned. This close agreement, however, does not always hold for the molecular extinction coefficients of the maxima.

TABLE III

MOLECULAR EXTINCTION COEFFICIENTS OF THE ABSORPTION MAXIMA OF THE VITAMINS D_2 AND D_3, OBTAINED DIRECTLY AND FROM THE SUBTRACTION CURVES OF THEIR ESTERS, λ264–5 mμ

	Solvent	
Vitamin D_2	Alcohol	Hexane
Pure crystals	18200	18200
3,5-Dinitrobenzoate	18150	17800
3,5-Dinitro-4-methylbenzoate	18000	17950
3-Nitro-4-methylbenzoate	17960	18000
4-Nitrobenzoate	17000	17100
Vitamin D_3		
Pure crystals	18200	18200
3,5-Dinitrobenzoate	17750	17650
3,5-Dinitro-4-methylbenzoate	18000	17950
4-Nitrobenzoate	17600	18100

It has been our experience that ergosterol is best purified by recrystallization from a benzene–ethanol (95%) mixture. A large number of moisture determinations made on material dried in vacuum over calcium chloride at room temperature indicate the presence of one mole of water, which makes a difference of about 4.6% in the molecular weight. In the case of ergosterol the extinction values for all the esters determined by the subtraction method are in close enough agreement with the theory to make a choice for practical analytical purposes simply a matter of availability and convenience. We have found the 3,5-dinitrobenzoate and the 3,5-dinitro-4-methylbenzoate to be the easiest to prepare and purify.

In our experience, 7-dehydrocholesterol is somewhat less stable than ergosterol and is not improved in purity by recrystallization. It is, therefore, prepared from the rigidly purified dinitrobenzoate (see experimental part) by saponification and the free alcohol is used as it crystallizes from the methanol-water-benzene mixture. Ma-

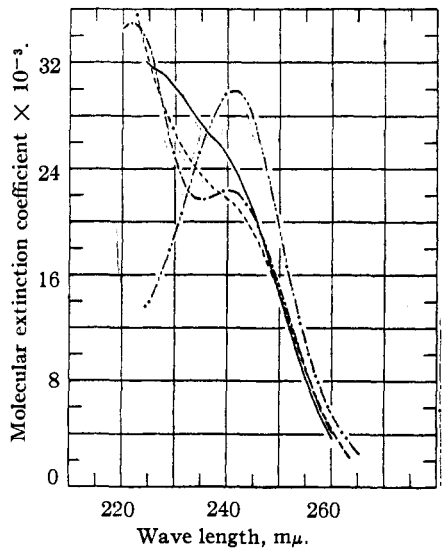


Fig. 8.—Absorption spectra of vitamin D_2 esters in alcohol, (a) 3,5-dinitrobenzoate ——, (b) 3,5-dinitro-4-methylbenzoate — — — —, (c) 3-nitro-4-methylbenzoate — · — · —, (d) 4-nitrobenzoate — · · — · · —.

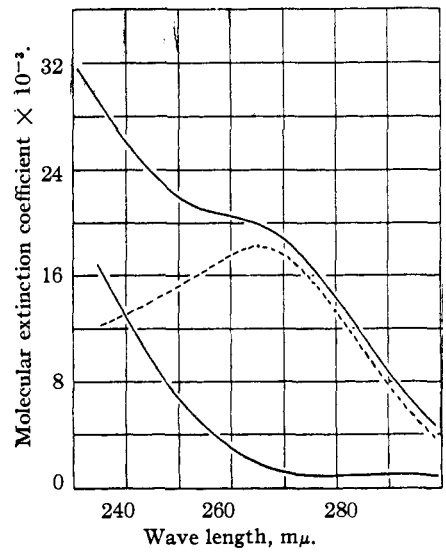


Fig. 9.—Absorption spectra, in hexane, of (a) vitamin D_2 3,5-dinitro-4-methylbenzoate, (b) cyclohexyl 3,5-dinitro-4-methylbenzoate, and (c) (— — — —) the curve for vitamin D_2 obtained by subtracting graphically (b) from (a).

terial prepared in this way and dried as in the case of ergosterol also contains one mole of water which makes a difference of about 4.7% in the molecular weight. It can be seen from Table II that the extinction values of 7-dehydrocholesterol as determined by the subtraction method are uniformly higher than the values as determined on the free sterol itself. This holds true regardless of ester and solvent used. This is probably due to slight decomposition of the sterol and we feel that the evidence from the ester subtraction curves indicates the true extinction values of 7-dehydrocholesterol to be about 10% above the values as determined on the free sterol.

The extinction values of the vitamins D_2 and D_3 as obtained by the subtraction method (see Table III) agree within the limit of experimental error with the values obtained for the purest samples of the free sterols. This holds true regardless of ester or solvent, except for vitamin D_2 4-nitrobenzoate, for which subtraction values are lower than those of any other vitamin ester. The explanation of this behavior lies in the fact that this compound is particularly difficult to purify and we have as yet not been able to remove the last traces of impurities in order to obtain a spectrochemically pure sample.

We are, of course, aware that our samples of crystalline vitamins D_2 and D_3 were obtained from aqueous solvents and that there is a possibility that the resulting crystals are hydrated. It is hoped that an independent check on water of hydration can be obtained through the use of the Karl Fischer reagent.[18] The results of this work are not yet available.

(18) See for example, Ernimont and Hopkinson, *Ind. Eng. Chem., Anal. Ed.* **15**, 272 (1943); McKinney and Hall, *ibid.*, **15**, 460 (1943),

Experimental Part

Absorption Spectra.—All spectra were determined with a Beckman quartz spectrophotometer.[19] The spectral band width isolated[11] for each density reading was within the range of approximately 0.9 to 3.5 mμ. Density readings were never further apart than 5 mμ intervals, while in regions where the extinction values are changing rapidly the interval was reduced to 2 mμ and in the immediate neighborhood of maxima and minima to 1 mμ. The individual points are not shown on the graphs since they are so numerous as to detract from clarity. The absorption cells were of silica; the thickness of each was 1.000 ± 0.002 cm. The concentrations employed varied with the degree of absorption, but were all within the range 10 to 35 mg./liter. All determinations were repeated enough times to obtain checks.

In the cases of certain of the sterol esters it was found necessary, because of the limited solubilities, to dissolve the sample in a small amount of ether or chloroform, then to dilute this rapidly to its final concentration with alcohol or hexane, as the case might be. In each such case the final concentration of ether or chloroform was not greater than 1% and usually 0.5%. In all cases the reference liquid used as a blank in the spectrophotometer was identical with the solvent used in the blank run, each component being taken from the same batch.

Solvents—(1) **Alcohol.**—The alcohol used was 95% grain alcohol, U. S. P., which was found by test to be perfectly satisfactory for this purpose without special purification. In a few cases solubilities made it necessary to use absolute alcohol, and for these an alcohol spectrophotometrically free of benzene was used.

(2) **Hexane.**—The material used was prepared from Skellysolve "B" by successive treatment with fuming sulfuric acid and alkaline potassium permanganate, followed by distillation through a fractionating column. The various fractions obtained were examined in the spectrophotometer and all which were satisfactory (transparent to as low as about 225 mμ) were combined.

(3) **Chloroform.**—Commercial chloroform was found to be only slightly improved in transparency by fractiona-

(19) Manufactured by the National Technical Laboratories, Pasadena, California. See Cary and Beckman, *J. Opt. Soc. Am.*, **31**, 682 (1941).

TABLE IV

PHYSICAL DATA FOR VITAMIN AND PROVITAMIN ESTERS AND RELATED COMPOUNDS

Compound	M. p. uncor., °C.	Rotation (1.6% in $CHCl_3$)	N Analyses, % Calcd.	Found	Solvent	Appearance	Lit. refs.
Ergosterol							
Monohydrate	162–164	−133.0				Needles	20
Benzoates:							
3,5-dinitro-	198–199	− 40.8	4.83	4.86	Acetone	Orange platelets	[a]
3,5-dinitro-4-methyl-	213–214	− 49.0			Acetone	Yellow needles	21
3-nitro-4-methyl-	191–193	− 47.2	2.67	2.57	Acetone	Needles	[a]
4-nitro-	182	− 49.5			Alcohol	Yellow platelets	22
7-Dehydrocholesterol							
Monohydrate	148–150	−112.5			MeOH-Bz	Fine needles	20
Benzoates:							
3,5-dinitro-	210–212	− 45.7			Acetone–Bz	Orange needles	13
3,5-dinitro-4-methyl-	191–192	− 37.5	4.76	4.74	Acetone	Fine orange needles	[a]
3-nitro-4-methyl-	174–176	− 46.8	2.32	2.56	Acetone	White needles	[a]
4-nitro-	153–154	− 49.8	2.70	2.63	Acetone	Yellow needles	[a]
Vitamin D_2							
Crystals	116–117	+ 48.2			Dil. MeOH	Fine clustered needles	5
Benzoates:							
3,5-dinitro-	146–148	+ 89.0			Acetone	Canary yellow prisms	23
3,5-dinitro-4-methyl-	113–114	+ 91.0 (acetone)			EtOAc + EtOH	Light yellow needles	21
3-nitro-4-methyl-	119–120	+106.8	2.20	2.57	Hexane	Pale yellow needles	5
4-nitro-	94.5–95	+105.2			Acetone	Bright yellow prisms	24
Vitamin D_3							
Crystals	84–85	+ 51.9			Dil. acetone	Fine needles	5
Benzoates:							
3,5-dinitro-	129[b]	+100.0			Acetone	Yellow needles	24
3,5-dinitro-4-methyl-	128–129	+106.6	4.95	4.73	Alcohol	Light yellow needles	5
3-nitro-4-methyl-	Light yellow resin, would not crystallize						
4-nitro-	125–126	+114.2			Acetone	Light yellow prisms	24
Cyclohexyl 3,5-dinitrobenzoate	112–113				Hexane	Fine pale yellow needles	25
Cyclohexyl 3,5-dinitro-4-methylbenzoate	120–121.5		9.12	9.21	Hexane	White needles	[a]
Cyclohexyl 3-nitro-4-methylbenzoate	59–60		5.35	5.32	Hexane	White needles	[a]
Cyclohexyl 4-nitrobenzoate	51–52		5.62	6.04	Hexane	Needles	26

[a] New compound. [b] This material is dimorphous. When crystallized from ether, a modification is obtained which melts at 141°. See ref. 24.

tion, so it was usually used directly as received. It should be tested for the absence of traces of hydrochloric acid.

Ergosterol.—The material obtained commercially[9] was recrystallized by the following method. The ergosterol was dissolved in a mixture of one part of alcohol to two of benzene, with refluxing. The solution was filtered hot, then allowed to cool with constant agitation. The crystallized ergosterol was filtered by suction and washed with alcohol followed by pentane. It was then air dried for one hour and kept in a vacuum drier at room temperature for forty-eight hours. The yield was about 82%, m. p., 162–164°, $[\alpha]_D$ −133° (1.6% in chloroform).

7-Dehydrocholesterol.—A solution of 5.78 g. (0.01 mole) of 7-dehydrocholesteryl 3,5-dinitrobenzoate in 8.97 g. (0.115 mole) of benzene and 24 ml. of 5% methanolic potassium hydroxide (0.021 mole) was refluxed with stirring for thirty minutes under an atmosphere of nitrogen. A purple color developed due mainly to the formation of potassium 3,5-dinitrobenzoate. The reaction mixture, handled under nitrogen, was filtered and washed with 7.2 g. (0.925 mole) of warm benzene. Water (3.0 g., 0.167 mole) was then added and 12 ml. of the solvent removed by distillation from a steam-bath. The remaining solution was then allowed to stand at 2° for fifteen hours. The 7-dehydrocholesterol was filtered and washed thoroughly, first with methanol, then with water, and dried in vacuum at room temperature over calcium chloride: yield, 3.42 g. (90%), m. p. 148–150°, $[\alpha]_D$ −112.5° (1.6% in chloroform). For the absorption spectrum see Fig. 2 and Table I.

Vitamin D_2.—Vitamin D_2 3,5-dinitrobenzoate (5.91 g., 0.01 mole) was refluxed with 58 ml. of 5% methanolic potassium hydroxide (2.96 g., 0.053 mole) for fifteen minutes under an atmosphere of nitrogen. The solution developed a deep purple color. The reaction mixture was filtered and the filter cake washed with 5 ml. of warm methanol. The vitamin crystallized upon the addition of 1 ml. of water and subsequent chilling. It was filtered and washed thoroughly, first with methanol, then with water. The crystals were dried in vacuum over calcium chloride; yield, 3.28 g. (83.3%), m. p. 116–117°, $[\alpha]_D$ +48.2°

(20) Sobotka, "Chemistry of the Sterids," Williams and Wilkins, Baltimore, Md., 1938.

(21) Windaus, von Werder and Gschaider, *Ber.*, **65**, 1006 (1932).

(22) Emerson and Heyl, THIS JOURNAL, **52**, 2015 (1930).

(23) Windaus, Linsert, von Lüttringhaus and Weidlich, *Ann.*, **492**, 226 (1932).

(24) Windaus, Deppe and Wunderlich, *ibid.*, **533**, 118 (1937).

(25) Reichstein, *Helv. chim. acta*, **9**, 802 (1926).

(26) Huntress and Mulliken, "Identification of Pure Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1941, Vol. I, p. 6415.

(1.6% in chloroform). For the absorption spectrum, see Fig. 7 and Table I.

Vitamin D_3.—Vitamin D_3 3,5-dinitrobenzoate (5.78 g., 0.01 mole) was refluxed with 22 ml. of 10% methanolic potassium hydroxide (0.04 mole, 2.2 g.) for ten minutes under an atmosphere of nitrogen. The reaction mixture was stirred to insure homogeneity. The saponification being completed, the deep purple mixture was cooled quickly to room temperature and poured into 35 ml. of water and extracted twice with 30 ml. of hexane. The combined hexane extracts were washed with distilled water until free of alkali and dried over anhydrous sodium sulfate. The solvent was removed in a vacuum, leaving a stiff resinous residue. The resinous vitamin was dissolved in 7 g. of acetone and cooled in an ice–salt-bath. Water was added to the cold solution until cloudiness developed, and the solution was then allowed to stand at 2° for three days, during which time the vitamin slowly crystallized out. After this time water was again added until the solution was cloudy, after which it was allowed to stand for three days at 2°. The crystalline vitamin D_3 was filtered and washed repeatedly with small portions of cold 50% aqueous acetone and then dried in vacuum over calcium chloride; yield, 1.9–3.0 g. (50–80%), m. p. 84–85°, $[\alpha]_D$ +51.9° (1.6% in chloroform). For the absorption spectrum, see Fig. 7 and Table I.

Provitamins and Vitamin Esters.—One one-hundredth of a mole of the crystalline alcohol was dissolved in 0.18 mole of pyridine (C. P., freshly distilled and dried over barium oxide) and the mixture treated with 0.012 mole of the freshly prepared acid chloride. The mixture was agitated until no further heat was liberated and then allowed to stand at room temperature for fifteen hours. The reaction mixture (an orange to red solution over precipitated pyridine hydrochloride) was poured into excess water and exhaustively extracted with benzene. The benzene layer was then extracted, twice each, successively, with 10% aqueous oxalic acid and cold 10% sodium carbonate, and washed with water until neutral. The solution was then dried over sodium sulfate and the solvent removed by vacuum distillation. The solid residue was recrystallized until there was no further change in melting point, rotation and ultraviolet absorption.

Vitamin D_3 3-nitro-4-methylbenzoate prepared by this method was a resin which could not be crystallized.

The vitamin D_2 4-nitrobenzoate was quite difficult to purify.

Cyclohexyl Esters.—These esters were prepared from C. P. cyclohexanol by the method described above.

Physical data, excluding ultraviolet absorption, for all the esters are given in Table IV.[27]

Acknowledgments.—The authors wish to express their appreciation to Mr. J. R. McCormick for frequent assistance, to Miss E. Rossi for technical work, and to the Misses E. Bass, P. Curran and A. Rainey for the microanalyses.

Summary

1. The ultraviolet absorption spectra of several nitrobenzoic esters of the provitamins and vitamins D_2 and D_3 have been determined and compared with the spectra of the corresponding free sterols and acids and also the cyclohexyl esters of the same acids. The comparisons were made by a graphical subtraction method.

2. Several previously unreported esters have been prepared.

(27) The ultraviolet absorption data for all the materials studied are available in the form of tables and graphs (with the exception of those presented in the text) as a supplement to this paper, in a document of the American Documentation Institute, 1719 N Street, Washington 6, D. C.

RENSSELAER, N. Y. RECEIVED NOVEMBER 29, 1944

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Studies on Double Refraction of Flow. II. The Molecular Dimensions of Zein

BY JOSEPH F. FOSTER AND JOHN T. EDSALL

The production of birefringence in certain normally isotropic liquids through the action of shearing stress has long been known. Not until about 1916, however, was the phenomenon associated with the orientation of asymmetric particles. Recent theoretical and experimental advances, of which two comprehensive reviews have recently been published,[1,2] have nevertheless clearly demonstrated the potentialities of this method in the elucidation of molecular size and shape.

In the case of the very elongated protein molecules such as myosin and tobacco mosaic virus, orientation can be brought about by low gradients (of the order of 100–500 sec.$^{-1}$), and a relatively simple apparatus suffices. Snellman and Björnståhl,[3] and Sadron, *et al.*,[4] through the use of instruments providing much higher gradients, have been able to extend the method to less elongated protein molecules, such as helix hemocyanin and horse antipneumococcus serum globulin, obtaining results which agree very satisfactorily with sedimentation and diffusion data.

The recent development[5] in this Laboratory of an instrument providing velocity gradients as high as 30,000 sec.$^{-1}$, together with the use of solvents of high viscosity, has made possible the extension of the method to some corpuscular proteins of even lower molecular weight. The present paper presents some results obtained with zein, the prolamine of corn.

Theoretical

The phenomenon of flow birefringence is best observed when the liquid is placed between two concentric cylinders, one of which is rotated while the other is held fixed. If the field between the cylinders is observed between crossed Nicol prisms, with light traveling parallel to the cylinder

(1) J. T. Edsall, in "Advances in Colloid Science," Interscience Publishers, New York, N. Y., 1942, Vol. 1, p. 269.

(2) J. T. Edsall, in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 506.

(3) O. Snellman and Y. Björnståhl, *Kolloid-Beihefte*, **52**, 403 (1941).

(4) C. Sadron, *J. phys. radium*, [7] **7**, 263 (1936); C. Sadron, A. Bonot and H. Mosimann, *J. chim. phys.*, **36**, 78 (1939).

(5) J. T. Edsall, C. G. Gordon, J. W. Mehl, H. Scheinberg and D. W. Mann, *Rev. Sci. Instruments*, **15**, 243 (1944).