The effects of high dietary supplements of copper sulphate on pantothenic acid metabolism in the chick

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1. The effects of incorporation of copper sulphate supplying 250 mg copper/kg semi-purified diet with graded amounts of calcium pantothenate (CaPa) were studied in chicks.

2. When the doses of CaPa were marginally adequate or less the Cu supplementation induced severe signs of pantothenic acid (PaA) deficiency.

3. Livers of the Cu-treated birds given low doses of PaA had lower concentrations of total and bound PaA than those of the corresponding control birds. The bound:total PaA value was also reduced.

4. The amount and concentration of coenzyme A (CoA) were significantly less in the livers of Cu-treated chicks. Fatty acid synthetase activity was not reduced.

5. It is suggested that high dietary supplements of $CuSO_4$ induce PaA deficiency through interference in the biosynthesis of CoA.

Although copper sulphate is widely used as a dietary supplement to improve the growth of pigs, its efficiency as a growth-promoter for poultry is doubtful. Whereas some workers have claimed small improvements in chick growth resulting from supplements providing up to 300 mg copper/kg diet others have reported no effect or even a growth depression (see review by Smith, 1969). In earlier studies briefly reported by Lloyd *et al.* (1971) signs of pantothenic acid (PaA) deficiency were observed in chicks given a practical diet supplemented with 250 mg Cu in the form of $CuSO_4 . 5H_2O$ and 39 g maize oil/kg. When the maize oil was omitted the deleterious effects of the Cu supplement were much less severe. In chicks given a semi-purified diet containing marginally-sufficient PaA the Cu supplement induced severe signs of deficiency of the vitamin and caused a reduction in bound:total PaA in the liver. This suggested possible interference in the metabolic pathways of conversion of PaA to its biologically-active forms, coenzyme A (CoA) and the fatty acid synthetase complex (FAS). The experiments reported here were done to investigate some of the metabolic consequences of high dietary Cu supplementation which might lead to external manifestation of PaA deficiency in the chick.

EXPERIMENTAL

Chicks and diets

The chicks were the progeny of Rhode Island Red cocks and Light Sussex hens maintained in the Institute's specified pathogen-free breeding unit. They were housed from 1 d of age in tiered brooders with wire-floored compartments. The initial temperature in the brooding area was 36° and was gradually reduced to room temperature, approximately 21°, during the following 3 weeks. Food and water were continuously available and artificial light was provided for 14 h/d.

The semi-purified basal diet had the following composition (g/kg): maize starch 602·5, casein (low vitamin, Calbiochem Ltd, Bishops Stortford) 180·0, gelatin 100·0, L-cystine 3·0, choline chloride 1·5, *myo*-inositol 1·0, maize oil 50·0, salt mixture 60·0, vitamin mixture in glucose 2·0. The salt mixture provided (/kg diet) $CaCO_3$ 17·1 g, $CaHPO_4 . 2H_2O$ 17·1 g,

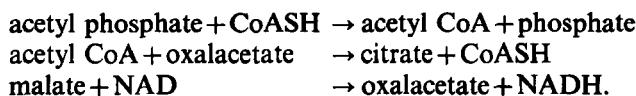KH_2PO_4 13·3 g, NaCl 8·67 g, $MgSO_4 . H_2O$ 2·67 g, $FeSO_4 . 7H_2O$ 670 mg, $MnSO_4 . 4H_2O$ 270 mg, $ZnSO_4 . 7H_2O$ 130 mg, KI 37 mg, $CuSO_4 . 5H_2O$ 16 mg. The vitamin mixture provided (/kg diet) pteroylmonoglutamic acid 1·5 mg, thiamin hydrochloride 3·0 mg, pyridoxin hydrochloride 4·0 mg, riboflavin 6·0 mg, nicotinic acid 40·0 mg, biotin 200 μg, cyanocobalmin 20 μg, Rovimix A-500 (Roche Products, Dunstable, Beds; providing 5·1 mg retinol) 34 mg, menaphthone 5·0 mg, DL-α-tocopheryl acetate 10·0 mg, cholecalciferol 40 μg, all dissolved in the maize oil. Where appropriate calcium pantothenate (CaPa) was included at doses between 0 and 20·25 mg/kg diet and in Cu-supplemented diets finely powdered $CuSO_4 . 5H_2O$ was incorporated at the rate of 1 g/kg, equivalent to 250 mg Cu/kg. After mixing, the diets were moistened with water, granulated by passing through a sieve of 5 mm mesh, then dried on stainless steel trays in a current of air at 42°.

Analytical procedures

Preparation and storage of liver samples. Chicks were killed by cervical dislocation and the livers were immediately removed and plunged into liquid nitrogen, then homogenized on ice for 3 min. The homogenized samples were divided among several vials, frozen and kept at −20° until taken for analysis.

Microbiological assays. PaA activity was measured microbiologically using *Lactobacillus plantarum* strain NCDO 82, ATAA 8014 by an assay procedure similar to that described by Bird (1963). Free PaA was determined in extracts of liver prepared by heating homogenized samples with 0·14 M-sodium phosphate buffer at pH 6·6 for 10 min at 115°, cooling and adjusting to pH 4·6, filtering and adjusting to pH 6·8. Total (free plus bound) PaA was determined after treatment of the liver homogenate with alkaline phosphatase and chick liver enzyme (Barton Wright, 1963).

Determination of CoA. Total CoA was measured by a kinetic enzymic assay based on that described by Allred & Guy (1969). It makes use of the fact that, when all other components of the reaction mixture are in excess, CoA is the rate-limiting substance in the following coupled enzyme system:

$$acetyl\ phosphate + CoASH \rightarrow acetyl\ CoA + phosphate$$
$$acetyl\ CoA + oxalacetate \rightarrow citrate + CoASH$$
$$malate + NAD \rightarrow oxalacetate + NADH.$$

Measurements were made on deproteinized liver samples prepared by homogenizing in perchloric acid, centrifuging at 4° for 5 min at 1500 *g*, decanting the supernatant fraction and adjusting to pH 3·5. After standing on ice for 1 h the samples were again centrifuged as described previously and the supernatant fraction collected. Before assay the deproteinized extracts and the standard CoA preparation were treated with dithiothreitol to ensure that the CoA was in the reduced form. The rate of formation of NADH was measured spectrophotometrically as an increase of absorbance at 340 nm. Test samples were compared with a standard preparation of CoASH (ChromatoPure, free acid, International Enzymes Ltd, Windsor, Berks).

Measurement of FAS complex activity. The assays were done on freshly-prepared supernatant fractions obtained by centrifuging the liver homogenates at 200000 *g* for 30 min at 4°. The method was based on that of Martin *et al.* (1961), in which the FAS in the test solution catalyses the incorporation of acetyl CoA into fatty acids, resulting in a decrease in absorbance. Since no standard sample of FAS complex was available the results were expressed in units suggested by Martin *et al.* (1961), i.e. the amount of enzyme required to give a decrease in absorbance of 0·100 optical density at 340 nm in 3 min was defined as 1 unit of activity.

Experimental design. Four experiments were done with chicks given graded amounts of

CaPa at and below the estimated dietary requirement, with or without a supplement of $CuSO_4.5H_2O$ providing 250 mg Cu/kg diet. The doses of CaPa were chosen in geometric progression in the proportion 3:2. In Expt 1 the doses were 6·0, 13·5 and 20·25 mg/kg diet, but as all three appeared to satisfy the chick's requirement for the vitamin the doses were reduced in the other three experiments to 2·67, 4·0 and 6·0 mg/kg diet. Microbiological assays made on several of the diets showed that there was no loss of PaA during the course of an experiment. Duplicate groups of ten birds were allotted to each treatment, usually five males and five females, at 1 d of age. They were weighed at weekly intervals and examined daily for signs of PaA deficiency, namely, lesions at the corners of the mouth, encrusted eyelids, sores on the upper surface of the feet and neurological disorders manifested by a staggering gait. Experiments were terminated during the fourth week, before the effects of the deficiency were so severe as to cause many deaths among birds on the lowest levels of PaA supplementation, and the livers were analysed for PaA or its derivatives.

The dietary supplements of CaPa, the analyses performed on the livers and the duration of the experiments were as follows:

Expt 1   6·0, 13·5 and 20·25 mg/kg; free and total PaA at 24 d.
Expt 2   2·67, 4·0 and 6·0 mg/kg; free and total PaA and CoA at 25 d.
Expt 3   2·67, 4·0 and 6·0 mg/kg; CoA at 21 d.
Expt 4   2·67, 4·0 and 6·0 mg/kg; FAS complex at 21 d.

Statistical treatment of results. In Expts 1, 3 and 4 the livers from all birds of the same sex within a cage were combined before analysis. In Expt 2 individual livers were analysed and the results averaged to give two values for each cage, one for each sex. Body-weights were similarly averaged to give two values for each cage. The values were subjected to analysis of variance, the variation being divided into between-cage and within-cage variation. The between-cage value was further subdivided into variation between dietary treatments and the pooled residual variation between cages, which was used to test the significance of the diet effects. The same residual value was used to calculate the standard errors of differences between diet means. The variation within cages was divided between sex, sex × dietary treatment interaction and the pooled residual variation within cages. This last value was used to test the significance of sex and sex × diet effect.

RESULTS
Growth rates and signs of deficiency
The effects of the different treatments on body-weights, incidence of signs of deficiency of PaA and mortality are summarized in Table 1. In all experiments at all levels of CaPa Cu supplementation resulted in lower body-weights than those of the unsupplemented chicks. At the higher doses of the vitamin chosen in Expt 1 the depression of growth was small, but in the other three experiments when the dietary supply of CaPa was near to or below the marginal requirement the reduction in growth of the Cu-fed chicks was highly significant ($P < 0.001$). In all experiments the clinical signs of deficiency appeared earlier and affected a larger number of chicks in the Cu-supplemented groups when the diet contained 6 mg CaPa or less/kg. Mortality was also higher in chicks given submarginal amounts of the vitamin, and the deaths occurred at an earlier age.

Concentration of free and bound PaA in the livers
As a consequence of the lower body-weight the livers of Cu-treated birds were smaller than those of the corresponding controls, the effect being more apparent at the lower doses of CaPa (Table 2). In Expt 1, in which high doses of the vitamin were used, whether Cu was supplemented or not, there was little difference in the concentration of total or bound

Table 1. *Mean body-weights (g), number of birds with signs of pantothenic acid deficiency and mortality in groups of chicks reared on different doses of dietary calcium pantothenate (CaPa) with or without a supplement of 250 mg copper as $CuSO_4.5H_2O/kg$ diet*

(Means of two × ten chicks)

Duration (d)		Cu in diet	Dose of CaPa (mg/kg diet)					SED	df	Statistical Significance of effects on body-wt
			2·67	4·0	6·0	13·5	20·25			
				Expt no. 1						
24	Body-wt	0	—	—	372	355	368 ⎫	11·12		⎧ Cu***
		+	—	—	248	336	335 ⎭		6 ⎨ CaPa**
										⎩ Cu × CaPa**
	Deficiency	0	—	—	0	0	0			
		+	—	—	19	0	1			
	Mortality	0	—	—	0	0	0			
		+	—	—	0	0	0			
				Expt no. 2						
25	Body-wt	0	192	248	283 ⎫	—	—	12·60		⎧ Cu***
		+	104	144	220 ⎭				6 ⎨ CaPa**
										⎩ Cu × CaPa**
	Deficiency	0	11	1	0	—	—			
		+	19	10	7	—	—			
	Mortality	0	5	1	0	—	—			
		+	2	3	0	—	—			
				Expt no. 3						
21	Body-wt	0	184	211	231 ⎫	—	—	7·27		⎧ Cu***
		+	77	80	98 ⎭				6 ⎨ CaPa**
										⎩ Cu × CaPa NS
	Deficiency	0	19	15	6	—	—			
		+	19	20	20	—	—			
	Mortality	0	0	0	0	—	—			
		+	6	2	1	—	—			
				Expt no. 4						
21	Body-wt	0	164†	201	212 ⎫	—	—	10·52		⎧ Cu***
		+	62†	77	106 ⎭				4 ⎨ CaPa**
										⎩ Cu × CaPa NS
	Deficiency	0	9†	17	11	—	—			
		+	9†	19	19	—	—			
	Mortality	0	0†	1	0	—	—			
		+	5†	2	1	—	—			

NS, not significant.
SED, standard error of differences of means.
CaPa, calcium pantothenate.
** $P < 0.01$, *** $P < 0.001$.
† Group of ten chicks discarded due to an accidental break in the water supply.

Table 2. *Concentration (nm/g) of total, bound and bound: total pantothenic acid (PaA) in livers of chicks reared on different doses of dietary calcium pantothenate (CaPa) with or without a supplement of 250 mg copper as CuSO₄.5H₂O/kg diet*

$$\text{Concentration (nm/g) of total, bound and bound: total pantothenic acid (PaA) in livers of chicks reared on different doses of dietary calcium pantothenate (CaPa) with or without a supplement of 250 mg copper as } CuSO_4.5H_2O/kg \text{ diet}$$

(Means of two × ten chicks)

Duration (d)	Dose of CaPa (mg/kg diet)	Cu	Mean wt of liver (g)	SED	df	Total	SED	df	Bound	SED	df	$\frac{\text{Bound}}{\text{Total}} \times 100$	SED	df
						Expt no. 1								
24	6·0	0	9·72			356·5			112·7			31·5		
		+	7·19			293·3			93·5			31·8		
	13·5	0	9·58			352·6			99·9			28·3		
		+	8·91			335·3			92·6			27·4		
	20·25	0	9·72			379·1			120·4			31·9		
		+	8·50	0·75	6	360·4	13·7	6	119·5	15·1	6	33·2	3·97	6
						Statistical significance of effects								
	Cu		•			**			NS			NS		
	CaPa		NS			•			NS			NS		
	Cu × CaPa		NS			NS			NS			NS		
						Expt no. 2								
25	2·67	0	7·49			236·3			41·9			17·9		
		+	3·13			207·2			34·2			13·6		
	4·0	0	8·24			274·4			95·9			34·9		
		+	4·82			202·6			26·6			11·8		
	6·0	0	8·43			297·3			129·5			45·4		
		+	7·28	0·43	6	262·6	13·7	6	62·3	13·5	6	22·7	5·54	6
						Statistical significance of effects								
	Cu		***			**			***			**		
	CaPa		***			**			**			•		
	Cu × CaPa		**			NS			•			NS		

NS, not significant.
CaPa, calcium pantothenate.
PaA, pantothenic acid.
SED, standard error of differences of means.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

PaA in the livers, except at the lowest supplement (6·0 mg/kg diet) of CaPa, when the concentration of both forms of the vitamin was somewhat reduced. In Expt 2, in which the supplements of CaPa were lower, total and bound PaA were reduced by the Cu treatment at all levels of the vitamin supplementation and bound: total PaA was significantly ($P < 0.01$) less in the Cu-treated groups. On this occasion the diet with 6·0 mg CaPa/kg supported poorer growth and the effect of Cu supplement on the concentration of bound PaA was more severe than had been observed in Expt 1.

Effect of Cu supplement on CoA concentration in the livers

The concentrations of CoA in the livers of chicks with and without the supplement of $CuSO_4.5H_2O$ are shown in Table 3. The concentration was not increased with increasing doses of dietary CaPa but was significantly reduced in both experiments by the supplement of $CuSO_4.5H_2O$.

Table 3. *Concentration of coenzyme A (CoA) in the livers of chicks reared on different doses of dietary calcium pantothenate (CaPa) with and without a supplement of 250 mg copper as $CuSO_4 . 5H_2O/kg$ diet*

(Means of two × ten chicks)

Duration (d)	Dose of CaPa (mg/kg diet)	Cu	CoA (nm/g liver)	SED	df	Statistical significance of effects	
			Expt no. 2				
25	2·67	0	71·0	13·8	6	Cu	*
		+	45·0			CaPa	NS
						Cu × CaPa	NS
	4·0	0	77·6				
		+	49·5				
	6·0	0	87·0				
		+	53·6				
			Expt no. 3				
21	2·67	0	68·7	9·3	6	Cu	***
		+	14·9			CaPa	NS
						Cu × CaPa	NS
	4·0	0	75·9				
		+	46·3				
	6·0	0	65·9				
		+	42·6				

NS, not significant.
SED, standard error of differences of means.
CaPa, calcium pantothenate.
CoA, coenzyme A.
* $P < 0.05$, *** $P < 0.001$.

Effect of Cu supplement on FAS activity

The FAS activity (Table 4) was not affected by the Cu treatment except at the lowest dose of dietary PaA (2·67 mg/kg) where there was a small significant increase ($P < 0.05$). No significant effect of increasing the dietary supplement of CaPa was observed.

DISCUSSION

There was considerable variation between experiments in the growth of chicks given similar supplements of CaPa. In particular, body-weights of chicks given 6·0 mg CaPa/kg diet were much greater in Expt 1 than in the later experiments. This presumably reflects differences in age and state of lay of the parent flock, which would be expected to influence the performance of the chicks and possibly the reserves of PaA carried over in their eggs. However, in none of the experiments was increased growth observed in any of the groups given the Cu supplement. On the contrary, on almost every occasion the body-weights achieved by the supplemented birds were significantly less than those of their controls, except

Table 4. *Fatty-acid synthetase activity (/g liver) of chicks reared to 21 d on different doses of dietary calcium pantothenate (CaPa) with or without a supplement of 250 mg copper as $CuSO_4 . 5H_2O/kg$ diet*

(Means from pooled samples of livers)

Dose of CaPa (mg/kg diet)	Cu	Fatty acid synthetase activity (units[†]/g liver)
2·67	0	22·6[‡]
	+	38·5*[‡]
4·0	0	18·4
	+	20·9
6·0	0	19·8
	+	21·1
Standard error of differences of means		3·46
df		4

* Significantly different from value for unsupplemented diet ($P < 0.05$).

† One unit of activity is the amount of enzyme required to give a decrease in absorbance at 340 nm of 0·100 in 3 min.

‡ Two missing values.

at the highest dose of dietary CaPa (20·25 mg/kg), when growth was approximately the same on the supplemented and unsupplemented diets.

The results of all four experiments confirmed the earlier findings of Lloyd *et al.* (1971) that a dietary supplement of 250 mg Cu as $CuSO_4 . 5H_2O$ consistently evoked signs of PaA deficiency in chicks. On diets containing marginally adequate amounts or less of CaPa, inclusion of the Cu salt induced a higher mortality and increased occurrence of clinical signs of deficiency, but the detrimental effects were not pronounced on diets containing 13·5 mg or more of CaPa/kg.

In the livers of the Cu treated birds the contents of total PaA (free plus bound) were less than in those of the corresponding control birds. This was partly due to the smaller size of the livers, but there was also a reduced concentration of PaA, particularly in Expt 2 on diets containing the Cu supplement and the lower doses of CaPa. The content of bound PaA was reduced even more than that of the total PaA, so that bound:total PaA value was significantly decreased. This effect was not apparent in Expt 1, in which higher dietary amounts of CaPa were used.

Although the signs of deficiency increased with decreasing amounts of dietary CaPa, and were more severe in the Cu-treated chicks, the pattern of clinical deficiency was not closely correlated with the concentrations of CoA or of PaA in the livers. This is not surprising since it is questionable whether liver content is a good indicator of vitamin status. It was observed in earlier work (Coates *et al.* 1968) that birds showing acute signs of deficiency of vitamins of the B complex, including PaA, nevertheless had appreciable liver reserves of the deficient vitamin. In rats CoA concentration in the liver was not significantly influenced by increasing dietary amounts of CaPa, nor was bound PaA concentration increased by supplements of PaA well above the dietary requirement (Nakamura, 1969; Nakamura *et al.* 1969). The bound forms of PaA that may occur in liver include CoA, the

15-2

FAS complex and intermediates in their biosynthesis. In the two experiments in which CoA was measured, a considerable reduction both in total amount and in concentration was observed in the livers of Cu-treated chicks. On some occasions concentration of bound PaA was apparently less than that of CoA measured on the same samples (Expt 2). These discrepancies could have been due to imprecision of measurement, since the methods of assay for both substances are subject to large errors and only small amounts of sample were available. FAS was not reduced, and even showed a small significant increase in the group receiving the lowest dose of dietary CaPa, which cannot be readily explained. Since several birds in this group had already died from deficiency exacerbated by the Cu-treatment the result was obtained on only a few survivors and may thus have been spuriously high.

In micro-organisms the biosynthesis of CoA and the acyl carrier protein (ACP), an essential component of the FAS complex, follows a common pathway from PaA via 4′-phosphopantothenyl cysteine to 4′-phosphopantetheine. The subsequent formation of dephospho-CoA and its phosphorylation to CoA have been reasonably well established but the metabolic sequence from 4′-phosphopantetheine to ACP and FAS has not yet been elucidated. In animal tissues the biosynthesis of CoA follows a similar pathway and it is known that 4′-phosphopantetheine is an essential component of FAS. From the greatly reduced concentration of CoA, and the lack of inhibition of FAS activity, it might be inferred that Cu supplementation interferes on the metabolic pathway between 4′-phosphopantetheine and CoA. However, in the light of findings with micro-organisms and in rats the inference is open to question. For example, Das & Toennies (1969) showed that *Streptococcus faecalis* produced both CoA and ACP when grown with adequate PaA, but when the PaA content of the medium was restrictive the CoA disappeared. Other workers observed that rats on the verge of death from PaA deficiency nevertheless had normal liver levels of FAS activity (Larrabee *et al.* 1965). In both these systems it appears that FAS activity was maintained at the expense of other PaA-containing compounds. If the same is true for the chick, limitation in supply of any of the intermediates on the common pathway could explain the observed results. One possible effect of the Cu treatment might be interference in the formation of 4′-phosphopantothenyl cysteine. Studies in vitro by Hanaki & Kamide (1975) showed that autoxidation of cysteine was catalysed by cupric ions under aerobic and anaerobic conditions. Robbins & Baker (1980) suggested that although cystine is completely utilized by the normal bird, in the Cu-supplemented chick formation of cysteine–copper or glutathione–copper complexes or both would necessarily limit available cysteine. If the processes suggested by these authors are proved to be correct, the reaction of cysteine with phosphopantothenic acid, during CoA formation, could be adversely affected through the limited availability of cysteine. The findings of Nakamura *et al.* (1967) that hepatic CoA was greatly reduced in methionine deficiency in the rat and of Verma & Motzok (1979) who reported enhanced CoA synthesis in chicks by increasing DL-methionine or SO_4 as $CaSO_4 . 2H_2O$ in the diet lend further support to the suggested possibility of interference of Cu in the formation of CoA.

The poorer growth of the Cu-supplemented chicks was inevitably accompanied by a reduced intake of food and thus of CaPa. This is unlikely to have been the cause of the increased incidence of deficiency as the requirement for vitamins is generally considered to be related to the amount of food eaten (Agricultural Research Council, 1975). No significant loss of CaPa from the diet occurred during the course of the experiment, but the possibility remains that the presence of Cu caused some destruction of PaA in the gut.

From earlier reports it appears that the deleterious effects of high dietary supplements of $CuSO_4$ are influenced by other components of the diet. Coates & Harrison (1959) observed little or no growth depression when a diet low in animal protein was supplemented with 1 g $CuSO_4 . 5H_2O$/kg but significantly poorer growth with the same supplement in a diet

of normal animal protein content. Lloyd *et al.* (1971) reported that the presence of maize oil in the diet considerably exacerbated the harmful effects of CuSO$_4$ and, more recently, Jensen & Maurice (1979) demonstrated the protective effect of sulphur amino acids against Cu toxicity in chicks. These possible influences are being separately investigated. None of them could be examined in the experiments now reported since the diet contained ample casein and cystine, with maize oil as the source of fat. In these circumstances it is clear that the harmful effects of including high levels of CuSO$_4$ in a chick diet are due to induction of a deficiency of PaA, probably because the synthesis of CoA from PaA is inhibited.

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