The recorded observations are ones selected at random from experiments using several different dicoumarol plasmas.

When normal plasma is added to dicoumarol plasma, the prothrombin content of the mixture is increased more than can be explained by the simple mixing of prothrombin solutions of different concentrations. Conley and Morse (*1*) recognized this when they added incoagulable plasma from a dog receiving large quantities of dicoumarol to normal human plasma.

This observation could be explained by the presence of a prothrombin accelerator in normal plasma which is deficient in dicoumarol plasma. When observed values for the mixture are plotted against arithmetically expected values, a logarithmic curve is obtained.

The accelerator is removed from the plasma by Seitz filtration. This distinguishes it from Owren's (*4*) factor V, which passes through Seitz filters.

The accelerator disappears when normal plasma is treated with thrombin. It is present, however, in serum.

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Isolation of Pure Vitamin A_2[1]

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Since the discovery in 1937 of vitamin A_2 (*1*, *4*), a compound related to vitamin A and found chiefly in the livers of certain species of fresh-water fish, there has been much disagreement among various investigators with respect to its structure and biological activity. Much of this dissension arises from the lack of criteria by which to measure vitamin A_2. The purpose of this paper is to establish some of these criteria by reporting the isolation of pure (though noncrystalline) vitamin A_2 alcohol through a crystalline ester.

During the fall of 1946, 150 lbs of "pike" livers were collected from local fish markets. Because the origin of the livers could not be definitely assured, each liver was examined under an ultraviolet lamp. Livers showing the brilliant yellow fluorescence of vitamin A_1 were discarded, while those showing the characteristic brownish-orange fluorescence of vitamin A_2 were pooled, ground, and extracted with ethyl ether. Slightly over 4 kg of rather low-potency oil was obtained.

In the following brief description of the concentration of vitamin A_2 from this oil, the symbol E stands for E (1%, 1 cm) at 351 mμ, the main ultraviolet absorption maximum of vitamin A_2.

The original oil (E = 4.0) was distilled in a centrifugal molecular still to give a distillate containing the natural vitamin A_2 esters (E = 24.2). Redistillation further increased the potency (E = 65.0), and the triglycerides were removed by saponification (E = 182). Sterols were crystallized out in acetone at – 30° C (E = 260) and the residue chromatographed on sodium aluminum silicate (E = 482). The chromatographed concentrate was distilled in a high-vacuum pot still (E = 848) and desterolated again in ethyl formate at – 30° C (E = 862). The concentrate was then chromatographed on magnesium oxide (E = 1,030), on zinc carbonate (E = 1,250), again on zinc carbonate (E = 1,320), and a third time on zinc carbonate (E = 1,350). The extinction could not be increased by further chromatography. This is about the same extinction as that obtained by Karrer and Bretscher (*3*) in their investigations of vitamin A_2. However, other data reported by these workers (main ultraviolet absorption maximum at 345 mμ instead of 351 mμ, along with relatively low extinctions for the subsidiary band at 287 mμ and for the $SbCl_3$ blue product at 695 mμ) indicate that their preparation probably contained appreciable quantities of vitamin A_1.

Since attempts to crystallize this material from various solvents (methyl alcohol, methyl formate, ethyl formate, Skellysolve "F") were unsuccessful, 1.25 gm of the concentrate was esterified with 1.4 gm of phenylazobenzoyl chloride by allowing it to stand for 4 hrs at room temperature in 20 ml of methylene chloride containing 2 ml of pyridine.

The vitamin A_2 phenylazobenzoate thus obtained was chromatographed twice on zinc carbonate. The ester formed a weakly adsorbed reddish-orange band which was eluted from the column with petroleum ether (Skellysolve "F"). The ester concentrate (1.9 gm) was allowed to stand in 10 ml of Skellysolve "F" at – 30° C. After 3 days, crystals formed in hemispherical orange rosettes on the walls of the vessel. Three recrystallizations gave a final yield of 329 mg of tiny orange prisms which melted at 76°–77° C (Fisher-Johns apparatus, uncorr.). *Analysis*: required for $C_{33}H_{38}N_2O_2$ (vitamin A_2 phenylazobenzoate using Karrer's open-chain formula)—C, 80.12; H, 7.74; N, 5.66; *Found*: C, 80.1; H, 7.8; N, 5.9. The compound had an ultraviolet absorption maximum at 341 mμ with E (1%, 1 cm) = 1,190. This shift to a lower wave length is due to the acid moiety, which has its own strong absorption band at 330 mμ.

Careful saponification under nitrogen of 258 mg of crystals gave 152 mg (theory, 150 mg) of vitamin A_2 alcohol. Adsorption on a small column of zinc carbonate showed a single homogeneous band with the exception of some strongly adsorbed red material (2 mg) at the top of the column and a tiny, weakly adsorbed orange band (about 1 mg) at the bottom of the column. These impurities were removed, and the vitamin A_2 eluted from the rest of the column was considered to be pure.

This preparation was a viscous, orange-yellow oil which showed two absorption maxima in the ultraviolet, one

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at 351 mμ with E = 1,460 and a subsidiary peak at 287 mμ with E = 820 (Fig. 1). A chloroform solution of $SbCl_3$ gave a blue color with a single absorption maximum at 693 mμ with E = 4,100 (Fig. 2).

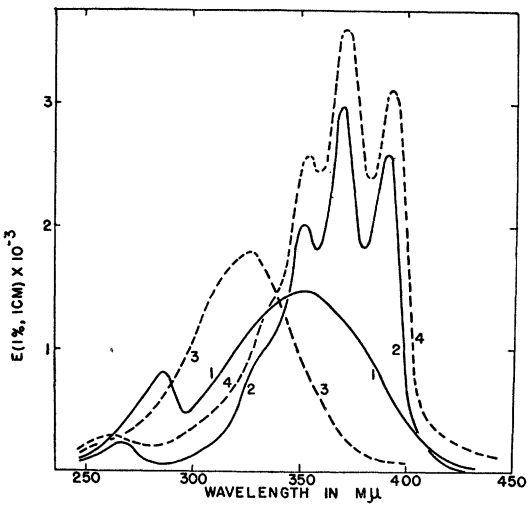


FIG. 1. Ultraviolet absorption spectra in ethanol of pure vitamin A_2 alcohol (curve 1) and crystalline anhydro vitamin A_2 (curve 2) compared with those of crystalline vitamin A_1 alcohol (curve 3) and crystalline anhydro vitamin A_1 (curve 4).

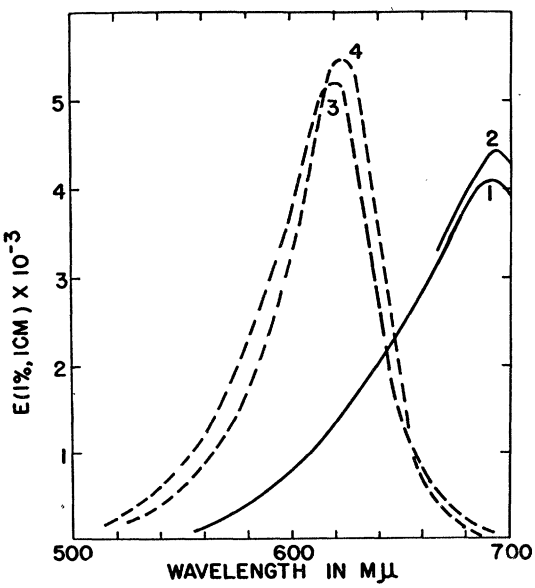


FIG. 2. Absorption spectra of the antimony trichloride products of pure vitamin A_2 alcohol (curve 1) and crystalline anhydro vitamin A_2 (curve 2) compared with those of crystalline vitamin A_1 alcohol (curve 3) and crystalline anhydro vitamin A_1 (curve 4).

Much of the existing confusion over the biological activity of vitamin A_2 has arisen from the fact that the $SbCl_3$ blue product of vitamin A_2 has appreciable absorption of its own at 620 mμ, although the $SbCl_3$ blue product of vitamin A_1 has very little absorption at 693 mμ (Fig. 2). Some workers have erroneously attributed this 620-mμ absorption of vitamin A_2 to vitamin A_1. In an earlier paper (*7*) the figure 3.06 was proposed as the ratio of E(693 mμ)/E(620 mμ) for vitamin A_2 preparations considered to be free of vitamin A_1. In the pure vitamin A_2 alcohol prepared above, this ratio was actually found to be 3.05.

Some of the slightly less potent vitamin A_2 alcohol fractions obtained in this work were combined and treated with N/30 alcoholic HCl to form anhydro vitamin A_2, a hydrocarbon analogous to anhydro vitamin A_1 (*13*). Although vitamin A_1 and A_2 alcohols are extremely difficult to separate chromatographically, their anhydro derivatives are readily separable (*2*). In the purification of the above anhydro vitamin A_2 concentrate by adsorption on zinc carbonate, no trace of anhydro vitamin A_1 was found, showing the reliability of the separation of livers under ultraviolet light.

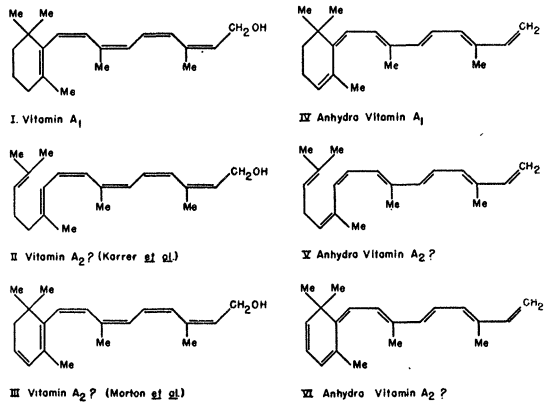
The chromatographically concentrated anhydro vitamin A_2 was readily crystallized at −30° C from a 20% solution in light petroleum (Skellysolve "F"). Two recrystallizations showed no change in melting point (89.5° C, uncorr.). *Analysis*: required for $C_{20}H_{28}$ (open-chain formula)—C, 89.49; H, 10.51. *Found*: C, 89.2; H, 10.7. Microhydrogenation in an apparatus described by the author (*12*) showed the presence of 7 double bonds. The ultraviolet absorption spectrum of this compound is almost identical with that of crystalline anhydro vitamin A_1 (Fig. 1) with maxima at 352, 370, and 391 mμ having extinctions of 2,040, 2,980, and 2,620, respectively. The antimony trichloride blue color, however, is the same as that of the original vitamin A_2 alcohol with an absorption maximum at 693 mμ and a slightly higher extinction of 4,400 (Fig. 2).

The above data shed but little light on the structure of vitamin A_2. The first proposed structure of Gillam, *et al.* (*3*), containing an extra vinylene group in the vitamin A chain, has been discarded on the basis of the distillation data of Gray and Cawley (*5*). Two other possibilities of merit are the open-chain formula of Karrer, *et al.* (*9*), relating vitamin A_2 to vitamin A_1 as lycopene is related to β-carotene, and the formula of Morton, *et al.* (*11*), containing a dehydroionone ring. Karrer's proposal is based chiefly on oxidative studies of vitamin A_2 concentrates, while Morton's is based on the claim that the "C_{20} aldehyde" of Haworth, *et al.* (*6*), obtained by Oppenauer oxidation of vitamin A_1 with diethylketone, is, in fact, the aldehyde of vitamin A_2 and is convertible to vitamin A_2 *in vivo*. This compound is supposedly the aldehyde of compound III.

Meunier, *et al.* (*10*) have postulated the structure of anhydro vitamin A_1 to be that given as formula IV. This possibility was given in a paper from this laboratory at about the same time (*13*), and the author is in agreement with this proposal. If such is the case, then by analogy the corresponding structure for anhydro vitamin A_2 would be formula V if structure II is correct and formula VI if structure III is correct.

The fact that the two anhydro vitamins have almost identical ultraviolet absorption spectra but give totally

different blue colors has been disregarded by most investigators, although an account of this seeming anomaly was first published in 1940 (*2*). Compounds IV and VI could hardly have the same ultraviolet spectra, but com-



pounds IV and V might be very similar. The surprisingly large difference in the blue colors could then be accounted for by assuming that antimony trichloride brings the isolated double bond of compound V into play. On the other hand, the shift in ultraviolet absorption maximum of compound II to 351 mμ from that of vitamin A_1 (I) at 326 mμ is rather large to be accounted for by mere opening of the ring. In the author's opinion, the evidence slightly favors structure II for vitamin A_2, if a choice must be made between these two proposals.

Biological tests indicate that the vitamin A_2 alcohol described above has a potency of approximately 1,300,000 U.S.P. units/gm, or about 40% of the activity of crystalline vitamin A_1. The biological experiments will be reported in greater detail elsewhere.

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Some Quantitative Aspects of the Feulgen Reaction for Desoxyribose Nucleic Acid

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The Feulgen reaction, originally described by Feulgen and Rossenbeck (*2*), has been used for the demonstration of desoxyribose nucleic acid (or thymonucleic acid) in tissue sections. The reaction involves the hydrolysis of sectioned material in 1N HCl at 50–60° C for about 10 min, followed by the application of the leuco-fuchsin (Schiff's) reagent for 1–2 hrs. Stowell (*5*) and Dodson (*1*) presented strong evidence that the reaction is specific for thymonucleic acid. The Feulgen technique has been applied in various ways for the quantitative estimation of the desoxyribose nucleic acid (DNA) in the cell. In order to establish its possible value as a quantitative method, the Feulgen reaction was tested on purified preparations of DNA.

It was impossible to separate the components of the reaction in liquid medium; therefore, it was necessary to find a solid medium into which the DNA could be incorporated. Weighed amounts of DNA were dissolved in hot 5% agar as previously described by Hillary (*3*). Eight different samples of agar without DNA, however, gave a positive Feulgen reaction following the usual hydrolysis. The fixatives used by Hillary were tried, but none was able to inhibit the Feulgen reaction with hydrolyzed agar. It became evident that Hillary's methods involving agar were unsatisfactory and that his conclusions on the effect of fixatives on the Feulgen reaction were open to question.

The problem was to find a medium that would gelate or solate at appropriate temperatures and which, after fixation and hydrolysis, would be negative to the Feulgen reaction. Eastman Kodak ash-free and Difco-Bacto gelatin were satisfactory. After fixation of a 20% gel in 6% formalin for 1½ hrs, there was no appreciable hydrolysis of the gelatin by the HCl. The gelatin was Feulgen negative after long periods of hydrolysis provided the formalin was thoroughly washed out. Formalin itself gives an intense Feulgen reaction even at very low concentration. Sodium DNA, at the concentrations used, readily dissolved in the 20% gelatin sol and was not appreciably lost from the gel during fixation, washing, hydrolysis, and the subsequent reaction with the leuco-fuchsin reagent (prepared by the Rafalko technique, *4*).

The procedure was to add 5 cc of the 20% gelatin sol, at 32–35° C, to small test tubes containing weighed amounts of sodium DNA. Careful agitation produced a uniform mixture of the gelatin sol with the DNA. Drops of the warm gelatin-DNA preparations, approximately 0.1 ml in volume, were then placed on 1″ × 3″ glass slides (previously coated with albumin fixative and heat dried) and allowed to gel. The gelatin drops were hardened in 6% formalin for 1½ hrs and washed in running tap water for 2–3 hrs to remove the formalin. The