Table 2. *Synthesis of nicotinamide nucleotides by nucleated erythrocytes*

The enzymic reaction mixture with nicotinic acid as substrate contained 20 μ moles of glutamine. Experimental details are given in the text. The results are given as means $\pm$ S.E.M.

Species	No. of observations	Amount of substrate (μ moles)	Synthesis of nicotinamide nucleotides (μ g./ml. of whole blood)	
			From nicotinic acid	From nicotinamide
Pigeon	6	0.5	25.3 $\pm$ 1.585	0.0
	6	1.0	32.9 $\pm$ 1.917	5.9 $\pm$ 0.665
	6	10.0	3.3 $\pm$ 0.393	9.1 $\pm$ 1.059
	6	50.0	4.3 $\pm$ 0.475	19.7 $\pm$ 0.615
	6	100.0	0.0	39.2 $\pm$ 1.812
	6	300.0	0.0	52.7 $\pm$ 1.880
Frog	6	0.5	18.4 $\pm$ 1.202	0.0
	6	1.0	26.2 $\pm$ 1.416	4.6 $\pm$ 0.580
	6	10.0	4.8 $\pm$ 0.217	8.3 $\pm$ 0.623
	6	50.0	0.0	17.4 $\pm$ 0.684
	6	100.0	0.0	28.6 $\pm$ 1.591
	6	300.0	0.0	41.6 $\pm$ 1.718

frog were capable of synthesizing nicotinamide nucleotides from both nicotinamide and nicotinic acid. On the other hand, rat blood was unable to form nicotinamide nucleotides from either of these substrates.

3. Human, guinea-pig, pigeon and frog erythrocytes synthesized nicotinamide nucleotides from nicotinamide and glucose. Monkey erythrocytes synthesized nicotinamide nucleotides from nicotinamide only in the presence of glutamine, suggesting that in this species the conversion of nicotinamide into nicotinic acid was an essential step in the formation of nicotinamide nucleotides.

4. Human, monkey, pigeon and frog erythrocytes could synthesize appreciable amounts of nicotinamide nucleotides from nicotinic acid, glucose and glutamine, whereas this did not occur in guinea-pig erythrocytes. Nicotinic acid was an effective precursor of nicotinamide nucleotides only at low concentrations.

The author thanks Dr C. Gopalan, M.D., Ph.D., Director, Nutrition Research Laboratories, Hyderabad 7, India, for his interest and encouragement.

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Biochem. J. (1963) **87**, 305

Studies on Metabolism of Vitamin A

1. THE BIOLOGICAL ACTIVITY OF VITAMIN A ACID IN RATS

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(Received 17 September 1962)

Since Dowling & Wald (1960) demonstrated that rats grow normally on continued dosage of vitamin A acid, but that they become blind, it has become

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increasingly apparent that vitamin A may exist in multiple forms with different biological functions. This view has further been strengthened by claims that the acid fails to meet the reproductive requirements of either male or female rats (Thompson,

Howell & Pitt, 1961*a, b*). Since acid-fed rats are blind, it has been argued that the animal cannot convert the acid into the aldehyde. Vitamin A acid or a compound derived from it appears to function specifically in growth. The activity of the alcohol or ester in this respect is unknown. In the present work the activity of the vitamin A acid in stimulating growth of vitamin-A-deficient rats has been compared with that of vitamin A acetate, and it is shown that, when given in higher doses, vitamin A acid is far superior to vitamin A acetate in this.

MATERIALS AND METHODS

Synthetic all-*trans*-vitamin A acetate and vitamin A acid were obtained as gifts from Hofmann-La Roche and Co. Ltd., Basle. Vitamin A acetate was dissolved in light petroleum (b.p. 40–60°), subjected to chromatography in an alumina column and its extinction measured in the Beckman model DU spectrophotometer to ascertain the purity and concentration. The acid was used as supplied. The ester had λ_{max} 326 m μ in light petroleum (b.p. 40–60°) with $E_{1\%}^{1\text{cm}}$ 1590, and the acid had λ_{max} 351 m μ in ethanol with $E_{1\%}^{1\text{cm}}$ 1540, and its m.p. was 179–180°.

For oral administration, vitamin A acetate was dissolved in pure groundnut oil containing 0.5% of α -tocopherol, with the aid of a small amount of light petroleum (b.p. 40–60°), the solvent being removed by a gentle stream of nitrogen. Different concentrations of the ester in oil were prepared so as to contain 0.25, 0.5 and 0.75 $\mu\text{g.}/0.1$ ml. and were stored in brown bottles at -20° until used. Control samples of the oil, containing all the ingredients except vitamin A, were similarly prepared and stored. For intraperitoneal injections, stock solutions of the ester, dissolved in light petroleum, were stored at -20° . Immediately before use, required amounts of this solution were withdrawn and the requisite quantities of Tween 20 and α -tocopherol were added. The light petroleum was then removed under suction and the mixture dispersed in 0.1M-basic sodium phosphate with its pH adjusted to 10.5. The final dispersion for injection contained 0.05 mg. of α -tocopherol, 0.2 mg. of Tween 20 and the requisite amount of vitamin A/0.1 ml.

For both oral and intraperitoneal administration, weighed samples of vitamin A acid were dissolved in small volumes of pure ethanol and neutralized with 0.1N-NaOH. The ethanol was evaporated off under suction and different volumes of the phosphate buffer (0.1M, pH 10.5) were added to give the required dilutions. For intraperitoneal injections, Tween 20 and α -tocopherol were added as with the ester. The various solutions were prepared immediately before use.

Commercial casein, as supplied by Polson and Co. (Bombay), was refluxed for 12 hr. on a boiling-water bath with approximately 3 vol. of ethanol acidified with HCl to pH 4.5. The casein was filtered off while still hot and re-extracted by refluxing with light petroleum (b.p. 40–60°) for 12 hr. The extracted casein was then filtered, left overnight in an oven at 90° and finally stored at room temperature until used.

Animals. Weanling male rats of this Institute strain, weighing 30–40 g., were placed on a vitamin-A-free diet composed of 20 parts of the solvent-extracted casein, 60

parts of corn starch, 10 parts of cane sugar, 5 parts of groundnut oil and 5 parts of salt mixture (Steenbock & Nelson, 1923). Choline chloride 5 g., *p*-aminobenzoic acid 1 g., thiamine hydrochloride 25 mg., pyridoxine hydrochloride 25 mg., riboflavin 50 mg., calcium pantothenate 100 mg., biotin 2 mg., folic acid 10 mg., and inositol 500 mg. were dissolved in 250 ml. of water, and each rat was given 0.5 ml. of this solution daily. In addition, each rat received, once a week, 2 mg. of α -tocopherol, 10 $\mu\text{g.}$ of vitamin K and 1.5 $\mu\text{g.}$ of vitamin D. Diet and water were given *ad lib*. The rats were weighed daily and a rat was considered depleted of vitamin A when its net weight gain on 4 successive days did not exceed 1 g., provided that on 2 of these days the animal did not gain in weight. It is our experience that on this diet the weight of the rats is constant within 4 weeks, and acute deficiency sets in within 6 weeks. In every experiment, litter mates were assigned to the different groups, as far as possible. The bioassay was carried out according to U.S.P. XIV (1950) specifications.

RESULTS

Biopotency of vitamin A acid as compared with that of vitamin A acetate. (a) Oral administration. The results of the experiment where growth responses to the acid and the acetate were compared in vitamin-A-deficient rats are summarized in Table 1, and plotted in Fig. 1 as logarithm of dose against gain in weight. Growth of the rats fed with vitamin A acid as doses of 0.05 and 0.1 $\mu\text{g.}$ was very poor. In fact, most of the animals in either group did not survive to the end of the experiment. On the other hand, those rats receiving 1.5 and 10 $\mu\text{g.}$ showed growth response that was well outside the critical range of the bioassay. However, the two curves obtained with 0.25, 0.5 and 0.75 $\mu\text{g.}$ doses of the acid and the acetate were parallel to each other (Fig. 1) and the biopotency of the acid, as calculated from these, was 49.8% of that of the

Fig. 1. Relationship of gain in weight to logarithm of daily dose of all-*trans*-vitamin A acetate (continuous line) and of vitamin A acid (broken line) given orally to male rats. A, B, C, D and E represent the projections of the growth of rats receiving 0.25, 0.5, 0.75, 1.5 and 10 $\mu\text{g.}$ of vitamin A acid respectively on the growth curve for all-*trans*-vitamin A acetate.

acetate. This value agrees closely with the figures of 42% quoted by Kofler & Rubin (1960).

Most of the rats receiving 10 μ g. of the acid/day showed a big increase in weight within 24 hr. of the dose (Fig. 2). It was noticed incidentally that rats that showed such growth invariably drank unusually large volumes of water. However, this effect was observed only when the dose was given to rats that were not severely deficient, but had maintained the weight for 4 days.

(b) Intraperitoneal injection. Previous work had indicated that the growth response of rats given

the acid can vary considerably, depending upon the route and form of administration (Dorp & Arens, 1946). The relative growth responses shown by rats after intraperitoneal injection of the acid or the acetate are summarized in Table 2, and plotted in Fig. 3. Here the acid was 141% as active as the acetate.

A comparison of the growth response in this experiment with that in (a) clearly reveals that the acid is decidedly more effective when injected, because the average weight gain for 4 weeks on 0.25, 0.50 and 0.75 μ g. doses were 17.3, 28 and 50 g.

Table 1. *Effect of oral administration of vitamin A acid and ester on growth of rats*

Body weights and total gains in weight are shown for male rats receiving synthetic vitamin A acid and synthetic all-*trans*-vitamin A acetate orally, and for controls receiving no supplement. Figures in parentheses indicate the numbers of rats that survived.

Supplement	Dose (μ g./day)	No. of rats	Depletion period		Assay period (28 days)			Biopotency (synthetic all- <i>trans</i> -vitamin A acetate = 100)
			Average duration (days)	Average final wt. (g.)	Average lowest wt. (g.)	Average final wt. (g.)	Average gain (g.)	
Vitamin A acid	0.05	6	28.2	71.0	—	60.0	-11.0 (1)	45.92 47.98 55.48 Average 49.8
	0.10	10	29.3	79.5	75.5	81.5	6.0 (2)	
	0.25	10	19.0	89.2	78.0	85.2	7.2 (8)	
	0.50	10	25.7	76.9	72.5	97.1	24.6 (8)	
	0.75	10	26.6	72.0	69.8	106.8	37.0 (9)	
	1.50	10	26.8	65.3	62.4	105.0	42.1 (9)	
	10.00	6	29.7	81.7	81.7	135.7	54.0 (5)	
Synthetic all- <i>trans</i> -vitamin A acetate	0.25	10	28.6	84.1	80.3	105.8	25.5 (7)	
	0.50	10	29.2	77.3	72.0	113.5	41.5 (9)	
	0.75	10	27.1	70.0	67.4	119.7	52.3 (9)	
None (control)	—	11	26.7	76.0	—	57.0	-19.0 (2)

Fig. 2. Growth curves of vitamin-A-deficient rats given orally 0.75 (Δ), 1.5 ($\square$) or 10 μ g. ($\circ$) of vitamin A acid or 0.75 μ g. of vitamin A acetate ($\bullet$) every day for 28 days. Arrow indicates the day on which supplementation was stopped. All the rats were weighed daily until they became moribund.

Table 2. *Effect of intraperitoneal injection of vitamin A acid and ester on growth of rats*

Body weights and total gains in weight are shown for male rats receiving synthetic vitamin A acid and all-*trans*-vitamin A acetate intraperitoneally, and for controls, which received only the phosphate buffer containing Tween 20 and α -tocopherol. Figures in parentheses indicate the numbers of rats that survived.

Supplement	Dose ($\mu\text{g./day}$)	No. of rats	Depletion period		Assay period (28 days)			Biopotency (synthetic all- <i>trans</i> -vitamin A acetate = 100)
			Average duration (days)	Average final wt. (g.)	Average lowest wt. (g.)	Average final wt. (g.)	Average gain (g.)	
Vitamin A acid	0.10	6	29.1	73.0	—	70.0	-3 (1)	
	0.25	8	28.6	78.2	78.0	95.3	17.3 (8)	145
	0.50	8	27.9	79.3	79.0	107.0	28.0 (8)	140
	0.75	8	28.8	75.6	74.9	125.0	50.1 (8)	139
								Average 141.3
Vitamin A acetate	0.25	8	29.8	74.3	72.0	84.0	12.0 (6)	
	0.50	8	27.6	77.8	75.8	95.8	20.0 (8)	
	0.75	8	28.3	79.2	78.2	114.45	36.25 (8)	
Control	—	8	27.9	78.3	—	61.0	-17.3 (1)

Fig. 3. Relationship of gain in weight to logarithm of daily dose of all-*trans*-vitamin A acetate (continuous line) and of vitamin A acid (broken line) given by intraperitoneal injection to male rats. A, B and C represent the projections of the growth of the rats receiving 0.25, 0.5 and 0.75 $\mu\text{g.}$ of the acid respectively on the growth curve for all-*trans*-vitamin A acetate.

respectively when injected, as against 7.2, 24.6 and 37 g. respectively when given orally. The acetate, on the other hand, was less effective when injected.

Effect of increased doses of the acid and the acetate on growth, food consumption and water balance in the deficient rats. An experiment was designed to measure the growth, food consumption, water intake and urine excretion of rats receiving 10 $\mu\text{g.}$ of the acid or the acetate. The acid-fed rats consistently showed a more pronounced gain in

Fig. 4. Daily weight gain (a), water intake (b), urine excretion (c) and apparent water retention (d) over a period of 7 days, in vitamin-A-deficient rats receiving orally 10 $\mu\text{g.}$ of vitamin A acid or vitamin A acetate daily. □, Vitamin A acid; ▨, vitamin A acetate.

weight on the first day, and on the second and third days also their growth was considerably greater than that of the acetate-fed rats (Fig. 4). Thus within 24 hr. of the dose the acid increased growth by 8.1 g., compared with only 0.9 g. with the acetate. The water intake of the rats given acid was also considerably higher than that of the acetate-

fed ones (Fig. 4). In contrast, the urine excretion of the animals of the two groups followed a reversed pattern, as a result of which the apparent retention of water was more pronounced in the rats given the acid. The net gain in weight, food consumption, water intake, urine excretion and apparent water retention over the entire period of 7 days are summarized in Table 3, which shows that there was a net difference in weight of 11 g. between the two groups, with a difference in the net water retention of 96 ml., but with practically no change in their food consumption.

These results showed that the acid is distinctly superior to the acetate in initiating and maintaining the growth of vitamin-A-deficient rats. The results of another experiment, where one single

dose of 500 μ g. of the acid or the acetate was given orally, are summarized in Fig. 5. Here again, the acid-fed rats gained 10 g. on the first day after supplementation, as against only 2 g. by the acetate-fed rats. On the second and third days also the acid-fed rats gained 7 and 5.6 g., as against 3 and 4.2 g. respectively by the other group. From this point onwards the rate of growth of the acid-fed group gradually slowed down, so that by the fourteenth day the acetate-fed group overtook it and thereafter continued to show a prolonged linear growth rate. The animals given the acid, on the other hand, continued to grow, although at a slower rate, until the twenty-sixth day, after which they began to lose weight. It is significant that a single dose of 500 μ g. of vitamin A acid was

Table 3. *Effect of vitamin A acid and ester on weight, food consumption and water balance of rats*

Average net weight gain, food consumption, water intake, urine excretion and apparent water retention over a period of 7 days in vitamin-A-deficient rats receiving orally 10 μ g. of vitamin A acid or acetate daily. Values represent averages from six male rats in each group.

Supplement	Net gain in wt. (g.)	Net diet consumed (g.)	Net water intake (ml.)	Net urine excreted (ml.)	Net apparent water retention* (ml.)
Vitamin A acid	28.7	70	216	57	159
Vitamin A acetate	17.7	72	141	78	63

* Difference between net water intake and urine excreted.

Fig. 5. Growth curves of vitamin-A-deficient rats given a single dose of 500 μ g. of vitamin A acid or vitamin A acetate. O, Vitamin A acetate; ●, vitamin A acid.

able to sustain the rate of growth of the rats for 4 weeks, over which period the net gain in weight was 60 g. However, in another experiment (not included here), in which a similar amount of the acid was given orally in oily solution, the growth response was poor. Dorp & Arens (1946) have also observed that the acid administered in oil is less potent. It has been our consistent observation that the xerophthalmic symptoms of the vitamin-A-deficient rats receiving orally 0.25 μ g. or less of the acid or the acetate per day are not usually cured, whereas doses of either derivative above this amount are equally effective in rapidly curing these symptoms.

DISCUSSION

The stereoisomers apart, all-*trans*-vitamin A exists in animal tissues in several forms, namely the ester, the alcohol and the aldehyde. Recent work on the biochemistry of vitamin A strongly suggests, as outlined in the introduction, that there are at least three well-defined functions of vitamin A that are carried out by its three apparently different forms; the aldehyde, retinene, takes part in the photoreceptive system of vision and the acid in general growth. If the acid by itself cannot meet the reproductive requirements and at the same time cannot be converted into the aldehyde, it is obvious that some other compound derived from vitamin A or retinene, and certainly not from the acid, meets this particular requirement.

If the acid cannot be converted into the aldehyde, and if there were to be any active form of vitamin A for general growth, it should be either the acid itself or some compound derived from it. One would therefore expect the acid to be more effective than the acetate in this respect, and the results of the present study demonstrate that, when given in higher doses, the acid is superior to the acetate in regard to growth. At the lower oral doses, as in the bioassay experiments, however, the acetate was superior. It is possible that the acid is either less stable in the gastrointestinal tract or is rather poorly absorbed, so that, when larger amounts are present, some of it can survive the destruction, thereby being available for absorption; or, alternatively, the presence of the acid in larger concentrations would obviously make it overcome more effectively any barrier to its absorption that might be present in the intestine. These possibilities are further strengthened by our demonstration that, at the lower doses, intraperitoneal injection of the acid is more effective than oral administration.

Since the work of Dorp & Arens (1946), Arens & Dorp (1946), Sharman (1949) and Dowling & Wald (1960) it has been generally believed that the acid is not stored in the animal body. There seem to be several possible explanations for this. The acid

might actually be poorly absorbed and stored, or it may be well absorbed but rapidly metabolized. We have shown here that, after a single dose of 500 μ g. of the acid, the rats continued to grow for nearly 26 days, and those given the acetate in similar amounts maintained their rate of growth for a much longer period. The fact that the effect of a single dose of the acid persisted for 26 days indicates that there was considerable absorption. However, the more prolonged effect of the ester may mean that the classical vitamin A is far more efficiently absorbed and stored, and at the same time is more stable in the animal body. It may also mean that the ester can act as a reservoir from which the animal draws economically to make the acid, so that if the acid is rapidly metabolized, the ester can be a constant source of supply of the acid.

The steps involved in the conversion of the natural ester of vitamin A (which is normally the palmitate) into the acid are hydrolysis, followed by oxidation of the alcohol to aldehyde, which in turn is oxidized to the acid; separate enzymes carry out all these reactions (Ganguly, 1960; Futterman & Saslaw, 1961; Mahadevan, Murthy & Ganguly, 1962). Therefore when the animal receives the acid it has the advantage of not going through all the steps that would have been necessary with the ester. Moreover, it is quite possible that in the deficient rats these enzyme activities are considerably reduced. These effects, singly or combined, would then readily explain the immediate increase in weight caused by the acid, but not by the ester, in the vitamin-A-deficient rats.

Vitamin-A deficiency causes damage to the cell structure (Moore, 1957) and consequently, perhaps, a loss of the forces regulating cell permeability. Many of the endocrine glands also are affected during vitamin-A deficiency (Lowe, Morton & Harrison, 1953; Wolbach & Howe, 1925; Truscott, 1947; Lipsett & Winzler, 1947). In this connexion, the observations of Grangaud & Conquy (1958*d*), that when aldostenedione or progesterone is administered to vitamin-A-deficient rats, the animals grow and live for a longer period than unsupplemented deficient rats, would be pertinent, because this would indicate that the loss of weight during vitamin-A deficiency might, at least partly, be due to the lack of hormones. In addition to this, ample evidence exists to demonstrate that the biosynthesis of many of the steroid hormones is markedly reduced during vitamin-A deficiency (Johnson & Wolf, 1960; Grangaud & Conquy, 1958*a, b, c*); some of these are known to regulate water balance in animals. It therefore seems quite conceivable that vitamin-A deficiency leads to reduced synthesis of such hormones, which causes loss of water, thereby leading to shrinkage

of the cells, which might be one of the reasons for the rapid loss of weight in the deficient state. We have noted that (P. Seshadri Sastry & J. Ganguly, unpublished work), as compared with the pair-fed animals, not only are the livers of the vitamin-A-deficient rats smaller in size, but their protein and nucleic acid concentrations are proportionately higher.

The marked weight gain during the first few days after oral administration of vitamin A acid was not due to increased food intake. There was, however, a most significant difference in the water intake, urine excretion and apparent water retention in the groups. The acid therefore seems to be more effective in causing a more pronounced water retention, leading to a rapid rehydration of the cells, which probably accounts for the marked growth response at the initial stages of supplementation.

Since preformed vitamin A acid is several steps ahead of the ester, it seems reasonable that it will act at a cellular level, either on the cells of the endocrine glands or on all cells in general, more quickly than the ester, and thereby stimulate the immediate growth. Our results therefore indicate that if the acid itself is not the active form of vitamin A for growth it is at least nearer to it than the ester. A similar conclusion was reached by Thompson & Pitt (1960), who found that vitamin A acid is much more effective than the acetate in producing hypervitaminosis A in rats.

It has been shown that enzymes exist in animal tissues that can esterify the alcohol or hydrolyse the ester (Ganguly, 1960), as well as others that can oxidize the alcohol to the aldehyde and the aldehyde to the acid (Futtermann & Saslaw, 1961; Mahadevan *et al.* 1962). As well as in the retina, vitamin A aldehyde has been demonstrated to be present in several animal tissues including rat liver (Plack & Kon, 1961; Winterstein & Hegedus, 1960; Mahadevan *et al.* 1962). However, no vitamin A acid has yet been demonstrated in animal tissues. Nevertheless, the possibility does exist that, starting with the ester, the animal can normally generate the acid in required amounts, since it possesses the necessary enzymes and substrates to do so.

SUMMARY

1. Vitamin A acid was biologically 48.9% as active as all-*trans*-vitamin A acetate in rats when given orally, and 141% as active when injected intraperitoneally.

2. When vitamin-A-deficient rats were fed with 10 μ g. of vitamin A acid or the acetate/day, the acid-fed rats showed a more pronounced growth response within 24 hr. of the dose, drank consider-

ably more water and excreted less urine than the acetate-fed group.

3. A single dose of 500 μ g. of the acid could sustain the growth rate of vitamin-A-deficient rats for 4 weeks.

4. The implications of these results are discussed and it is suggested that the marked growth in response to vitamin A acid may be due to a more rapid correction of water imbalance in the deficient animals.

We wish to thank Dr O. Isler of Hofmann-La Roche and Co. Ltd., Basle, for checking the purity of the vitamin A acid. P.S.S. and K.S.R. wish to thank the Council of Scientific and Industrial Research (India) for the award of Senior and Junior Research Fellowships to them respectively, and P.M. thanks the Lady Tata Memorial Trust for a scholarship. Financial assistance from the Indian Council of Medical Research, New Delhi, and of the Rockefeller Foundation, New York, is recorded.

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