pure methyl β-methylglutaconate[6] (0.42 mole) in 20 ml. of methanol, there was added a solution of 111 g. of 85% potassium hydroxide (1.68 moles) in 450 ml. of methanol. After 24 hr. at room temperature the mixture was cooled to 5° and the potassium salt of II (R = *p*-$Me_2NC_6H_4$) filtered; the yield was 60.3 g. (49.0%). This was dissolved in 240 ml. of water and approximately two equivalents of hydrochloric acid were added. The resulting solid (41.7 g.) was filtered and the filtrate made nearly acid to congo red, precipitating an additional 21.2 g. of product. The combined solids were crystallized thrice from 1:1 ethanol–acetone to give 27.8 g. of red-orange laths, m.p. 200–201°. The analytical sample was again crystallized from dioxane. The filtrates contained only a red-brown gum.

Preparation of Ethyl 5-(*p*-Aminophenyl)-3-methyl-2,4-pentadienoate.—Dry hydrogen chloride was added to a suspension of 4.9 g. (0.02 mole) of Ic (R = *p*-$AcNHC_6H_4$) in 160 ml. of ethanol until 3.7 g. (0.1 mole) had been absorbed. The mixture was then refluxed for 3 hr., complete solution occurring at once. Most of the ethanol was removed by evaporation under nitrogen, water was added to the residue, and, after ether extraction, the aqueous layer was made alkaline with solid potassium hydroxide. The oil which separated was extracted with ether, whose evaporation left 3.8 g. (82.5%) of crude product. This was chromatographed from benzene on a column of Alorco grade F-20 alumina. The main golden yellow zone was separated and eluted with ether to give 3.3 g. (72%) of the product as a yellow viscous oil. It was characterized as the picrate, prepared in ethanol in 82% crude yield, which formed yellow leaves, m.p. 142.5–143.5° after recrystallization from methanol.

Anal. Calcd. for $C_{20}H_{20}N_4O_9$: N, 12.2. Found: N, 12.3.

It is possible that the hydrogen chloride caused stereoisomerization, so that no configuration is assigned to this compound.

Rearrangements of the 4-*cis* Acids Ic to their *trans* Isomers Ib.—The several 4-*cis* acids examined were found to vary in their stability to iodine and light, so that in no case can the described procedure be considered the optimum.

The compounds Ic (R = *p*-$MeOC_6H_4$, 3,4-$CH_2O_2C_6H_3$, 3,4-$(MeO)_2C_6H_3$ and 2,6-$Cl_2C_6H_3$) were treated thus: the compound was dissolved in 2 volumes of methanol and 5 volumes of benzene (10 volumes for R = 3,4-$CH_2O_2C_6H_3$) containing 5% of the weight of the compound of dissolved iodine, and the solution was irradiated for 30 min. from below with a mercury vapor lamp at a distance of 3 cm., the heat from the lamp being sufficient to maintain a gentle reflux. The product which separated at 5° was crystallized from ethanol. The yields varied from 84% for R = 3,4-$CH_2O_2C_6H_3$ to 25% for R = 3,4-$(MeO)_2C_6H_3$.

The compounds Ic (R = C_6H_5 and $C_6H_5CH{=}CH$) were destroyed under the above conditions. The former was dissolved in 20 volumes of ether and 20 volumes of benzene containing 1% of the weight of the compound of dissolved iodine. After 4 hr. at room temperature in diffuse daylight an additional 1% of iodine in a small amount of benzene was added, and after 2 hr. more the solution was washed with thiosulfate and water, the ether removed by distillation, and ligroin added to the distilland to crystallization at the b.p. Two recrystallizations of the resulting solid from benzene gave 50% of Ib (R = C_6H_5), m.p. 157–158°; the mixed m.p. with Ic (R = C_6H_5) was 123–146°. The m.p. can be raised to 160.5–161° by further crystallizations from benzene and ethanol.

Ic (R = $C_6H_5CH{=}CH$) was treated with 1% of iodine in 75 volumes of ether and 37.5 volumes of benzene for 2 hr. at room temperature in diffuse daylight, then 1% more of iodine was added and, after a further 1.5 hr., the mixture was worked up by washing with thiosulfate and water and removing most of the ether by distillation. There crystallized from the distilland 80% of Ib (R = $C_6H_5CH{=}CH$), m.p. 196–198°, raised to 199.5–200.5 by recrystallization from ethanol. The compound crystallizes beautifully in pale yellow, large, very thin, rhombic plates, exactly as described by Kuhn and Hoffer.[2]

ROCHESTER, NEW YORK

[COMMUNICATION NO. 209 FROM THE RESEARCH LABORATORIES, DISTILLATION PRODUCTS INDUSTRIES, DIVISION OF EASTMAN KODAK COMPANY]

Biochemical Studies on Vitamin A. XIV. Biopotencies of Geometric Isomers of Vitamin A Acetate in the Rat[1]

BY STANLEY R. AMES, WILLIAM J. SWANSON AND PHILIP L. HARRIS

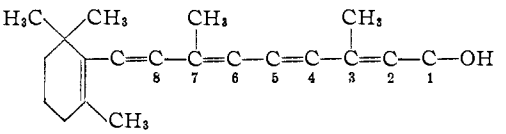
RECEIVED AUGUST 24, 1954

The physiological potencies of three new isomers, 6-mono-*cis*, 2,6-di-*cis* and 2,4-di-*cis*, of vitamin A acetate as well as of neo (2-mono-*cis*) vitamin A acetate have been determined using standard vitamin A bioassay procedures. Neovitamin A acetate (2-mono-*cis*) has a biopotency of 2,190,000 units/gram. The 6-mono-*cis*-vitamin A acetate has a biopotency of 634,000 units/gram; the 2,6-di-*cis* isomeric acetate has a biopotency of 688,000 units/gram; and the 2,4-di-*cis* isomeric acetate has a biopotency of 679,000 units/gram. The 6-mono-*cis*, 2,6-di-*cis* and 2,4-di-*cis* isomeric vitamin A acetates are about 23% as active as all-*trans*-vitamin A acetate.

Five isomers of vitamin A (all-*trans*, neo (2-mono-*cis*), 6-mono-*cis*, 2,6-di-*cis* and 2,4-di-*cis*) have now been characterized chemically and physically.[2] All-*trans*-vitamin A acetate has been isolated in crystalline form.[3] Neovitamin A acetate was first described by Baxter and Robeson[4] and was prepared from crystalline neovitamin A alcohol. The comparative biopotencies of all-*trans* and neovitamin A acetates have been previously reported by Harris, Ames and Brinkman.[5] Robeson, *et al.*,[6] have recently synthesized the 6-mono-*cis*- and 2,6-di-*cis*-vitamin A acetates. The aldehyde corresponding to the 2,4-di-*cis*-vitamin A was first described by Hubbard and Wald[7] and more recently by Dieterle and Robeson.[8] The present report summarizes the results of rat bioassays of the five isomeric vitamin A acetates.

(1) Presented in part before the Division of Biological Chemistry at the 126th Meeting of the American Chemical Society, New York, New York, September, 1954.

(2) The steric configurations of the isomers can be identified according to the following numbering system



(3) J. G. Baxter and C. D. Robeson, THIS JOURNAL, **64**, 2407 (1942).

(4) J. G. Baxter and C. D. Robeson, *ibid.*, **69**, 136 (1947).

(5) P. L. Harris, S. R. Ames and J. H. Brinkman, *ibid.*, **73**, 1252 (1951).

(6) C. D. Robeson, J. D. Cawley, L. Weisler, M. H. Stern, C. C. Eddinger and A. J. Chechak, *ibid.*, **77**, 4111 (1955).

(7) R. Hubbard and G. Wald, *J. Gen. Physiol.*, **36**, 269 (1952–53).

(8) J. M. Dieterle and C. D. Robeson, *Science*, **120**, 219 (1954).

Experimental

The pure compounds used in this investigation were synthesized in the Organic Chemistry Department of Distillation Products Industries as recently described.[6] The 2,4-di-*cis*-vitamin A acetate was prepared from the corresponding crystalline 2,4-di-*cis*-vitamin A aldehyde recently described.[8] They were supplied as pure materials or as solutions of pure compounds in refined cottonseed oil at approximately 1% concentration. The all-*trans*-vitamin A acetate used was crystalline material dissolved in refined deodorized cottonseed oil and distributed as the USP Reference Standard[9] at a defined potency of 10,000 units per gram (identical with the International Standard[10]).

Spectrophotometric determinations of specific absorbancy ($E^{1\%}_{1\,cm.}$) of the isomeric vitamin A acetates were made using calibrated Cary or Beckman spectrophotometers and employing isopropyl alcohol as the solvent.

Bioassays from which the data were obtained were of two types, rat growth and rat liver storage. The rat growth bioassays compared two levels of the test material with two similar levels of the USP Reference Standard using a modified USP XIII procedure. All growth bioassays were set up according to the suggestions of Bliss.[11] The liver storage bioassays were conducted by modifications[12,13] of previously described methods.[14,15]

The bioassay potency is expressed in units per gram of test material, independent of any chemical or spectrophotometric analysis. The bioassay potency of each sample was divided by its specific absorbancy and the resulting value is termed the conversion factor.[10] The conversion factors were averaged and, when the number of determinations justified, a standard error of the mean was calculated.

Results

The results of both rat growth and rat liver storage bioassays of the geometric isomers of vitamin A acetate compared with the USP Vitamin A Reference Standard are given in Table I. The average conversion factor determined by individual bioassays was multiplied by the specific absorbancy of the pure material, according to Robeson, *et al.*,[6] in order to give the best estimate of the biopotency of the geometric isomer. The biopotencies of these geometric isomers were calculated and the results are shown in Table II. All-*trans*-vitamin A acetate has a defined biopotency of 2,907,000 units per gram.[9,10]

Neovitamin A acetate or the 2-mono-*cis* isomer has been evaluated by a number of liver storage and growth bioassays. The mean conversion factor based on 7 liver storage bioassays was 1433 and the biopotency was 2,060,000 units per gram. The growth bioassay data given by Harris, *et al.*,[5] recalculated as described above, gave a mean conversion factor of 1627, and using an $E^{1\%}_{1\,cm.}$ of 1435 yielded a biopotency of 2,340,000 units per gram. As previously indicated, growth and liver storage bioassays yield results which are not statistically different, so the mean conversion factor of neovitamin A acetate on the basis of all of the available rat growth and rat liver storage bioassays is 1523 and the biopotency is 2,190,000 units per gram, 75.2% the biopotency of all-*trans*-vitamin A acetate.

TABLE I

BIOASSAYS OF GEOMETRIC ISOMERS OF VITAMIN A ACETATE
(Bioassay potency in terms of USP Reference Standard.)

Type of bioassay	Bioassay potency, u./g.	$E^{1\%}_{1\,cm.}$	Conversion factor
	Neo-(2-mono-*cis*)-vitamin A acetate		
		λ_{max} 328 mμ	
LS	1,790,000	1337	1336
LS	1,860,000	1397	1334
LS	1,750,000	1082	1619
LS	2,390,000	1284	1861
LS	1,770,000	1380	1282
LS	1,790,000	1384	1290
LS	1,660,000	1264	1312
			Mean = 1433 ± 84 S.E.
G	6 bioassays (5)		Mean = 1627 ± 114 S.E.
	6-Mono-*cis*-vitamin A acetate		
		λ_{max} 323 mμ	
LS	762,000	1206	632
LS	596,000	1200	497
G	529,000	1163	455
			Mean = 528
	2,6-Di-*cis*-vitamin A acetate		
		λ_{max} 324 mμ	
LS	710,000	1167	608
LS	577,000	1114	518
G	775,000	1059	732
			Mean = 619
	2,4-Di-*cis*-vitamin A acetate		
		λ_{max} 322 mμ	
LS	815,000	895	911
LS	464,000	867	535
G	606,000	867	699
			Mean = 715

The bioassays of the 6-mono-*cis*-vitamin A acetate gave a mean conversion factor of 528 and a biopotency of 634,000 units per gram, about 21.8% that of all-*trans*-vitamin A acetate. A similar series of bioassays on the 2,6-di-*cis* isomeric acetate gave a mean conversion factor of 619 and a biopotency of 688,000 units per gram, about 23.7% that of the all-*trans* isomer. The two "6-*cis*" isomers of vitamin A acetate have similar activities with an average biopotency of 661,000 units per gram, 22.7% that of all-*trans*-vitamin A acetate.

The bioassays of the 2,4-di-*cis*-vitamin A acetate gave an average conversion factor of 715. Using an estimated $E^{1\%}_{1\,cm.}$ (max.) of 950 a biopotency of 679,000 units per gram was calculated which is approximately 23.4% that of the all-*trans* acetate. This figure is in good agreement on a molar basis with the biopotency of a sample of the 2,4-di-*cis*-vitamin A alcohol.[13]

Discussion

The discovery of several new isomeric vitamin A acetates with only a fraction of the bioactivity of the all-*trans* isomer is of both scientific and commercial interest. The 6-mono-*cis*- and 2,6-di-

(9) Pharmacopeia of the United States of America, "USP Vitamin A Reference Standard, Instructions for Use," May 18, 1948.

(10) *World Health Organization Technical Report Series*, **3**, 4 (1950).

(11) Suggested Revision of the U.S.P. Biological Assays for Vitamins A and D Submitted to the U.S.P. by the Animal Nutrition Research Council through Dr. C. I. Bliss, November 15, 1948.

(12) S. R. Ames, H. A. Risley and P. L. Harris, *Anal. Chem.*, **26**, 1378 (1954).

(13) S. R. Ames and P. L. Harris, to be published.

(14) K. Guggenheim and W. Koch, *Biochem. J.*, **38**, 256 (1944).

(15) J. R. Foy and K. Morgareidge, *Anal. Chem.*, **20**, 304 (1948).

TABLE II

BIOPOTENCIES OF GEOMETRIC ISOMERS OF VITAMIN A ACETATE

Isomer	Bioassays	Mean CF	$E^{1\%}_{1\,cm.}$ [6]	Biopotency, units/g.	Relative biopotency, %
All-*trans*	By definition	(1900)	(1530)	(2,907,000)	(100)
Neo (2-mono-*cis*)	13	1523	1435	2,190,000	75.3
6-Mono-*cis*	3	528	1200	634,000	21.8
2,6-Di-*cis*	3	619	1112	688,000	23.7
			Mean "6-*cis*"	661,000	22.7
2,4-Di-*cis*	3	715	950 (est.)	679,000	23.4

cis-vitamin A acetates appear on the basis of both structure and biopotency to constitute a "6-*cis*" class of vitamin A isomers. The rat can utilize the "6-*cis*"-vitamin A acetates equally well for both growth and for liver storage but only about 23% as well as the all-*trans* isomer. Chick bioassays have resulted in a similarly low bioactivity for the "6-*cis*" isomeric acetates.[13] Since both the rat and the chick respond poorly to the "6-*cis*" isomers and the rat uses the 2,4-di-*cis* isomer poorly, it is probable that humans would have similar difficulty in efficiently utilizing vitamin A other than the all-*trans* and neo isomers.

Vitamin A isomers of low biological activity may occur naturally in certain instances. Fisher, Kon and Thompson[16] have reported on the occurrence of vitamin A in certain marine Crustacea. The physicochemical potencies of such concentrates were reported to be 2–3 times higher than their biological potencies. A few sources of natural vitamin A have been observed in these laboratories to possess physicochemical potencies substantially in excess of their biological activities. Such discrepancies can be accounted for by the presence of isomers other than all-*trans* and neo-vitamin A.

From a practical point of view, the presence of the "6-*cis*" or 2,4-di-*cis* isomers in vitamin A preparations would be difficult to detect by physical and chemical assays. The use of a bioassay, either growth or liver storage, is the best procedure available at present for readily indicating the presence of significant amounts of these isomers. The use of a biological assay is recommended for the evaluation of natural or synthetic vitamin A concentrates of questionable composition.

Acknowledgment is made to N. D. Embree and R. W. Lehman for advice, to H. A. Risley for the performance of vitamin A analyses of livers, to the Manufacturing Control Laboratory for spectrophotometric analyses, and to the Organic Chemistry Department for furnishing the pure vitamin A compounds.

ROCHESTER, N. Y.

(16) L. R. Fisher, S. K. Kon and S. Y. Thompson, *J. Marine Biol. Assoc. United Kingdom*, **31**, 229 (1952).

[COMMUNICATION NO. 210 FROM THE RESEARCH LABORATORIES, DISTILLATION PRODUCTS INDUSTRIES, DIVISION OF EASTMAN KODAK COMPANY]

Biochemical Studies on Vitamin A. XV. Biopotencies of Geometric Isomers of Vitamin A Aldehyde in the Rat[1]

BY STANLEY R. AMES, WILLIAM J. SWANSON AND PHILIP L. HARRIS

RECEIVED AUGUST 24, 1954

The physiological potencies of five geometric isomers (all-*trans*, neo (2-mono-*cis*), 6-mono-*cis*, 2,6-di-*cis*, and 2,4-di-*cis*) of vitamin A aldehyde and of α-vitamin A aldehyde have been determined. All-*trans* and neo-(2-mono-*cis*)-vitamin A aldehydes have the same biopotency of 3,070,000 u./g., about 91% the molar bioactivity of all-*trans*-vitamin A acetate. The 6-mono-*cis*- and 2,6-di-*cis*-vitamin A aldehydes have the relatively low biopotencies of 637,000 and 581,000 u./g., respectively, about 18% the molar bioactivity of all-*trans*-vitamin A acetate. The 2,4-di-*cis*-vitamin A aldehyde has a biopotency of 1,610,000 u./g., about 48% the molar bioactivity of all-*trans*-vitamin A acetate. The α-ionone analog of vitamin A aldehyde has less than 2% the potency of vitamin A acetate and during metabolism it is converted to the corresponding α-vitamin A alcohol which is stored in the liver.

Five crystalline geometric isomers of vitamin A aldehyde have now been characterized chemically and physically. Crystalline all-*trans*-vitamin A aldehyde[2–4] was reported by Wendler *et al.*[5] to be substantially as active as vitamin A in the growth test. Ames, *et al.*,[6] have recently reported both the all-*trans*- and neo-(2-mono-*cis*)-vitamin A aldehydes[3,4] to have biopotencies of about 3,000,000 u./g. The "*cis*-vitamin A aldehyde" of Graham, *et al.*,[7] was reported to be as active as vitamin A. An isomer with similar chemical properties has since been characterized as the 6-mono-*cis* aldehyde.[4] The 2,6-di-*cis*-vitamin A aldehyde was recently synthesized and crystallized by Robeson,

(1) Presented in part before the Division of Biological Chemistry at the 126th Meeting of the American Chemical Society, New York, New York, September, 1954.

(2) S. Ball, T. W. Goodwin and R. A. Morton, *Biochem. J.*, **42**, 516 (1948).

(3) R. Hubbard and G. Wald, *J. Gen. Physiol.*, **36**, 269 (1952–53).

(4) C. D. Robeson, W. P. Blum, J. M. Dieterle, J. D. Cawley and J. G. Baxter, THIS JOURNAL, **77**, 4120 (1955).

(5) N. L. Wendler, C. Rosenblum and M. Tishler, *ibid.*, **72**, 234 (1950).

(6) S. R. Ames, W. J. Swanson, H. A. Risley and P. L. Harris, *Federation Proc.*, **13**, 174 (1954).

(7) W. Graham, D. A. VanDorp and J. F. Arens, *Rec. trav. chim.*, **68**, 609 (1949).