The influence of microorganisms and of stress on the chick's requirement for pantothenic acid

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1. The responses of germ-free (GF) and conventional (CV) chicks to suboptimal amounts of dietary calcium pantothenate (CaPa) were compared. At the lower levels of supplementation the signs of deficiency were more severe in the CV chicks.

2. Calculations based on mean body-weights of chicks given graded dietary supplements of calcium pantothenate (CaPa) in both environments showed that, to achieve the same body-weight and freedom from signs of deficiency, the GF birds required only approximately two-thirds the amount of CaPa as was needed by corresponding CV birds.

3. Increasing the dietary supplement of CaPa resulted in increased contents of PaA in the livers. GF birds had more PaA per liver than their CV counterparts. In both instances the effect was due to the larger liver size. Concentration of PaA in the liver was not affected by dietary supplement nor by the environment.

4. CV birds were deemed to be under greater stress than GF birds because their adrenal glands were heavier and contained less cholesterol.

5. CV chicks deliberately stressed by injections of adrenocorticotrophic hormone had heavier adrenals with lower cholesterol contents than unstressed controls, but the condition was not altered by increasing the dietary supply of CaPa.

In studies with germ-free (GF) and conventional (CV) chicks Coates *et al.* (1968) showed that substantial amounts of most vitamins of the B complex were synthesized by microbial action in the lower gut. Nevertheless the effects of deficiency were as severe in the CV as in the GF birds, implying that the synthesised vitamins were not absorbed. On a diet deficient in pantothenic acid (PaA) mortality was higher in the CV environment, and the amount of PaA in the livers was significantly less, which led the authors to suggest that the requirement for PaA was increased in the presence of the gut microflora. Since coenzyme A (CoA) has a role in adrenocortical function, the activity of which is increased by stress, they postulated that the apparently greater demand for PaA might be the result of stress imposed by the microbial burden. The experiments now reported were done to verify the higher requirement for PaA by CV birds and to investigate the hypothesis that it was brought about by stress.

MATERIALS AND METHODS

Chicks

Chicks were the progeny of the Institute's specified-pathogen-free flock of Rhode Island Red cocks × Light Sussex hens. For the experiments with GF birds clean eggs were incubated in the usual way for 18 d, then disinfected externally with peracetic acid vapour (Harrison, 1969) and taken into sterile Gustafsson stainless steel isolators where they were left to hatch. Their corresponding CV controls were taken from the same batch of eggs but after the disinfection process were returned to the incubator to hatch. The chicks were housed in groups of four in cages with wire-mesh floors, either inside the isolators or in a room in which temperature, lighting and humidity could be matched to the conditions in the isolators. In the last experiment, using CV chicks only, groups of ten birds were maintained in electrically-heated tiered brooders in a standard brooder room. In all

experiments the birds were distributed so that initial body weights and sexes were as evenly disposed as possible among the experimental treatments. Sterility checks were performed on the GF birds soon after hatching and at the end of the experiments.

Diets

The basal diet of purified ingredients had the following composition (g/kg): maize starch 596·5, 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 8·0. The salt mixture provided (/kg diet): $CaCO_3$ 17·1 g, $CaHPO_4.2H_2O$ 17·1 g, KH_2PO_4 13·3g, 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. Vitamin supplementation was higher than normal to allow for possible losses during sterilization of the diet. The vitamin mixture provided (/kg diet): pteroylmonoglutamic acid 6·0 mg, thiamin hydrochloride 12·0 mg, pyridoxin hydrochloride 16·0 mg, riboflavin 24·0 mg, nicotinic acid 160·0 mg, biotin 0·8 mg, cyanocobalamin 80 μg, retinol 20·4 mg (as Rovimix A-500, Roche Products, Dunstable, Beds), menaphthone 20 mg, DL-tocopheryl acetate 40 mg and cholecalciferol 0·16 mg. The last three items were dissolved in the maize oil. Where necessary calcium pantothenate (CaPa) was included at doses between 0 and 6·25 mg/kg diet. After mixing the diets were granulated by moistening with water, passing through a 5 mm mesh sieve then drying on stainless-steel trays in a current of warm air at 42°. For Expts 1–4 the diets were sterilized by gamma-radiation at 5 Mrad, and the sterilized diets were given both to the GF chicks and their CV controls.

Analytical methods

Preparation and storage of samples. At the end of the experiments the chicks were killed by cervical dislocation and the required tissues immediately removed, blotted and weighed. Adrenals were plunged into liquid nitrogen and stored at $-20°$ until analysed. Livers were also rapidly cooled in liquid N_2 then cut into smaller pieces and maintained ice-cold while being homogenized for 3 min. The homogenized samples were divided between a number of vials and kept at $-20°$ until analysed.

Microbiological assays. PaA activity was measured by a microbiological assay using *Lactobacillus plantarum* strain NCDC 82, ATAA 8014 (Bird, 1963). Homogenized liver samples were extracted with 0·14 M-sodium phosphate buffer at pH 6·6 for 10 min at 115°, cooled and adjusted to pH 4·6 then filtered and readjusted to pH 6·8. Free PaA was determined in the extracts. Total (free plus bound) PaA was determined after treatment of the homogenate with alkaline phosphatase and chick liver enzyme as described by Barton-Wright (1963).

Determination of cholesterol. Cholesterol was determined in homogenates of adrenals by the method of Zlatkis *et al.* (1953).

Experimental design. Four experiments were done to compare the response of chicks in a GF or CV environment to suboptimal graded dietary supplements of CaPa. The experimental diets were fed from the first day of age. The birds were weighed at weekly intervals and examined daily for signs of deficiency, namely, lesions at the corners of the mouth and on the feet, incoordinated movements and death. They were killed during the fourth week of life, when the signs of deficiency among those on the lower supplements were severe but while most of the birds were still surviving the treatment. In Expt 3 the livers were analysed for bound and total PaA. In Expt 4 cholesterol was measured in the adrenal glands. The dietary supplements of CaPa, the duration of the experiment and the number of birds per treatment (in parentheses) were as follows:

Expt 1. 0, 2·67, 4·0 and 6·0 mg/kg 25 d (4),
Expt 2. 0, 2·67, 4·0 and 6·0 mg/kg 25 d (4),

Expt 3. 3·02, 3·62, 4·34, 5·21 and 6·25 mg/kg 21 d (16),

Expt 4. 2·67 and 4·0 mg/kg 25 d (24).

Expt 5 was done with CV birds only, to investigate a possible relationship between stress and the chick's requirement for PaA. Four groups of ten chicks received the basal diet supplemented with either 2·67 or 4·0 mg CaPa/kg. Half the birds on each dietary treatment were 'stressed' by intramuscular injection three times weekly of a long-acting preparation of adrenocorticotrophic hormone (ACTH; Corticotrophin gelatin injection, B.P., Armour Pharmaceutical Co. Ltd, Eastbourne) at the rate of 120 i.u./kg body-weight. The remaining half of the chicks were injected at the same time with similar volumes of physiological saline (9 g sodium chloride/l). The experiment lasted for 24 d, when the birds were killed and cholesterol determined in the adrenals.

Statistical treatment of results. In Expts 1 and 2 analysis of variance was performed on body-weights of individual chicks and in Expt 3 on mean body-weights of cages of four birds. In Expt 3 the livers from all birds of the same sex within a cage were combined before further treatment. In Expts 4 and 5 pairs of adrenal glands from individual chicks were analysed and the results averaged to give two values for each cage, one for each sex. Similarly the body-weights, liver weights and microbiological assay results were averaged to give two values per cage. These average values were subjected to analysis of variance, the variation being divided into that between and within cages. The variation between cages was further divided into variation between environment (GF and CV), treatments (dietary supplements of CaPa) and the pooled residual variation between cages. This residual variation was used to test the significance of treatment effects and to calculate the standard errors of differences between treatment means which are given in the figures and tables. The variation within cages was divided into variation due to sex, sex × dietary treatment interaction and the pooled residual variation within cages. This second residual variation was used to test the significance of sex × diet effects. In the event, a few significant effects of sex were detected but as they were small in relation to the effects of the experimental treatments, and the sexes were evenly distributed, they have not been recorded here.

The requirement for PaA of chicks in the GF and CV environment was assessed from their responses to the graded dietary supplements of CaPa. Dose-response curves were constructed by plotting the mean body-weights *v.* logarithm of the dose of CaPa. If the requirement in both environments was the same the curves would be expected to coincide, but since they did not a quantitative estimate of the difference between them was made by applying the method of biological assay of PaA described by Coates *et al.* (1950). The curve constructed from the values from GF chicks was regarded as the standard and that from the mean weights of the CV chicks as a test curve. They were checked for linearity and parallelism, the common slope was calculated and the relative potency of the diet for CV chicks was estimated as the difference between the logarithmic doses of CaPa that produced a similar response in the two environments. Limits of error were calculated as recommended by the British Standards Institution for the biological assay of vitamin D_3 (British Standards Institution, 1940).

RESULTS

Sterility checks

In both Expts 1 and 2 an isolator became contaminated so that only one group of four chicks on each experimental treatment completed the test. In Expts 3 and 4 no contaminations were detected throughout the whole experimental period.

Incidence of deficiency

The mean body-weights, mortality and numbers of birds with signs of PaA deficiency are shown in Table 1. Body-weights were higher in the GF environment except at the higher

Table 1. *Mean body-weights, relative numbers of birds showing signs of deficiency and mortality in groups of germ-free (GF) and conventional (CV) chicks given suboptimal amounts of dietary calcium pantothenate (CaPa)*

(Duration of tests was 25, 25 and 21 d for Expts 1, 2 and 3 respectively)

Expt no.	No. of birds per treatment	Dose of CaPa (mg/kg)	Body-wt (g)		No. of birds showing signs of deficiency		No. of dead birds	
			GF	CV	GF	CV	GF	CV
1	4	0	108	90	0/3	2/2	1/4	2/4
2	4	0	112	95	0/3	2/2	1/4	2/4
1	4	2·67	204	157	0/3	3/3	1/4	1/4
2	4	2·67	208	182	0/3	3/4	1/4	0/4
1	4	4·0	214	160	0/4	0/3	0/4	1/4
2	4	4·0	258	265	0/3	0/4	1/4	0/4
1	4	6·0	294	302	0/4	0/4	0/4	0/4
2	4	6·0	314	340	0/4	0/4	0/4	0/4
3	16	3·02	177	137	3/15	9/14	0/15	1/15
3	16	3·62	207	146	5/16	9/16	0/16	0/16
3	16	4·34	224	154	0/16	12/16	0/16	0/16
3	16	5·21	281	202	0/16	3/16	0/16	0/16
3	16	6·25	281	233	0/15	0/16	0/15	0/16

		Standard error of differences of means (df)						
1		15·0 (10)						
2		12·8 (11)						
3		11·9 (30)						

levels of CaPa supplementation in Expts 1 and 2. On diets containing low doses of CaPa the incidence of deficiency signs was much greater among the CV birds, and the mortality generally higher. The differences were less apparent at levels of supplementation of 4·0 or more mg CaPa/kg diet.

Liver content of PaA

The results from Expt 3 are recorded in Table 2. The total PaA content of whole livers was significantly higher ($P < 0·001$) in the GF chicks and so was that of bound PaA ($P < 0·05$). This effect was due to the larger livers of the GF chicks, which had grown more than their corresponding CV controls. When liver weight was calculated in terms of metabolic body-weight ($BW^{0·75}$) the difference between the two environments disappeared. Similarly, when the contents of total and bound PaA were calculated per g liver there were no differences between the values for GF or CV chicks.

Response to graded doses of CaPa

In Fig. 1 (Expt 3) the mean body-weights of groups of chicks in the two environments are plotted *v.* dose of dietary CaPa on a logarithmic scale. The response of the GF chicks was consistently higher than that of their CV counterparts, and the value for the requirement for PaA of GF: requirement of CV chicks was 62:100. This was deduced from the calculation that the response in CV chicks produced by, for example, 4·34 mg CaPa/kg diet

Table 2. *Expt 3. Liver weights and contents of total and bound pantothenic acid (PaA) in livers of germ-free (GF) and conventional (CV) chicks given suboptimal amounts of dietary calcium pantothenate (CaPa) for the first 21 d of life*

(Mean values for sixteen birds/treatment)

Dose of CaPa (mg/kg diet)	Environ-ment	Liver wt		PaA in whole liver (nm)		PaA concentration in liver (nm/g)	
		g	g/kg BW$^{0.75}$	Total	Bound	Total	Bound
3·02	GF	5·27	0·109	1569	564	300	107
	CV	4·73	0·118	1241	469	264	99
3·65	GF	6·07	0·111	1870	608	314	102
	CV	4·81	0·115	1246	368	260	79
4·34	GF	6·64	0·115	1921	780	291	118
	CV	4·96	0·113	1540	471	311	95
5·21	GF	8·37	0·122	2177	753	265	91
	CV	6·79	0·127	1984	679	293	100
6·25	GF	8·79	0·129	2499	903	288	105
	CV	7·15	0·120	2415	903	339	127
Significance of effects:							
Environment		**	NS	***	*	NS	NS
CaPa		***	*	***	***	NS	NS
CaPa × environment		NS	NS	NS	NS	*	NS
Standard error of differences of means (df 30)		0·55	0·007	86	114	21	20

BW, body-weight; NS, not significant.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3. *Expt 4. Weights and cholesterol contents of pairs of adrenal glands from germ-free (GF) and conventional (CV) chicks given suboptimal amounts of dietary calcium pantothenate (CaPa) for the first 25 d of life*

(Mean values for two groups of twelve birds/treatment)

Dose of CaPa (mg/kg diet)	Environment	Body-wt (g)	Wt of pairs of adrenal glands		Total cholesterol content of adrenals (mg)
			mg	mg/kg BW$^{0.75}$	
2·67	GF	167	35·3	763	0·397
	CV	132	30·8	798	0·330
4·0	GF	240	36·5	598	0·612
	CV	170	33·4	713	0·480
Significance of effects:					
Environment		***	**	*	**
CaPa		***	NS	***	***
CaPa × environment		**	NS	NS	NS
Standard error of differences of means (df 20)		7	1·8	42	0·39

BW, body-weight; NS, not significant.
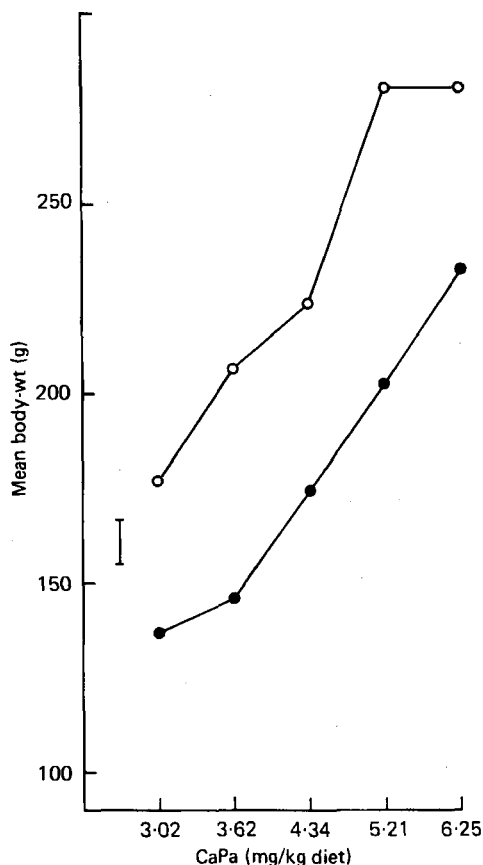* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 1. Mean body-weights of germ-free (GF; O—O) and conventional (CV; ●—●) chicks reared to 21 d on different dietary supplements of calcium pantothenate (CaPa). Points represent mean values for sixteen chicks and the vertical bar represents the pooled standard error of differences of means.

was attained with only 2·69 mg CaPa/kg, with limits of error of 2·28–3·17 mg CaPa/kg, in GF chicks. The results of Expt 4 lend support to these values. On that occasion the PaA requirement of GF:requirement of CV chicks was 68:100, but as the response curves were each based on two points only, and did not pass the test for parallelism, no limits of error could be calculated.

Effects on the adrenal glands

Table 3 shows that the adrenal glands from CV birds were significantly larger than those of the equivalent GF birds, in terms both of actual weight ($P < 0.01$) or as a proportion of metabolic body-weight ($P < 0.05$). The adrenal weights in both environments were significantly affected by the dose of CaPa, being larger at the lower levels of supplementation. The total cholesterol content of the adrenals was significantly less in CV chicks, and in both environments increased with increasing doses of CaPa.

Effects of ACTH on CV chicks

Results from Expt 5 are given in Table 4. There was a small non-significant depression in body-weights of the birds injected with ACTH. Their adrenals were heavier and the content of cholesterol lower ($P < 0.05$). Neither factor was affected by the dose of CaPa.

Table 4. *Expt 5. Weights and cholesterol contents of pairs of adrenals from conventional chicks given suboptimal amounts of dietary calcium pantothenate (CaPa) for the first 25 d of life, with or without injections of adrenocorticotrophic hormone (ACTH)*

(Mean values for two groups of ten birds/treatment)

Dose of CaPa (mg/kg diet)	ACTH	Body-wt (g)	Weight of pairs of adrenal glands		Total cholesterol content of adrenals (mg)
			mg	mg/kg BW$^{0.75}$	
2·67	0	193	32·7	638	588
	+	182	36·0	734	335
4·0	0	240	33·2	549	589
	+	202	39·4	738	413
Significance of effects:					
ACTH		NS	•	*	*
CaPa		0·05	NS	NS	NS
ACTH × CaPa		NS	NS	NS	NS
Standard error of differences of means (df 4)		16	2·3	78	103

BW, body-weight; NS, not significant.
* $P < 0.05$.

DISCUSSION

In the four experiments done to compare the chick's response in a GF or CV environment to suboptimal amounts of dietary PaA the body-weights attained by the GF birds were almost always significantly greater than those of their CV counterparts. Several authors using a variety of diets have observed that GF chicks grow better than their CV controls. In the present experiments the poorer growth was not merely a general response to the presence of a gut microflora but was apparently also due to a greater demand for PaA, since the clinical signs of deficiency were more prevalent and more severe in the CV chicks. When growth response curves to graded doses of CaPa in the two environments were constructed a quantitative estimate could be made which showed that GF chicks needed approximately two-thirds of the dose required by their CV controls to achieve comparable body-weights and freedom from signs of deficiency.

In earlier work (Coates *et al.* 1968) GF birds deprived of PaA had a higher content of PaA in the liver than did their CV controls. In both environments the liver contained relatively high amounts of the vitamin, even in birds showing severe signs of deficiency. Similar observations were made in Expt 3 now reported. Except at the highest rate of dietary supplementation with CaPa the amounts both of total and bound PaA in whole livers were greater in GF than in CV birds. However, this effect was entirely due to the greater weight of liver in the GF birds, which in turn was a function of the increased body-weight. Similarly an increase in the dietary supply of CaPa brought about an increase in body-weight, liver weight and hence in PaA content of the liver, but neither the environment nor the dietary supplement of CaPa had any effect on concentration of PaA in the liver, which remained fairly constant even in severely deficient chicks. Thus it appears that liver concentration of PaA is not an indicator of PaA status, and it is perhaps not surprising that little difference was found between GF and CV chicks in this respect.

The meaning of the term 'stress' is a matter of controversy, as discussed by Freeman

(1971), who defined the condition in terms of the complex changes that occur in plasma or adrenal constituents following an adverse stimulus. Accepted indicators of stress in the chicken include increased adrenal weight, depletion of adrenal cholesterol and elevation of plasma corticosterone. Only the first two factors were measured in our experiments. The third was considered unreliable in the circumstances, since the level of circulating corticosterone can respond rapidly to a sudden stimulus such as might be imposed while taking blood samples in the restricted conditions of the GF isolators. The CV chicks had significantly heavier adrenal glands with significantly lower contents of cholesterol, so by both criteria they were deemed to be under greater stress, as defined previously, than were their GF counterparts. Increasing the dietary supply of CaPa reduced the size of the adrenals and increased their content of cholesterol in both environments, suggesting that deficiency of PaA was itself a form of stress.

To investigate the possible relationship between PaA status and stress the condition was deliberately induced by injection of ACTH in CV chicks maintained on diets containing inadequate PaA. Adrenal weights were increased and cholesterol content decreased in the injected birds, but neither factor was significantly altered by increasing the supplement of CaPa. These results are thus somewhat at variance with those of the previous experiment, in which signs of stress were considerably more marked at the lower dose of dietary CaPa. It is possible that the more spacious housing of the CV chicks used in the last experiment was less stressful than the smaller cages used in the GF and CV comparison, and that the effects of PaA inadequacy were consequently less marked. Alternatively the condition induced by injection of ACTH may not have been strictly comparable to that imposed by the presence of a microflora. Whatever the reason for the discrepancy, the findings of the last experiment lend no support to the suggestion that stress increases the chick's requirement for PaA, and other explanations for the apparently greater need of the CV chick for dietary PaA must be considered.

It is possible that part of the dietary vitamin supply is used by gut microorganisms at the expense of the host, but since the difference was observed even on diets containing no CaPa it seems more likely that tissue reserves of the vitamin are used at a faster rate by CV compared with GF birds. Analogous findings have been reported in mammals with other water-soluble vitamins. For instance, GF guinea-pigs deprived of ascorbic acid lived longer and developed signs of scurvy later than did their CV counterparts (Levenson et al. 1962) and a greater need for thiamin was shown by CV compared with GF rats (Wostmann et al. 1963). In the presence of microorganisms it might be expected that the need to metabolize end-products of microbial activity would increase the host's metabolic burden and so create a greater demand for the vitamin co-factors concerned in the appropriate pathways. Since CoA plays a role in many biochemical reactions it is not unlikely that the host's requirement for PaA would be increased in this way.

REFERENCES

Barton-Wright, E. C. (1963). *Laboratory Practice*. London: United Trade Press.
Bird, O. D. (1963). In *Analytical Microbiology*, p. 497 [F. Kavanagh, editor]. New York and London: Academic Press.
British Standards Institution (1940). *Stand. Specif. Br. Stand. Inst.* no. 911.
Coates, M. E., Ford, J. E. & Harrison, G. F. (1968). *Br. J. Nutr.* **22**, 493.
Coates, M. E., Kon, S. K. & Shepheard, E. E. (1950). *Br. J. Nutr.* **4**, 203.
Freeman, B. M. (1971). *Wld's Poult. Sci. J.* **27**, 263.
Harrison, G. F. (1969). *Lab. Anim.* **3**, 51.
Levenson, S. M., Tennant, B., Geever, E., Laundy, R. & Daft, F. S. (1962). *Arch int. Med.* **110**, 693.
Wostmann, B. S., Knight, P. L., Keeley, L. L. & Kan, D. F. (1963). *Fedn Proc. Fedn Am Socs exp. Biol.* **22**, 120.
Zlatkis, A., Zak, B., Boyle, E. J. & Mich, D. (1953). *J. Lab. clin. Med.* **41**, 486.