Studies in Vitamin A

18. THE EFFECT OF VITAMIN A DEFICIENCY ON THE PYRUVATE AND α -KETOGLUTARATE LEVELS OF RAT BLOOD

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The problem of the mode of action of vitamin A in the general metabolic processes of mammals (i.e. other than in specialized functions such as night vision) has not yet been solved. From this point of view, the work of von Euler & Högberg (1939, 1940) is important, for it suggests that vitamin A may function in phosphorylation processes. They found that the blood pyruvate levels of vitamin A-deficient rats were more than three times greater than those of rats maintained on the same diet supplemented with vitamin A (43 $\mu\text{g./ml.}$ compared with 12 $\mu\text{g./ml.}$). On feeding the A-deficient rats either vitamin A or cocarboxylase, their blood pyruvate levels returned to normal; on the other hand, massive doses of aneurin (10 mg.) only reduced the levels to 24 $\mu\text{g./ml.}$ It was thus concluded that vitamin A controls the phosphorylation of aneurin to cocarboxylase. Other brief reports of a vitamin A-B₁ synergism exist (Takagi & Saga, 1940; Mitalo, 1942; Cimino, 1944), but other investigators have found no interrelationship (Scheunert & Svreenivasa Rau, 1937; Scheunert & Wolf-ganger, 1938; Scheunert & Wagner, 1938; Dye, Porter & Kelly, 1947) and there is indeed one report that the two vitamins antagonize each other (Namikawa, 1940).

Because of the potential importance of the claims of von Euler & Högberg, and the contradictions in the literature, it was decided to repeat and extend their original investigation. In particular, assuming that the reported increases in blood pyruvate levels in vitamin A deficiency could be confirmed, it was important to find out whether the increase was due to pyruvate and not to α -ketoglutarate, the other main keto acid present in blood, for the method (Lu, 1939) used by von Euler & Högberg (1939, 1940), is not specific for pyruvate and would also measure the α -ketoglutarate present. A spectrophotometric method for determining both components simultaneously has been devised; it is based on the measurement at two wavelengths of the intensity of the colour produced by the 2:4-dinitrophenylhydrazones in alkaline solution. The preliminary extraction procedure follows closely, but not identically, that of Friedemann & Haugen (1943).

EXPERIMENTAL

Determination of blood pyruvate and α -ketoglutarate. The freshly drawn blood sample (about 1 ml.) is run into a 15 ml. centrifuge tube containing 2 ml. of 10% (w/v) trichloroacetic acid and centrifuged at 3000–3500 rev./min. for 5 min. The supernatant is decanted, a further 1 ml. of trichloroacetic acid added to the precipitate, the two mixed thoroughly and then recentrifuged. The supernatant is again collected, added to the first fraction and the combined extracts placed in a 25° bath; after temperature equilibration is complete (10 min.), 1 ml. of 0.1% (w/v, in 2N-HCl) 2:4-dinitrophenylhydrazine (also at 25°) is added. The mixture is incubated for 10 min., transferred quantitatively to a 25 ml. separating funnel and extracted with diethyl ether by shaking for 2 min. This procedure was found to give better reproducibility than the method recommended by Friedemann & Haugen (1943), who mixed the two phases by bubbling N₂ through them. This is not satisfactory when ether is the organic solvent, because excessive evaporation of the ether occurs, although it is quite satisfactory when toluene is used, as in the original method of Friedemann & Haugen (1943). In the present experiment, ether was preferred to toluene (Friedemann & Haugen, 1943) or ethyl acetate (von Euler & Högberg, 1939, 1940). The first solvent extracts only pyruvate, whilst the second, although extracting both pyruvate and α -keto-glutarate, is unsatisfactory because the degree of colour development varies from batch to batch (Long, 1942).

When the layers have completely separated, the aqueous layer is drawn off and discarded. The ethereal layer is then extracted with 6 ml. of 15% (w/v) Na₂CO₃ which extracts only the hydrazones of keto acids, leaving in the ether layer excess 2:4-dinitrophenylhydrazine and neutral hydrazones. The Na₂CO₃ layer is drawn off and 5 ml. transferred to a 10 ml. standard flask, which is then made up to volume with 1.5N-NaOH. A red colour quickly develops, which is then examined spectrophotometrically, using in the compensating cell a blank obtained by carrying out the full procedure on distilled water. In most experiments the blank was almost colourless, but occasionally a more marked colour appeared; this was generally traced to the sample of trichloroacetic acid in use at the time. The strength of the NaOH used in the development of the red colour is somewhat critical. The colour, especially that due to α -ketoglutarate, does not develop fully with N-NaOH, whilst it is unstable and rapidly fades in 3N-NaOH. With 1.5N-NaOH, development is complete and the colour is stable over 1–2 hr.

A colorimetric method for the simultaneous determination of the two keto acids in an extract prepared essentially as described above has been described by Friedemann & Haugen (1943); it is based on extinction measurements at two wavelengths, 420 and 540 $m\mu$, and has given satisfaction in the hands of Coxon, Liébecq & Peters (1949), Buffa & Peters (1949) and Coxon & Peters (1950). The two main objections to this method are both related to the fact that the E values measured at 420 and 540 $m\mu$ always differ considerably; this may lead (a) to error owing to the measurements being made on different regions of the extinction scale of a spectrophotometer; and (b) to lack of sensitivity owing to the E_{540} being so much less intense. One of the simplest ways of calculating the amount of each component in a binary mixture which possesses an isobestic point is that described by Goodwin & Morton (1946). This method, which requires the measurement of the extinctions at two wavelengths, one being the isobestic point, has been successfully applied to the present problem.

Fig. 1. The absorption spectra in 0.75N-NaOH of the 2:4-dinitrophenylhydrazones of pyruvic acid and α -ketoglutaric acid. The isobestic point (λ 412 $m\mu$, ϵ 1100) is indicated —, pyruvic acid; ---, α -ketoglutaric acid.

The absorption curves of the 2:4-dinitrophenylhydrazones of pyruvic and α -ketoglutaric acids are recorded in Fig. 1. The pyruvic acid used was obtained by repeated fractional distillation under reduced pressure, the fraction distilling between 70 and 75° at 25 mm. Hg being used. The concentration of pyruvic acid in the solutions was determined by the bisulphite-binding power method of Clift & Cook (1932), it being impossible to weigh out accurately this intensely hygroscopic substance. The specimen of α -ketoglutaric acid used was kindly supplied by Prof. H. A. Krebs, F.R.S. It should be emphasized that the values recorded in Fig. 1 are obtained after putting the acids through the procedure just described; they were not obtained by dissolving the hydrazones directly in 0.75N-NaOH.

By referring all analyses to these standard curves, any slight systematic losses during the extraction procedure are eliminated.

The curves recorded here (Fig. 1) are somewhat different from those of Friedemann & Haugen (1943). This is because they did not use a spectrophotometer, but a colorimeter in conjunction with a series of filters; their curves, therefore, cannot be considered exact, for extinctions at wavelengths intermediate between those at which the filters transmitted maximally could not be measured.

It will be seen from Fig. 1 that the isobestic point is at 412 $m\mu$, and that the molar extinction (ϵ) at this wavelength is 1100. Taking into account the considerations discussed by Goodwin & Morton (1946), it was decided that maximal accuracy would be achieved by using as the second wavelength 440 $m\mu$, where $\epsilon_{\text{pyruvic acid}}$ is 1515 (A) and $\epsilon_{\alpha\text{-ketoglutaric acid}}$ is 1055 (B). If x is the total molar concentration of keto acids and y the molar concentration of pyruvic acid, then, from the equations of Goodwin & Morton (1946), x is $E_{412 m\mu}/1100$ and

$$y = [E_{440 m\mu} - 1055 E_{412 m\mu}/1100]/460.$$

Animals. In all experiments not involving the stock diet, weanling rats were used. In order to prevent coprophagy, by which means they could supplement their aneurin intake, animals on experiment were kept in separate cages of mesh recommended by U.S. Pharmacopoeia XII (see Hawk, Oser & Summerson, 1947).

Diets. Two experimental diets were used and their constituents are recorded in Table 1. Each animal received 10 g. diet/day. The animals receiving a sufficient amount of

Table 1. Composition of the experimental diets

(Diet no. 1 was made up as near as possible (as far as the information was available) to that used by von Euler & Högborg, 1940.)

Constituent	Diet no. 1 (parts by wt.)	Diet no. 2 (parts by wt.)
Casein (light white)	100	100
Gluten (wheat)	300	0
Hardened arachis oil (i.v. 32)*	65	0
Arachis oil (i.v. 120)*	0	30
Starch	0	325
Salt mixture* (Hawk & Oser, 1931)	25	25
Vitamin supplement*	See Table 2	

* von Euler & Högborg did not indicate the i.v. of their hardened arachis oil, the type of salt mixture used, or the potency of their vitamin supplement.

vitamin A + aneurin generally ate all their food; those on vitamin A and aneurin-deficient diets generally ate all their food until within 4–6 days of the end of the experiment, when, with the onset of deficiency symptoms, their appetite decreased. Diet no. 1 was made as near as possible to that of von Euler & Högborg (1939, 1940), although their published details are insufficient to be certain that the two diets were exactly the same in every detail. The uncertainties arose from the fact that von Euler & Högborg did not state (a) the iodine value (i.v.) of their hardened arachis oil, (b) the type of salt mixture used, or (c) the aneurin potency of the dried yeast. This last omission was particularly important, because it was likely to be a critical factor in the experiments.

It was eventually concluded that their diet contained between 2.2 and 4.4 $\mu\text{g.}$ aneurin/10 g., because they state that 100 $\mu\text{g.}$ of aneurin plus 180 $\mu\text{g.}$ of cocarboxylase represents 50–100 times the daily requirement of a growing rat. This was confirmed when, in a preliminary experiment, diet no. 1 was made up containing the amount of dried yeast used by von Euler & Högberg (1940), it was found to contain, by chemical analysis (kindly carried out by Dr E. Kodicek), 3.6 $\mu\text{g.}$ aneurin/10 g. As the fat-soluble vitamins and those of the B complex are now available commercially in crystalline form, it was decided that a much better controlled experiment was possible using mixtures of these, rather than continuing to use dried yeast. The major components of the diet were, therefore, autoclaved at 15 lb. for 5 hr. to destroy any aneurin present in the diet (Elvehjem, Kline, Keenan & Hart, 1932). The appropriate amounts of vitamins (Table 2) were added daily to each 10 g. portion of diet. In addition to the vitamins recorded in Table 2, the following, often considered non-essential for rats (Brown & Sturtevant, 1949) were added: nicotinic acid (250 $\mu\text{g.}$), inositol (700 $\mu\text{g.}$), *p*-aminobenzoic acid (1 mg.) and 2-methyl-1:4-naphthoquinone (800 $\mu\text{g.}$); (the amounts are per 10 g. diet).

Table 2. *Vitamin allowance (other than aneurin) to rats on the experimental diets*

Vitamin	Allowance ($\mu\text{g.}/10\text{ g. food}$)	Recommended allowance* ($\mu\text{g.}/\text{day}$)
Pyridoxin	25	10
Calcium pantothenate	200	100
Choline chloride	10000	10000
Riboflavin	33	25
α -Tocopheryl acetate	800	300
Vitamin A	33	1.3

* Brown & Sturtevant (1949).

The stock diet consisted of rat cubes supplied by Lever Bros. and Unilever Ltd.

Collection of blood samples. In order to obtain sufficient blood for duplicate determinations, about 1 ml. is required; to obtain this amount it is necessary to kill the experimental animals. All the accepted routine procedures were unsuitable in this case, because any slight struggle of the animal

raised its blood keto acid level considerably. Stotz & Bessey (1942), in their studies on blood pyruvate levels in rats, resorted to cord resection, whilst Handler (1945), using rabbits, found the use of light barbiturate anaesthesia most suitable. The latter procedure appeared more suitable for the present purpose, but it remained to be seen if the barbiturate which it was intended to use (veterinary nembutal supplied by Abbott and Co.) affected the blood pyruvate levels or the chemical determination or both. No effect was observed on the determination, but it was found that nembutal anaesthesia gradually reduces blood pyruvate levels (Goodwin & Williams, 1952). If, however, blood is drawn within 2–3 min. of injection of the anaesthetic, no significant error is involved.

The following procedure was found to be most effective: the rat, previously fasted for 18 hr., was injected intraperitoneally with 0.2 ml. of nembutal and then held firmly to prevent movement until anaesthesia was sufficiently advanced to allow the thorax to be opened. As soon as this was possible, the heart was exposed, the aorta cut, and the blood allowed to run into a 5 ml. beaker, which had been previously smeared with citrate. Citrate is the anticoagulant of choice because it tends to stabilize the pyruvate in the blood (Long, 1944), whilst oxalate causes rapid losses (Friedemann & Haugen, 1942). As quickly as possible, samples of the blood are poured into trichloroacetic acid, for in spite of the possible stabilizing action of citrate, there are many reports of the instability of pyruvate in blood (Wilkins, Weiss & Taylor, 1938; Bueding & Wortis, 1940; Bueding & Goodhart, 1941). The procedure is then as described under the section 'Determination of blood pyruvate and α -ketoglutarate' (p. 708).

RESULTS

The simultaneous determination of pyruvate and α -ketoglutarate levels in blood

Accuracy of the method. This was tested by preparing a series of known mixtures of the two components, treating them as if they were blood, and observing the analytical recovery. The results of a typical series are recorded in Table 3. It will be seen that, whatever the relative amounts of the two

Table 3. *Determination of pyruvate and α -ketoglutarate in prepared mixtures of the two*

Keto acids present in mixture ($\mu\text{g.}/\text{ml.}$)		Molar ratio: pyruvate/ α -keto- glutarate	Keto acids found in mixture ($\mu\text{g.}/\text{ml.}$)		Recovery	
Pyruvate	α -Keto- glutarate		Pyruvate	α -Keto- glutarate	Pyruvate (%)	α -Keto- glutarate (%)
7.9	32.0	0.415	8.2	31.8	104	99
16.0	33.3	0.81	16.0	32.2	100	97
7.9	16.0	0.83	7.8	16.1	99	101
4.9	7.5	1.08	5.1	7.5	104	100
24.4	25.0	1.59	25.4	22.2	104	89
7.9	8.0	1.66	8.3	7.1	105	89
29.4	20.0	2.42	29.7	20.6	101	103
8.2	4.2	3.24	8.4	3.3	102	79
7.9	4.0	3.32	7.8	4.6	99	115
7.9	4.0	3.32	7.8	3.9	99	98
9.8	2.0	8.3	10.0	1.1	102	55
29.4	5.0	9.7	30.4	5.8	103	116
44.0	5.0	14.5	44.0	8.7	100	174
9.8	0.0	∞	10.1	-1.0	103	—

components present, the accuracy of the pyruvate determinations is excellent, the mean recovery being 102 %. The accuracy of the α -ketoglutarate determinations is still good (mean recovery 101 %, with a slightly greater scatter than with pyruvate)

a final check of the assumption that only two keto acids are involved, the following experiment was carried out: The complete absorption spectrum of the colour produced by the hydrazones extracted from a sample of blood was plotted. Assuming that only the two reported constituents were present, the amounts of these were calculated in the usual way. The complete spectrum which would be expected from these results was then computed and compared with the observed curve. It will be seen (Fig. 2), that the two curves are, within experimental limits, coincident over the spectral range examined. If any other keto acids had been contributing to any significant extent (e.g. > 1 %) to the observed spectrum, marked divergencies between this and the calculated curve would have been encountered.

Fig. 2. The absorption spectrum in 0.75N-NaOH of the 2:4-dinitrophenylhydrazones occurring in a specimen of normal rat blood, compared with that calculated according to the procedure outlined in the text, assuming only pyruvic and α -ketoglutaric acids are present. —, observed curve; ---, calculated curve.

provided that the molar ratio pyruvate: α -ketoglutarate is below 4. When this ratio is above 4, then the determination of α -ketoglutarate ceases to be reliable. As this ratio has never yet been observed in blood, this failure is of no practical consequence. If the blood filtrates contain keto acids other than pyruvate and α -ketoglutarate, then this method is

Blood pyruvate and α -ketoglutarate levels on various diets

Stock diet. The mean value of the pyruvate and α -ketoglutarate levels of rats of both sexes, drawn at random at different times of the year from the stock colony, are recorded in Table 4. It will be seen that there is no significant difference between the sexes, although von Euler, von Euler & Pettersson (1941), claimed that increased pyruvate levels were observed at oestrus.

Experimental diets. The results obtained in a typical experiment using a diet (diet 1, Table 1) simulating as nearly as possible that of von Euler & Högborg (1939, 1940) are recorded in Table 5. Weanling rats were used and the experiment continued until the group on the vitamin A-deficient diet began to lose weight (34–38 days). That this was a true sign of the onset of vitamin A deficiency was confirmed by analysing the livers of these rats

Table 4. Mean values for pyruvate and α -ketoglutarate of blood in male and female rats chosen at random and maintained on a stock diet

Sex	No. of animals	Pyruvate			α -Ketoglutarate		
		$\mu\text{g./ml.}$	S.D.	P	$\mu\text{g./ml.}$	S.D.	P
Mixed	20	9.14	± 2.31	—	5.76	± 2.70	—
Male	8	8.36	± 1.41	0.10–0.20	6.49	± 2.15	0.10–0.20
Female	8	9.94	± 2.63		4.70	± 2.01	

not applicable. There appears to be no reasonable doubt, however, that in normal blood these two ketoacids are the only ones present in detectable amounts (Friedemann & Haugen, 1943). The recent report (Calvallini, Frontali & Toschi, 1949) that four 2:4-dinitrophenylhydrazones arise from blood, still requires confirmation, for there is evidence that two of the 'spots' observed on the Italian workers' chromatogram are artifacts (see, for example, Altmann, Crook & Datta, 1951). As

for vitamin A; in no case, using the antimony trichloride method, could vitamin A be detected. It will be seen from Table 5 that no significant differences in the blood levels of either keto acid occurred between the two groups. A statistical comparison between these results and those obtained with animals on the stock diet indicates that, in both groups, the two parameters are significantly higher than those of the normal diet ($P < 0.001$ in both cases).

Table 5. *Mean blood levels of total keto acids, pyruvate and α -ketoglutarate in weanling rats on experimental diets*

(The rats were maintained for 34–36 days on diet 1 (Table 1), supplemented with 3.5 μ g. aneurin/day, Group A was given vitamin A, group B was not.)

	Group A (5 rats)		Group B (4 rats)		P (between groups)
	(μ g./ml.)	S.D.	(μ g./ml.)	S.D.	
Total keto acids	26.7*	± 5.2	29.7*	± 9.3	0.50–0.70
Pyruvate	13.9	± 3.2	16.2	± 5.1	0.30–0.50
α -Ketoglutarate	15.8	± 4.4	16.2	± 5.3	0.80–0.90

* Total keto acids are expressed as μ moles/100 ml.

Table 6. *Mean blood levels of total keto acids, pyruvate and α -ketoglutarate in groups of weanling rats (six per group) fed diet 2 (Table 1) and varying supplements of aneurin*

Aneurin intake (μ g./day)	Total keto acids			Pyruvate			α -Ketoglutarate		
	(μ moles/100 ml.)	S.D.	P	(μ g./ml.)	S.D.	P	(μ g./ml.)	S.D.	P
4	22.5	± 7.1	0.50–0.70	12.7	± 5.1	1.0	12.4	± 7.1	0.30–0.50
6	20.6	± 7.3		12.7	± 6.7		8.9	± 3.8	
8	18.6	± 6.8	0.50–0.70	12.1	± 2.9	0.80–0.90	9.7	± 5.2	0.70–0.80
10	13.0	± 1.9	0.05–0.10	7.9	± 0.6	0.001–0.01	6.0	± 2.7	0.001–0.01
12	14.9	± 1.1	0.10–0.20	8.0	± 1.5	0.90	8.5	± 1.5	0.10–0.20

When it was found impossible to reproduce the effect attributed by von Euler & Högborg (1939, 1940) to vitamin A deficiency, it was decided to carry out further similar experiments on a more balanced diet, for the diet used by these workers is virtually carbohydrate free. Using diet 2 (Table 1), a preliminary investigation was carried out to determine the aneurin intake at which blood keto acid levels begin to rise. It was thought that if the observations of von Euler & Högborg (1940) were due to an increased aneurin requirement in vitamin A deficiency, then the best way to demonstrate this would be to observe the blood levels of a group of rats fed a vitamin A-deficient diet containing just sufficient aneurin to prevent elevated blood keto acid levels on the same diet supplemented with vitamin A.

The results obtained on diets containing varying amounts of aneurin are recorded in Table 6. The growth curves for the various groups are given in Fig. 3. It will be seen from Table 6 that there is a significant difference between the blood levels of keto acid of rats receiving 8 μ g. aneurin/10 g. diet and those receiving 10 μ g. This conclusion is strengthened when it is noted that there is no statistical difference between the values obtained for rats on the stock diet and those on the diets containing 10 and 12 μ g. aneurin/10 g., whilst the values for the 4, 6 and 8 μ g. aneurin/10 g. diets are all significantly higher than those on the stock diet. Similarly with α -ketoglutarate levels, there is a significant difference between the 8 and 10 μ g. diet. An apparent inconsistency arises here, for the α -ketoglutarate levels on the 12 μ g. diet are above those on the 10 μ g. diet and not significantly different

from those on the 8 μ g. diet. As one extremely high value was encountered in this group, no real significance can be attached to this apparent

Fig. 3. The mean growth rates of groups of weanling rats maintained on diet 2 (Table 1) supplemented with varying amounts of aneurin. $\times \times \times$, 4 μ g. aneurin/day; ---, 6 μ g. aneurin/day;, 8 μ g. aneurin/day; - - - - , 10 μ g. aneurin/day; —, 12 μ g. aneurin/day.

anomaly, and in fact, the 10 and 12 μ g. values, when compared with values on the stock diet, are found not to be significantly different from normal. The growth curves (Fig. 3) confirm the findings on blood; it will be seen that after 30 days on the various diets, only those animals obtaining 10 and 12 μ g.

aneurin/10 g. diet, continue to gain weight; those on the lower levels begin to lose weight with varying rapidity after between 20 and 25 days on the diet.

As a result of this experiment it was decided to examine the effect of vitamin A deficiency on the blood keto acid levels of rats maintained on diet 2 (Table 1) and supplemented with either 8 or 10 μ g. aneurin/10 g. diet. Two groups of weanling rats were taken; one was given vitamin A and the other deprived of vitamin A. Each of these groups was divided into two sub-groups, one of which obtained 8 μ g., and the other 10 μ g. aneurin/10 g. diet. The experiment was continued until vitamin A deficiency was established as evidenced by cessation of growth followed by a decline in weight. The absence of vitamin A from the livers of the deficient groups was demonstrated in the usual way using the antimony trichloride method.

acid levels occur on this diet although it is virtually carbohydrate-free, and Banerji (1941) and Dann (1945) found that clinical signs of avitaminosis-B₁ did not occur on carbohydrate-free diets even in the complete absence of aneurin from the diet. They did, however, observe that in the absence of aneurin, rats on carbohydrate-free diets excreted more bisulphite-binding substances (keto acids) in the urine. Unfortunately, neither investigator measured the blood keto acid levels, but one would suppose that they would also be raised if increased urinary excretion of bisulphite-binding substances occurs. The present experiments on carbohydrate-free diets indicates that this is so, and that on a carbohydrate-free diet, a daily intake of 3.5 μ g. of aneurin is insufficient to prevent elevated blood keto acid levels although no typical clinical signs of aneurin deficiency existed. It is also important to notice

Table 7. Mean blood levels of total keto acids, pyruvate and α -ketoglutarate fed diet no. 2 with either 8 or 10 μ g. aneurin/day, and with or without vitamin A

(Groups of weanling rats, five per group were used.)

	Aneurin intake 8 μ g./day					Aneurin intake 10 μ g./day				
	No vitamin A supplied		Vitamin A supplied		P (between groups)	No vitamin A supplied		Vitamin A supplied		P (between groups)
	(μ g./ml.)	S.D.	(μ g./ml.)	S.D.		(μ g./ml.)	S.D.	(μ g./ml.)	S.D.	
Total keto acids	16.0*	± 2.7	15.0*	± 5.6	0.70-0.80	13.1*	± 3.2	13.2*	± 1.8	>0.90
Pyruvate	11.2	± 2.6	10.9	± 4.8	>0.90	10.4	± 2.1	10.0	± 2.6	0.80-0.90
α -Ketoglutarate	4.8	± 2.9	3.9	± 0.75	0.50-0.70	2.1	± 1.6	2.6	± 1.5	0.50-0.70

* Total keto acids are expressed as μ moles/100 ml.

It will be seen from the results recorded in Table 7 that no significant differences are apparent between the groups deficient in vitamin A and those receiving supplements of this vitamin. It is interesting also to note in this experiment that there were no significant differences between the blood keto acid levels in the groups fed 8 and 10 μ g. aneurin, the values on the 10 μ g. level being increased above normal. This indicates how close to the minimum daily requirement of aneurin this value is; the rise on the 10 μ g. diet may be due to the fact that the duration of this experiment was longer, 40 days compared with 30 days in the previous experiment.

DISCUSSION

Using a diet as comparable as possible with that of von Euler & Högborg (1939, 1940), it has not been possible to demonstrate that vitamin A deficiency has any effect on the blood levels of either pyruvate or α -ketoglutarate. On this diet, the keto acid levels were significantly raised compared with normals, but the increases were the same, irrespective of whether the rats were deficient in vitamin A or not. It is interesting to note that increases in blood keto

that the rise of α -ketoglutarate levels is relatively greater than that of pyruvate; this may well be due to the high content of gluten which contains considerable amounts of glutamic acid which could, by deamination, give rise to α -ketoglutaric acid. Oberdisse (1947) has shown that in dogs intravenous injection of alanine causes an increase of blood pyruvate. Using a normal carbohydrate-containing diet supplemented with borderline amounts of aneurin, it was also not possible to demonstrate any effect of vitamin A deficiency. These latter experiments must be considered critical, for any effect of vitamin A on aneurin metabolism should have revealed itself in these experiments. It must be concluded that the higher pyruvate levels recorded by von Euler & Högborg (1939, 1940) were not due to vitamin A deficiency; consequently their evidence that vitamin A controls the phosphorylation of aneurin to cocarboxylase cannot be valid.

It is obvious from the results recorded in Tables 6 and 7 and in Fig. 3 that the minimal aneurin requirement for growing rats lies between 8 and 10 μ g./day; this confirms previous estimates, e.g. Arnold & Elvehjem (1938), 10 μ g.; Yudkin (1941), 8 μ g. It is also not inconsistent with the only previous report

(Kratzing & Slater, 1950) on the maximal aneurin intake on which hyperpyruvaemia was observed, the figure given being 7.8 $\mu\text{g./day}$.

SUMMARY

1. A spectrophotometric method for the simultaneous determination of blood pyruvate and α -ketoglutarate levels is described.

2. Using a carbohydrate-free diet essentially similar to that of von Euler & Högberg (1940), the claims of these authors that vitamin A deficiency increases blood pyruvate levels in rats was not confirmed; α -ketoglutarate levels were also unaffected.

3. Using a diet containing a normal proportion of carbohydrate and supplemented so as to meet the minimal aneurin requirements of growing rats, it was again impossible to demonstrate any effect of

vitamin A deficiency on blood pyruvate and α -ketoglutarate levels.

4. On a carbohydrate-free diet a daily intake of 3.5 $\mu\text{g.}$ of aneurin is insufficient to prevent raised blood pyruvate and α -ketoglutarate levels, irrespective of the vitamin A status of the animals.

5. On a normal diet, the minimal daily aneurin intake required to prevent increased blood pyruvate and α -ketoglutarate levels in rats is between 8 and 10 $\mu\text{g.}$

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