The Effect of Vitamin A and other Dietary Constituents on the Activity of Adenosine Triphosphate Sulphurylase

By A. S. LEVI, S. GELLER, D. M. ROOT AND G. WOLF

Department of Nutrition and Food Science, Massachusetts Institute of Technology,
Cambridge, Mass. 02139, U.S.A.

(Received 23 February 1968)

1. ATP sulphurylase was assayed in various organs from vitamin A-deficient and pair-fed control rats at different stages of deficiency. Activity decreased slightly in the liver and markedly in the adrenal gland. Striking differences in liver activity were observed between pair-fed control and *ad libitum*-fed animals. This observation suggested that diet (apart from vitamin A) strongly influenced the activity of ATP sulphurylase. 2. Total starvation caused a severe decrease in activity in liver within 48 hr. This was due to a lack of protein intake. 3. By feeding groups of vitamin A-deficient and pair-fed control rats on a diet containing 80% protein, the specific activity of the liver ATP sulphurylase was maintained in the pair-fed control group at the normal level of an *ad libitum*-fed rat, whereas it decreased by 25% (statistically significant at $P < 0.01$) in the deficient rat. On a 20%-protein diet, there were no significant differences between vitamin A-deficient and pair-fed control rats. These relationships held also for enzyme activity expressed per g. of liver, per total liver and per g. of DNA. There were no differences in liver protein or DNA concentration between vitamin A-deficient and control rats on either protein intake. 4. Control rats on a 20%-protein diet had liver specific enzyme activities about one-half of those in control rats on an 80%-protein diet, as well as lower liver protein concentrations. 5. It is concluded that, when the effect of protein deprivation on ATP sulphurylase is separated from the effect of vitamin A deficiency, a lowering of the enzyme activity caused by the vitamin deficiency is demonstrable.

Beginning with the work of Fell & Mellanby (1952) on the release of chondroitin sulphate from chick bone rudiments grown in organ culture in the presence of excess of vitamin A, and the observations of Dziewiatkowski (1954) on the decreased uptake of sulphate into epiphyseal cartilage due to vitamin A deficiency, a complex, confusing and sometimes controversial picture of the relationship between vitamin A and sulphation emerged. The decreased sulphation in vitamin A deficiency was first ascribed to the lower activity of the sulphate-activating enzyme system (Varandani, Wolf & Johnson, 1960) and later specifically to the first enzyme, ATP sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4), in the sequence of reactions that lead to incorporation of inorganic sulphate into organic molecules (Sundaresan, 1966). These findings were confirmed (Subba Rao, Seshadri Sastry & Ganguly, 1963; Hall & Straatsma, 1966) and contested (Pasternak, Humphries & Pirie, 1963; Pasternak & Pirie, 1964; Mukherji & Bachhawat, 1967).

The aim of the present research is to clarify why different workers obtained different results. Our conclusion is that two variable factors, vitamin A and dietary protein intake, control the level of activity of the enzyme ATP sulphurylase. Difficulties arise when they are not varied separately. A preliminary report of this work has appeared (Levi & Wolf, 1965).

MATERIALS AND METHODS

Animals and diets. Male weanling albino rats of the Sprague-Dawley strain (40–50 g.) were obtained from Sprague-Dawley Inc., Madison, Wis., U.S.A., and from Charles River Breeding Laboratory, Wilmington, Mass., U.S.A. Holtzman rats (Holtzman Co., Madison, Wis., U.S.A.) were used in the experiment shown in Table 4. The animals were kept in individual cages in a constant-temperature room at 22°. Rats were separated into two groups of comparable weight. Both received the vitamin A-deficient diet (Wolf, Lane & Johnson, 1957) (prepared by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.)

(diet 1), and one group a supplement of 180 μ g. of retinol dissolved in 0.3 ml. of cottonseed oil orally three times/week (crystalline retinol was obtained from Distillation Products Industries, Rochester, N.Y., U.S.A.). Food intake of each unsupplemented animal was measured daily. The supplemented rat of each pair received an amount of diet equal to that eaten by the deficient rat the day before.

Rats, both on the unsupplemented and supplemented diet, usually reached a weight plateau in about 5 weeks. They were killed after they had lost weight for 4–5 days and had visible eye lesions (except as indicated in Table 4).

In the investigation of the effect of dietary protein intake on the enzyme, the diet was that described by Khairallah & Wolf (1965) with double the vitamin mix and a methionine supplement of 1.5% of the casein. Casein (obtained from General Biochemicals Inc.) was varied at the expense of carbohydrates (diet 2). Vitamin A-free casein, prepared by General Biochemicals Inc. as described by Wolf *et al.* (1957), was used in the diet (diet 2) of the vitamin A-deficient group on the 80%-casein diet.

Enzyme assays. Assay I was that of Wilson & Bandurski (1958) carried out on a supernatant fraction prepared as described by Sundaresan (1966). The concentration of ATP was kept at or above 10 μ moles for 1 mg. of protein/ml. of incubation medium for maximum activity.

Assay II was that of Carroll (J. Carroll, personal communication) somewhat modified. The method was originally used by Spencer & Harada (1960) to assay for [35 S]PAPS* formation. The assay is based on the presence in supernatant fraction of ATP sulphurylase and sulphokinase with the former enzyme activity assumed to be rate-limiting (Robbins & Lipmann, 1958). In the presence of ATP, MgCl_2 and labelled SO_4^{2-} , therefore, labelled PAPS forms. The danger of hydrolysis of PAPS is avoided by adding excess of sulphotransferase and *p*-nitrophenol, to which the sulphate is rapidly and quantitatively transferred. The radioactivity in the *p*-nitrophenyl sulphate formed is then assayed.

The concentration of *p*-nitrophenol used in the assay was optimum. The concentration of sulphate used did not give maximal conversion, but it was deemed adequate as it was well above the K_m concentration (K_m 1.5×10^{-4} M; Hall & Straatsma, 1966) since proportionality was obtained between amount of enzyme incubated and product formed. In fact, every enzyme assay was made in duplicate but with various amounts of enzyme protein, so that proportionality between enzyme and product was checked for every assay.

The assay procedure was as follows. To 0.5 ml. of an incubation mixture containing 100 μ moles of tris-HCl buffer, pH 7.6, 5 μ moles of MgCl_2 , 0.04 μ mole of *p*-nitrophenol, 10 μ moles of ATP and 0.25 μ mole of K_2SO_4 (containing about 1 μ C of $\text{Na}_2^{35}\text{SO}_4$, obtained from New England Nuclear Corp., Boston, Mass., U.S.A.) was added 0.05 or 0.1 ml. of the supernatant fraction (Sundaresan, 1966) to be assayed (about 3 mg. of protein/ml.), sulphotransferase preparation and water. The mixture was incubated at 37° for 20 min., heated at 100° for 90 sec. and centrifuged. The supernatant solution (0.025 ml.) was applied to Whatman no. 1 filter paper and chromatographed ascendingly for about 1 hr. in acetone–water (9:1, v/v). The labelled *p*-

nitrophenyl sulphate formed travelled with the solvent front, and the residual sulphate remained at the origin. Both spots were cut out, the paper was placed in liquid-scintillation phials, and the radioactivity of the ^{35}S counted in a liquid-scintillation counter (Nuclear-Chicago Corp. model 720) after addition of 15 ml. of a 0.3% solution of 2,5-diphenyloxazole in toluene. From the percentage of total radioactive sulphate in the *p*-nitrophenyl sulphate, the μ moles of sulphate converted could be calculated. Tests showed that all the radioactivity was in the supernatant solution after removal of the denatured protein by centrifugation and that it could all be recovered in the spots at the origin and the solvent front. Radioactive intermediates such as APS and PAPS remained at the origin together with SO_4^{2-} . By chromatography in isobutyric acid–0.5 N ammonia (5:3, v/v), it was possible to separate these from SO_4^{2-} and from *p*-nitrophenyl sulphate. However, as a result of some test incubations, it was found that their radioactivity was negligibly small. Cysteine, which has been used for sulphate activation (Mukherji & Bachhawat, 1966), had no effect on the conversion. Sulphotransferase was prepared by dialysing rat liver supernatant fraction (Sundaresan, 1966) for 24–48 hr. against frequent changes of water in the cold and by freeze-drying the resulting solution to 10–20% of the original volume. It was found that this preparation had no sulphurylase activity. The optimum amount used was determined for each batch and varied from 0.05 to 0.2 ml. Before use, the sulphotransferase was checked for absence of sulphurylase activity by incubating with the above mixture and showing the absence of labelled *p*-nitrophenyl sulphate.

The above assay for ATP sulphurylase has advantages over the more commonly used assay that depends on PAPS formation. For instance, on incubation of supernatant fraction with PAPS for 20 min., 16% of the PAPS was hydrolysed/mg. of protein by liver of vitamin A-deficient rats, whereas 26%/mg. of protein was hydrolysed by liver of pair-fed control rats. Therefore any differences between deficient and normal groups may be obscured by the lower rate of hydrolysis in supernatant fraction from deficient livers. *p*-Nitrophenyl sulphate, on the other hand, is hydrolysed only to the extent of 2%/mg. It is known that PAPS-hydrolysing activity resides in the supernatant fraction (Brunngraber, 1958), whereas *p*-nitrophenyl sulphate is split by a microsomal enzyme (Subba Rao *et al.* 1963). The valid criticism is made by Mukherji & Bachhawat (1966) that suboptimum concentrations of sulphate had been used by previous workers in the sulphurylase assay. In our assay II the concentration of sulphate was one-fifth of that used by Mukherji & Bachhawat (1966). However, when repeating some of our experiments with increased concentrations of sulphate, we obtained the same differences between the deficient and control groups.

[^{35}S]PAPS was prepared by the method of Varadani *et al.* (1960) and eluted from the electrophoresis paper; labelled *p*-nitrophenyl sulphate was eluted from the chromatography paper after a standard incubation and chromatography as described above. Each was separately incubated with supernatant fraction to determine its hydrolysis and reisolated by electrophoresis or paper chromatography.

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) and DNA by the method of Munro & Fleck (1966).

* Abbreviations: PAPS, adenosine 3'-phosphate 5'-sulphatophosphate; APS, adenosine 5'-sulphatophosphate.

RESULTS AND DISCUSSION

ATP sulphurylase activity during development of vitamin A deficiency in various organs. Total and specific activities of ATP sulphurylase in liver were measured weekly as the vitamin deficiency developed and are shown in Figs. 1 and 2. The differences between the vitamin A-deficient and pair-fed control rats at the deficient stage ($P < 0.05$ for Fig. 1; $P < 0.1$ for Fig. 2) are significant, but the most striking differences are between the specific activities in pair-fed control rats at 4 weeks, at the plateau stage of deficiency and at extreme deficiency (Fig. 2). These differences become more obvious when the specific activity of the enzyme in the pair-fed control is compared with that in the *ad libitum*-fed control rats (Fig. 2), and lead to the conclusion that the deprivation of food in the pair-fed controls (attendant on being given the same amount of diet as the vitamin A-deficient rats) severely lowers the activity of the sulphurylase. Geison (1965) made a similar observation on the effect of food deprivation (apart from vitamin A) on the activity of this enzyme.

The effect of the vitamin deficiency on sulphurylase activity in other organs was not extensively investigated in this study. However, in one experiment, only very slight differences due to vitamin A

deficiency were seen in the enzymic activity of pair-fed and deficient kidney, lung-spleen and colon preparations. On the other hand, the results with adrenal gland were startling: the activities in the pair-fed control animals rose steeply at the plateau stage and in the extreme deficiency whereas those in the deficient animals fell precipitously. The increased activity of adrenal glands of the pair-fed rats may have been in response to the stress of food deprivation and the possible involvement of sulphated intermediates in steroid biosynthesis. The pronounced decrease in sulphurylase activity in the adrenal glands of the deficient rats may have been the cause of the decreased glucocorticoid production observed in vitamin A deficiency by Johnson & Wolf (1960) and by Glick (1963).

ATP sulphurylase activity and dietary protein intake. Total starvation results in a marked decrease in liver ATP sulphurylase activity (Table 1). The principal cause of this decrease is lack of dietary protein. Thus when two diets provide the same amount of protein but differ in all other constituents by one-half (vitamin A in both cases being adequate) there is no difference in the enzyme activity (columns 1 and 2, Table 2). Activity decreases to almost one-half (column 3, Table 2) when protein is largely replaced by carbohydrates. With this dietary protein content, again, when protein intake is kept constant (column 4) and all other dietary constituents are halved, there is no change in the enzyme activity (column 4, Table 2). It is noteworthy that, with between 40% and 20% protein in the diet, there is no significant change in enzyme

Fig. 1. Total enzyme activity of ATP sulphurylase/liver. Each value represents an average of four rats. Assay I was used. The abscissa indicates weeks on deficient diet (diet 1). □, Control rats; ▨, deficient rats. Standard errors are indicated.

Fig. 2. Specific activity of ATP sulphurylase in liver supernatant of deficient, pair-fed control and *ad libitum*-fed control. Each value represents an average of three animals. Assay I was used. Zero time: 4 days after weaning. The abscissa indicates time on deficient diet (diet 1). □, Control (pair-fed) rats; ▤, control (*ad libitum*-fed) rats; ▨, deficient rats. Standard errors are indicated.

activity, whereas a sharp decrease occurs when protein is cut to 5% (all rats fed *ad libitum*).

Supplementation of a 5%-protein diet with methionine, cysteine or tyrosine (Table 3) does not

prevent the decrease in enzyme activity and it is our hypothesis that it is a complete mixture of amino acids that keeps the enzyme activity at the normal value. On a low-protein diet the rate of liver protein synthesis is lower than on a high-protein diet (Clark, Naismith & Munro, 1957). One might speculate that the sulphurylase turns over rapidly in liver and that its activity therefore decreases faster than that of other proteins, assuming that protein degradation rates remain unchanged.

Liver ATP sulphurylase activity in vitamin A-deficient rats on a high-protein or low-protein diet. The stage was now set for a test of the hypothesis that, with a 20%-protein diet, the food intake of a pair-fed control rat is too low to provide sufficient protein to maintain the liver sulphurylase activity at the normal value. Such an animal is therefore not an adequate control for investigation of the effect of vitamin A deficiency on the enzyme activity.

Table 1. *Effect of total starvation on liver ATP sulphurylase in the rat*

Assay II was used. Diet 1, supplemented with vitamin A, was given from weaning for 6 weeks.

Time starved (hr.)	Specific enzyme activity (mμmoles of <i>p</i> -nitrophenyl sulphate/mg. of supernatant protein/hr.)	
	Expt. 1	Expt. 2
0	16.4	17.1
48	3.23	3.18
48	3.55	3.82
72	6.36	6.61
96	6.51	5.86

Table 2. *Effect of varying the casein content and quantity of the diet on ATP sulphurylase activity in rat liver*

Four male rats were in each group (99–136 g. from Charles River Breeding Laboratories). Diet 2 (vitamin-supplemented) was given for 1 week. Assay II was used. Values per mg. of protein are means $\pm$ s.e.m. Statistical significance of difference between columns (1) and (3) is $P < 0.01$, between columns (3) and (5) is $P < 0.05$. Differences between columns (1) and (2) and between columns (3) and (4) are not statistically significant. Average daily food intake: 14.3 g. (column 1); 13.0 g. (column 3).

Diet ...	Enzyme activity (mμmoles of <i>p</i> -nitrophenyl sulphate formed/hr.)				
	40% Casein*	80% Casein†	5% Casein*	10% Casein‡	20% Casein*
Column ...	(1)	(2)	(3)	(4)	(5)
Per mg. of supernatant protein	24.2 $\pm$ 1.67	20.1 $\pm$ 1.46	13.6 $\pm$ 1.77	13.1 $\pm$ 0.83	22.4 $\pm$ 2.03
Per mg. of liver	2.03	1.91	0.78	1.14	1.57
Per total liver $\times 10^3$	17.0	11.0	4.6	4.6	13.4

* Fed *ad libitum*.

† Pair-fed to one-half of animals used for column (1).

‡ Pair-fed to one-half of animals used for column (3).

Table 3. *Effect of amino acid supplement to the 5%-casein diet on ATP sulphurylase activity in rat liver*

Four male rats were in each group (75–84 g. from Charles River Breeding Laboratories). Diet 2 was given for 1 week. Assay II was used. Values per mg. of protein are means $\pm$ s.e.m.

Diet ...	Enzyme activity (mμmoles of <i>p</i> -nitrophenyl sulphate formed/hr.)				
	6% Casein	6% Casein + 1% methionine	6% Casein + 1% cysteine	6% Casein + 0.5% tyrosine + 0.5% methionine	20% Casein
Per mg. of supernatant protein	14.6 $\pm$ 0.83	15.4 $\pm$ 1.29	14.1 $\pm$ 1.13	15.3 $\pm$ 1.15	28.9 $\pm$ 1.60
Per mg. of liver	1.18	1.17	1.03	1.07	2.50
Per total liver $\times 10^3$	3.3	4.6	4.0	4.1	14.7

Animals were killed at the plateau stage of the vitamin deficiency, before their food intake had fallen to less than 5 g./day (average daily food intake over the last 48 hr. of life of all animals, 5.9 g.); those eating less were discarded. With a 20%-protein diet, the enzyme activity of the control animals (column 2, Table 4) was lower than the specific activity of 22 μ moles of SO_4^{2-} activated/mg. of protein/hr. (column 5, Table 2) for an *ad libitum*-fed rat, which ate about 16 g./day. On the 20%-protein diet the activities in control rats and deficient rats were the same (columns 1 and 2, Table 4). On the other hand, the pair-fed control rat on the 80%-protein diet, eating 5 g./day, ate about as much protein as an *ad libitum*-fed control rat on a 20%-protein diet. The specific activity of the sulphurylase (column 4, Table 4) was at about that found in such an *ad libitum*-fed rat. But vitamin A-deficient rats eating the same amount of food had a significantly lower enzyme activity (column 3, Table 4). In this pair, food intake was therefore such that the pair-fed control animal had a normal enzyme activity. The deficient animal ate the same amount of the same diet (minus vitamin

A), but its specific enzyme activity was significantly lower. Similar relationships held for total liver enzyme, enzyme per g. of DNA and per g. of liver.

These results show that the separate effects of protein and vitamin A on liver ATP sulphurylase activity can be clearly distinguished: the increase in enzyme activity when replacing a 20%-protein diet with an 80%-protein diet is dependent on the presence of vitamin A. The results in Table 5 show that the effects of vitamin deficiency are not due to a decrease in protein synthesis in the vitamin-deficient compared with the normal rat since the protein content per liver or per cell is similar on the 80%-casein diet. The liver of the deficient rat weighs less, probably because of dehydration since the amount of protein and the number of cells do not change.

It is interesting to compare the control livers in rats on 20%- and 80%-protein diets: not only is the sulphurylase activity higher with an 80%-protein diet (Table 4), but liver weight, total liver protein and protein per cell are also significantly higher (Table 5).

In view of these results, it is now possible to

Table 4. Liver ATP sulphurylase activity as affected by vitamin A deficiency and dietary casein uptake

Animals were fed on a 20%-casein vitamin A-deficient diet (diet 1) (supplemented or unsupplemented with vitamin A) from weaning (3-4 weeks). They were then either continued on this diet or given an 80%-casein diet (diet 2) for 2 weeks. Animals were killed at the plateau stage of growth regardless of whether eye lesions had occurred, provided that food consumption did not fall below 5 g./day. Enzyme was assayed by assay II. Individual determinations are averages of duplicates. Figures in parentheses show numbers of animals used for determination of the mean values $\pm$ S.D. N.S., Not statistically significant.

	Enzyme activity (mμmoles of <i>p</i> -nitrophenyl sulphate/hr.)				<i>P</i> value	<i>P</i> value between (1) & (3)	<i>P</i> value between (2) & (4)
	20%-Casein diet		80%-Casein diet				
	Deficient (1)	Pair-fed (2)	Deficient (3)	Pair-fed (4)			

Per mg. of supernatant protein	13.1 ± 2.6 (10)	12.9 ± 2.4 (10)	15.7 ± 4.0 (16)	22.2 ± 3.4 (17)	< 0.01	N.S.	< 0.01
Per total liver × 10 ³	16.7 ± 3.7 (7)	17.7 ± 8.6 (7)	27.0 ± 7.6 (10)	46.6 ± 15.0 (12)	< 0.01	< 0.01	< 0.01
Per μg. of DNA	1.13 ± 0.19 (7)	1.18 ± 0.45 (8)	2.09 ± 0.99 (10)	3.03 ± 0.80 (12)	< 0.05	< 0.05	< 0.01
Per mg. of liver	2.85 ± 0.49 (8)	2.98 ± 0.88 (8)	4.47 ± 1.2 (10)	6.02 ± 0.86 (12)	< 0.01	—	< 0.01

Table 5. Analyses of livers of rats on vitamin A-deficient and control diets of different casein content

Livers were those of animals used in experiments described in Table 4. N.S., Not statistically significant.

	20%-Casein diet		80%-Casein diet		P value	P value between (2) and (4)
	Deficient (1)	Pair-fed (2)	Deficient (3)	Pair-fed (4)		
Weight (g.)	5.2 $\pm$ 0.9 (8)	5.2 $\pm$ 1.7 (8)	5.9 $\pm$ 1.5 (12)	7.8 $\pm$ 2.2 (12)	< 0.05	< 0.05
Protein (mg.)	1227 $\pm$ 128 (7)	1317 $\pm$ 376 (7)	1615 $\pm$ 389 (10)	1995 $\pm$ 485 (12)	N.S.	< 0.01
DNA (mg.)	12.6 $\pm$ 4.5 (8)	13.5 $\pm$ 3.5 (8)	13.2 $\pm$ 5.9 (10)	16.3 $\pm$ 5.9 (12)	N.S.	N.S.
Protein/DNA (mg./mg.)	106 $\pm$ 40 (8)	91 $\pm$ 18 (8)	144 $\pm$ 73 (10)	131 $\pm$ 33 (12)	N.S.	< 0.01

discuss previous work on the relation between ATP sulphurylase and vitamin A. No data are available on the food intake of the deficient and normal rats used. However, we assume that those workers who reported a difference between the groups succeeded in keeping up the food intake of their animals to the end of the experiments. The greater the differences reported, the greater one can assume to have been the food intake so that, for instance, Sundaresan (1966), who did not pair-feed, found very large differences. Mukherji & Bachhawat (1967) presumably used careful pair-feeding to such a degree of deficiency that food intake became very low and consequently differences between the groups disappeared.

The results in the earlier part of the present work (Figs. 1 and 2) can also be reconsidered in the light of the above hypothesis: the specific activity of the sulphurylase in the deficient and pair-fed control rats on the 20%-protein diet continues to decrease from the fourth week through the plateau stage to the deficient stage (Fig. 2); we believe that, as the food intake at the plateau stage declines, protein synthesis begins to decline, leading to lower concentrations in relation to other proteins of those enzymes, such as the sulphurylase, which we believe to have a rapid turnover. With normal food intake (*ad libitum*), no decline is seen (Fig. 2). One might then ask: why should total activity of liver sulphurylase increase if protein synthesis declines, when passing from the fourth week to the plateau stage (Fig. 1)? The answer is that the animal is still growing between the fourth week and the plateau stage, so that protein synthesis continues, leading to increased total activity. This increase, of course, would be much greater for an *ad libitum*-fed animal. Though there is more total protein synthesized between the fourth week and the plateau stage in the pair-fed animal, synthesis at the plateau stage has declined sufficiently for specific activity of a rapidly turning-over enzyme to show a decrease. The concentration of protein per g. of liver was found to decline slightly (10%) between the fourth week and the plateau stage, whereas liver weight increased greatly (26%).

One report remains unexplained (Rogers, 1967). Vitamin A-deficient newborn rats survive when given retinoic acid. Groups of such rats with and without a retinoic acid supplement were made to eat equal amounts of food by forced feeding. ATP sulphurylase activity remained at the same level in the unsupplemented as in the supplemented group. However, no indication is given of the assay used.

Our results suggest that vitamin A deficiency significantly lowers the activity of the enzyme

ATP sulphurylase in liver and to a small extent in some other tissues examined. The effect seems to be extreme in the adrenal gland. ATP sulphurylase should be added to the growing list of enzymes whose activities are lowered by vitamin A deficiency [gulonolactone oxidase (Ganguly, 1967), codeine demethylase (Yonemoto & Johnson, 1967), squalene oxidocyclase (Subba Rao & Olson, 1967)]. However, no evidence has been found for a direct coenzymic action of the vitamin on the sulphurylase (A. S. Levi & G. Wolf, unpublished work).

The authors are grateful for support of this work by the National Institutes of Health (Grants AM08732 and ES 00056) and to Mr J. C. Rylaarsdam for excellent technical assistance. This paper is contribution no. 1200 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

REFERENCES

- Brunngraber, E. G. (1958). *J. biol. Chem.* **233**, 472.
 Clark, C. M., Naismith, D. J. & Munro, H. N. (1957). *Biochim. biophys. Acta*, **23**, 587.
 Dziewiatkowski, D. C. (1954). *J. exp. Med.* **100**, 11.
 Fell, H. B. & Mellanby, E. (1952). *J. Physiol.* **116**, 320.
 Ganguly, J. (1967). *J. sci. industr. Res.* **26**, 110.
 Geison, R. L. (1965). Ph.D. Thesis: University of Illinois.
 Glick, B. (1963). *Poult. Sci.* **42**, 1022.
 Hall, M. O. & Straatsma, B. R. (1966). *Biochim. biophys. Acta*, **124**, 246.
 Johnson, B. C. & Wolf, G. (1960). *Vitam. & Horm.* **18**, 457.
 Khairallah, E. A. & Wolf, G. (1965). *J. Nutr.* **87**, 469.
 Levi, A. S. & Wolf, G. (1965). *Fed. Proc.* **24**, 562.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Mukherji, B. & Bachhawat, B. K. (1966). *Indian J. Biochem.* **3**, 4.
 Mukherji, B. & Bachhawat, B. K. (1967). *Biochem. J.* **104**, 318.
 Munro, H. N. & Fleck, A. (1966). *Analyst*, **91**, 78.
 Pasternak, C. A., Humphries, S. K. & Pirie, A. (1963). *Biochem. J.* **86**, 382.
 Pasternak, C. A. & Pirie, A. (1964). *Exp. Eye Res.* **3**, 365.
 Robbins, P. W. & Lipmann, F. (1958). *J. biol. Chem.* **233**, 686.
 Rogers, W. E. (1967). *Fed. Proc.* **26**, 636.
 Spencer, B. & Harada, T. (1960). *Biochem. J.* **77**, 305.
 Subba Rao, K. & Olson, R. E. (1967). *Fed. Proc.* **26**, 635.
 Subba Rao, K., Seshadri Sastry, P. & Ganguly, J. (1963). *Biochem. J.* **87**, 312.
 Sundaresan, P. R. (1966). *Biochim. biophys. Acta*, **113**, 95.
 Varandani, P. T., Wolf, G. & Johnson, B. C. (1960). *Biochem. biophys. Res. Commun.* **3**, 97.
 Wilson, L. G. & Bandurski, R. S. (1958). *J. biol. Chem.* **233**, 975.
 Wolf, G., Lane, M. D. & Johnson, B. C. (1957). *J. biol. Chem.* **225**, 995.
 Yonemoto, T. & Johnson, B. C. (1967). *Fed. Proc.* **26**, 635.