Though the available evidence is not adequate to permit an unequivocal decision, it is probable that much of the effect of these inorganic ions is a competitive one, *i.e.*, they compete with methyl orange for common binding sites on the protein. This conclusion is clearer in the case of thiocyanate because of its greater effectiveness and because of the studies of its binding in the reference cited above. A similar conclusion was reached in a study of the effect of bound dodecyl sulfate on the combination of an anionic azo dye with bovine albumin.[17]

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(17) F. Karush, THIS JOURNAL, **72**, 2714 (1950).

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Biochemical Studies on Vitamin A. IX. Biopotency of Neovitamin A in the Rat

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The physiological potencies of neovitamin A and of neovitamin A acetate have been compared with all-*trans* vitamin A and all-*trans* vitamin A acetate using standard vitamin A bioassay procedures. By rat-growth bioassay, neovitamin A has 85.3% the biopotency of all-*trans* vitamin A on an E-value basis and 80.7% on a molar basis. By liver-storage type bioassay, neovitamin A shows 75.6% the biopotency of the all-*trans* isomer on an E-value basis and 71.5% on a molar basis. Rats have the ability to convert ingested all-*trans* to neovitamin A and *vice versa*, and they tend to deposit in their liver a mixture of the two isomers containing approximately 12% of the neo form.

Pure crystalline neovitamin A (*cis–trans*) has been characterized chemically and physically[1,2] and has had assigned to it tentatively[2] a high biological potency, "substantially the same" as vitamin A (all-*trans*). Subsequent repeated bioassays of the two vitamin A isomers in pure form, both free and esterified, have confirmed their high activities, but have indicated a significant difference in their biological potencies. The present report summarizes the pertinent data obtained in this Laboratory during the past seven years.

Experimental

The pure compounds used in the study were prepared by the Organic Chemistry Department of Distillation Products Industries and were supplied as pure crystals in sealed ampoules or as solutions of the crystalline compounds in refined cottonseed oil at approximately 1% concentration. The all-*trans* vitamin A acetate used was in most instances crystalline material dissolved in cottonseed oil and distributed as the U.S.P. Reference Standard.

Spectrometric determinations of specific absorbancy ($E^{1\%}_{1\ cm.}$) of the vitamin A compounds were made using a Beckman spectrophotometer and employing isopropyl alcohol as the solvent. The estimated potencies of the neovitamin A compounds were obtained by multiplying their $E^{1\%}_{1\ cm.}$ 328 mμ by 1894. This conversion factor was that currently used to transpose *E*-value ($E^{1\%}_{1\ cm.}$ 325 mμ) of all-*trans* vitamin A to biological potency in terms of U.S.P. units per gram. Consequently, most comparisons in this report are made on a "per unit *E*-value" basis.

Bioassays from which the data were obtained were of two types, rat growth and liver storage. The rat-growth bioassays were routine tests in which two levels of a variety of substances were compared with two similar levels of vitamin A acetate, a modified U.S.P. XIII procedure. In many of the early bioassays, the design of the test was such that no estimate of the limits of uncertainty could be made. However, later bioassays were set up according to the suggestions of Bliss[3] and for these, standard errors have been calculated. The liver-storage bioassays, also used to compare the physiological activities of all-trans and of neovitamin A, were conducted by a modification[4] of previously described methods.[5,6] The results obtained were examined statistically to determine relative potency and standard error.

Results

The comparison of biopotencies, by rat-growth tests, of the two types of vitamin A alcohol are shown in the first portion of Table I. The evidence indicates that neovitamin A possesses less vitamin A potency per unit *E*-value than all-*trans* vitamin A. This indication is borne out by the results obtained by comparing the biopotency of neovitamin A acetate and all-*trans* vitamin A acetate as also shown in Table I. The over-all mean, 85.3% with a standard error of 2.58%, indicates that neovitamin A compared with all-*trans* vitamin A, per unit *E*-value, is less potent by about 15%.

Comparisons using the liver-storage type of bioassay yielded the results shown in Table II. The average of 75.6 ± 3.52% (S.E.) for the potency of neovitamin A compared with all-*trans* vitamin A determined by liver storage is somewhat lower than the 85.3 ± 2.58% relationship established for the rat-growth method. However, the difference between the two means is not statistically meaningful (t = 1.94, P = 0.05–0.1).[7] Thus, neovitamin A is probably as well utilized by the rat for storage as for growth.

A mixture of the two isomers, simulating naturally-occurring fish oils which contain vitamin A in the proportion, one-third neovitamin A and two-thirds *trans*-vitamin A, was bioassayed both by growth and by liver-storage methods. In this experiment (Table III), neovitamin A acetate

(1) C. D. Robeson and J. G. Baxter, *Nature*, **155**, 300 (1945).

(2) C. D. Robeson and J. G. Baxter, THIS JOURNAL, **69**, 136 (1947).

(3) 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.

(4) S. R. Ames, P. L. Harris and H. A. Risley, to be published.

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

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

(7) t = diff. of means $\sqrt{\frac{n_1\sigma_1^2 + n_2\sigma_2^2}{n_1 n_2}\left(\frac{n_1 + n_2}{n_1 + n_2 - 2}\right)}$

TABLE I

RELATIVE BIOPOTENCY OF ALL-*trans* VITAMIN A AND NEOVITAMIN A BOTH IN ALCOHOL AND ESTER FORM. (MULTILEVEL U. S. P. XIII BIOASSAY WITH A MINIMUM OF 9 RATS PER LEVEL)

Bioassay no.	Biopotency of neovitamin A relative to vitamin A: Percentage	Standard error,[a] %
Neovitamin A alcohol compared with vitamin A alcohol		
A-78-342	79.3	.....
P-81R-744	96.0	.....
A-167-844	75.9	.....
A-167R-1044	99.5	.....
A-193-145	86.6	.....
A-291-9-648	87.7	± 9.95
A-325-7-1148	84.9	±11.10
Mean for alcohol form	87.1 ± 3.25%[b]	
Neovitamin A acetate compared with vitamin A acetate		
A-291-9-648	82.7	± 9.89
A-325-7-1148	93.0	± 9.70
P-170-149	69.5	±21.30
A-339-449	71.7	±11.00
A-354-FRL	92.8	±13.20
A-356-749	89.6	±13.30
Mean for acetate form	83.2 ± 4.28%[b]	
Mean for all comparisons	85.3 ± 2.58%[b]	

[a] Percentage Standard Error of comparisons within assays[8] = $100 \text{ antilog}\left(\frac{\sigma}{b}\sqrt{\frac{1}{N_{ref.}} + \frac{1}{N_{assay}}}\right) - 100$. [b] Standard error of the comparisons between assays = $\sqrt{\frac{\Sigma d_M^2}{(n-1)(n)}}$.

TABLE II

RELATIVE LIVER STORAGE OF ORALLY ADMINISTERED ALL-*trans* VITAMIN A AND NEOVITAMIN A

(Vitamin supplements were fed in equal aliquots on days 1, 2 and 3 to vitamin A-deficient rats. Animals were killed on day 5, the entire liver removed and analyzed for vitamin A content)

Expt. no.	No. of animals	Relative storage of neovitamin A compared with vitamin A: Percentage	Standard error, %
P-146R-148[a]	32	69.6	...
A-311-848[a]	40	84.0	...
P-170-149[a]	20	84.5	±3.6
A-312-848[b]	40	70.5	...
P-170-149[b]	20	69.6	±3.5
Mean		75.6 ± 3.52%	

[a] The compounds compared in these experiments were vitamin A and neovitamin A as free alcohols. [b] The compounds compared in these experiments were vitamin A acetate and neovitamin A acetate.

was about 30% less active than *trans* vitamin A acetate by both bioassay procedures. The simulated concentrate was about 10% less active than the reference standard (*trans* vitamin A) in both bioassays. This is the result expected by calculation and, thus, it may be concluded that each isomer exerts its characteristic physiological effect independent of the presence of the other isomer.

The extent of interconversion of the neo- and all-*trans* forms of vitamin A *in vivo* was also examined. The relative amounts of the two isomers stored in the liver following supplementation with either crystalline neo- or crystalline all-*trans* preparations alone, were determined. The results detailed in Table IV show that the rat converts some neo- to *trans* vitamin A and *vice versa*, then stores a mixture of the two isomers in the liver as previously reported.[2] The relative abundance of neovitamin A in the alcohol fraction is about the same (approximately 26.5%) whether pure neo- or pure *trans* vitamin A is fed, but it is slightly different in the ester fraction. The ester fraction from the livers of animals fed *trans* vitamin A contained 9.2% neovitamin A and those fed the neovitamin form contained 16.7% neovitamin A. This difference, however, is probably not significant since analyses of liver oil from stock colony rats receiving a natural-type diet showed a range of values between 8.2 and 17.6% neovitamin A in the ester fraction. Consequently, the rat may attempt to store an equilibrium mixture of neo- and *trans* vitamin A, regardless of diet, which is approximately 1 to 2 parts of neo- to 10 parts of *trans* vitamin A. This is in contrast to the 1 part to 2 parts relationship found in the neo- and *trans* vitamin A in fish oils.

Discussion

The biopotencies of the two vitamin A isomers may also be compared on a molar basis by calculation from data reported by Robeson and Baxter[2] relative to the specific absorbancy of both compounds. The $E^{1\%}_{1\text{ cm.}}$ 325 mμ of pure all-*trans* vitamin A is 1740 and the $E^{1\%}_{1\text{ cm.}}$ 328 mμ of pure neovitamin A is 1645, both in ethanol solution. Therefore, the biopotency, by the rat-growth method, of neovitamin A relative to all-*trans* vitamin A, 85.3% on a per unit *E*-value basis, would be reduced by 1645/1740 to 80.7% on a per unit weight basis and, in this particular comparison, on a molar basis. Similarly, the biopotency of neovitamin A, by the liver-storage method, is 75.6% that of all-*trans* vitamin A per unit *E*-value but only 71.5% on a molar basis.

In terms of U.S.P. or of International Units, the potency of all-*trans* vitamin A is 3,333,333 U. per gram by definition. These units refer to potency determined by rat-growth bioassays. Consequently, neovitamin A possesses a biopotency of 2,690,000 U./g. ($3.33 \times 10^6 \times 80.7\%$).

The new finding that neovitamin A has less biological potency than all-*trans* vitamin A should be of both scientific and commercial interest. It permits for the first time a comparison of biopotency changes due to *cis-trans* isomerism in vitamin A with similar changes in carotenoids. Zechmeister[8] has recently summarized the relationship between biological activity and stereoisomerism in carotenes. For example, neo-α-carotene U (a mono-*cis*-α-carotene containing the *cis* double bond in a peripheral location) has about 25% the potency of all-*trans*-α-carotene. Also, neo-β-carotene U, a mono-*cis* compound, and neo-β-carotene B, a di-*cis*-carotene, possess only 38 and 53%, respectively, the potency of all-*trans*-β-carotene. Similarly, neocryptoxanthin U

(8) L. Zechmeister, *Vitamins & Hormones*, **7**, 57 (1949).

TABLE III

BIOLOGICAL RESPONSE TO THE FEEDING OF ALL-*trans* NEOVITAMIN A AND A MIXTURE OF THE TWO ISOMERS

Test material	Growth bioassay: Units fed/wk.	Growth bioassay: Response, g./wk.	Growth bioassay: Relative potency, % ± S. E.	Liver storage bioassay: Total units fed	Liver storage bioassay: Average units recovered per liver	Liver storage bioassay: Relative potency, % ± S. E.
U. S. P. vitamin A acetate	6	11.61	100	1000	389.8	100
reference standard (all-*trans*)	12	19.11	(By defin.)	2000	832.4	(By defin.)
Pure neovitamin A acetate	6	6.50	69.5 ± 21.3	1000	234.6	69.6 ± 3.5
	12	15.53		2000	586.2	
Mixture—2 parts of vitamin A acetate and 1 part of	6	7.99	91.3 ± 13.1	1000	330.8	91.8 ± 5.8
neovitamin A acetate	12	20.07		2000	775.0	

TABLE IV

INTERCONVERSION OF ALL-*trans* AND OF NEOVITAMIN A BY THE RAT

Vitamin A-depleted rats (3 animals per group) of about 100 g. weight were supplemented for 12 days and the livers removed. The combined ether-extractable lipids were analyzed for total vitamin A, alcohol/ester[9] and neo/*trans* ratio.[2]

Supplement	Total units fed	Total units recovered from livers	Relative potency, %	Relative amount of total stored in alcohol form, %	Alcohol fraction neo/*trans* ratio	Ester fraction neo/*trans* ratio	Total extract neo/*trans* ratio
Negative control	0	0	...	..	...	.....	.....
All-*trans* acetate	168,480	83,000	100	13.5	25/75	9.2/90.8	11.3/88.7
Neo-acetate	167,760	58,800	71.1	13.0	28/72	16.7/83.3	19.2/88.8

(probably a mono-*cis* isomer) has only 45% of the potency of all-*trans*-cryptoxanthin.[10] However, pro-γ-carotene (poly-*cis*) has been demonstrated to be biologically equivalent to all-*trans*-γ-carotene for the rat[11] and to be about 20% more potent for the chick.[12] With the exception of the γ-carotene series, the change from a *trans* to a *cis* configuration results in a decrease of physiological activity ranging from 25 to 75% in the carotenoids and from 20 to 28% in the vitamin A–neovitamin A stereoisomeric set.

Also, from a practical point of view, the differences between the biological activities of neovitamin A and *trans* vitamin A are large enough to merit consideration, especially in predicting biological potency from physicochemical assays of commercial sources of vitamin A, since such materials, either natural[2] or synthetic,[13] contain neovitamin A. The conversion factor, therefore, of a product with 1 part neovitamin A to 2 parts of *trans* vitamin A would be about 5 to 8% lower than the conversion factor of a similar preparation of all-*trans* vitamin A.

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(10) H. J. Deuel, Jr., S. M. Greenberg, E. Straub, T. Fukui, A. Chatterjee and L. Zechmeister, *Arch. Biochem.*, **23**, 239 (1949).

(11) L. Zechmeister, J. H. Pinckard, S. M. Greenberg, E. Straub T. Fukui and H. J. Deuel, Jr., *ibid.*, **23**, 242 (1949).

(12) S. M. Greenberg, C. E. Calbert, J. H. Pinckard, H. J. Deuel, Jr., and L. Zechmeister, *ibid.*, **24**, 31 (1949).

(13) J. Cawley, C. Robeson, L. Weisler, E. Shantz, N. Embree and J. Baxter, *Science*, **107**, 346 (1948).