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Growth response to dietary penicillin of germ-free chicks and of chicks with a defined intestinal flora

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In certain circumstances, antibiotics added to the diet increase the rate of growth of chicks (for review see Jukes, 1955). Coates, Dickinson, Harrison, Kon, Porter, Cummins & Cuthbertson (1952) and Coates, Davies, Harrison, Kon & Porter (1955) have suggested that this action of penicillin added to a diet complete in all known essential nutrients may be due to the suppression of an unidentified 'infection' that depresses growth. This view was recently confirmed in a series of experiments with germ-free and conventional chicks (Forbes & Park, 1958). Lev, Briggs & Coates (1956, 1957) observed that spores of *Clostridium welchii* type A were present in the caecums of chicks from the 'infected' premises 1 day after feeding, but not in those from the clean environment. A growth stimulation by penicillin occurred only in the 'infected' premises where the growth rate of chicks was depressed compared with that of the chicks in the clean quarters. Penicillin in the diet either eliminated the clostridia from the intestines of the chicks or reduced the lecithinase production of these organisms. Thus the presence of *Cl. welchii* type A in the caecums of chicks was associated with growth depression, and elimination of the organisms or reduction in their toxigenicity accompanied the reversal by penicillin of the growth depression. Because of the complex nature of the intestinal flora it was not possible by the usual bacteriological techniques to determine the role of clostridia in the growth of chicks. Germ-free chicks, or chicks in which a defined flora had been implanted, seemed to permit a direct approach to the problem.

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Experiments are reported here on the effect of certain species of bacteria on the growth of chicks free of all other bacteria and fungi and on the growth response of such birds to penicillin supplements in the diet. The strains of bacteria selected for these studies were from those predominant in the intestinal flora, such as *Escherichia coli*, *Lactobacillus lactis*, *Streptococcus liquefaciens* and *Cl. welchii* type A (Lev & Briggs, 1956*a*, *b*; Lev *et al.* 1957).

EXPERIMENTAL

Experimental design

The growth and the growth response to penicillin of germ-free chicks were compared with those of germ-free chicks deliberately implanted with (1) *Cl. welchii*, (2) *Strep. liquefaciens*, *E. coli* and *Lb. lactis* together, with and without *Cl. welchii*.

Chicks

White Leghorn chicks were used. The germ-free chicks and the chicks with a defined intestinal flora were raised in Reyniers germ-free units (Reyniers, Trexler, Ervin, Wagner, Luckey & Gordon, 1949). The method for rearing the germ-free chicks was modelled on that of Reyniers *et al.* (1949), and was that used in previous experiments (Forbes & Park, 1958).

Diet

A semi-synthetic casein–starch diet, C-1R (Forbes & Park, 1958), which allowed excellent growth of germ-free and conventional chicks, was used, and its composition is shown in Table 1. It was made up and mixed in 20 kg batches, sterilized and transferred to the germ-free units. For sterilization 1 kg portions were placed in gauze

Table 1. *Composition of chick diet C-1R (Forbes & Park, 1958)*

Ingredient	Quantity/100 g	Ingredient	Quantity/100 g
Maize starch (g)	57·1	Vitamin B_{12}, crystalline* (mg)	0·005
Casein, purified* (g)	25·0	Vitamin A acetate* (i.u.)	2600
Maize oil (g)	5·0	Vitamin D_3, crystalline* (i.u.)	100
Alphacel* (g)	3·0	DL-α-tocopherol* (mg)	5
Glycine (g)	1·5		
L-Arginine HCl (g)	1·0	$CaCO_3$ (g)	2·5
DL-Methionine (g)	0·5	K_2HPO_4 (g)	1·72
Choline HCl (g)	0·27	Na_2HPO_4 (g)	1·4
Thiamine HCl (g)	0·10	NaCl (g)	0·5
Calcium pantothenate (mg)	10	$MgSO_4.7H_2O$ (g)	0·45
Nicotinic acid (mg)	10	$MnSO_4.H_2O$ (mg)	30
Riboflavin (mg)	4	$FeSO_4$ (mg)	12
Pyridoxine HCl (mg)	2	$CuSO_4$ (mg)	1
Folic acid (mg)	1	$CoCO_3$ (mg)	1
Menaphthone (mg)	0·8	$ZnSO_4.7H_2O$ (mg)	1
Biotin (mg)	0·1	KI (mg)	0·3

* Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

bags in a layer not exceeding 1 in. in thickness in the jacketed autoclave of each germ-free unit. Steam was allowed to flow through the autoclave for 10 min, the pressure was then increased to 17 lb./sq. in. (122°), maintained for 25 min and gradually returned to normal. Procaine penicillin G sealed in glass ampoules was sterilized by

irradiation at 1,800,000 rep (roentgen equivalent physical), transferred to the units through a germicidal trap and mixed with the sterile diet. Irradiation did not affect the antibacterial activity of the penicillin as determined by the tube-dilution technique with *Staphylococcus aureus* (strain H) as a test organism. Random samples of the supplemented food taken from the food trays were assayed for penicillin content. Penicillin was always found to be present at the expected level.

As in previous experiments (Forbes & Park, 1958), the chicks were given canned sterilized water (MacDonald-Bernier Co., Boston, Mass.). Food and water were given *ad lib.* Food for each group of chicks was offered in a covered tray (about 20 in. long and $2\frac{1}{2}$ in. wide) provided with nine holes ($1\frac{3}{4}$ in. $\times$ $1\frac{1}{8}$ in.)

Rearing conditions

After hatching, the chicks in each germ-free unit were subdivided into two groups of seven to ten chicks of approximately equal weight. In each germ-free unit the two groups of chicks were kept in cages about 11 in. wide, 22 in. long, and 13 in. high (made of $\frac{3}{8}$ in. $\times$ $\frac{3}{8}$ in. wire mesh), one group serving as control, the other receiving the diet supplemented with penicillin. The chicks were weighed weekly. The temperature during the hatching of the chicks was 37°; it was gradually reduced to room temperature (24–26°) from the 1st day until the 14th day after hatching, and maintained there until the end of the experiment. The lights in the germ-free units were kept on continuously.

Bacteriological procedures

Sterility tests of the chicks were performed weekly as described previously (Forbes & Park, 1958). The various organisms used to make up the defined intestinal flora were isolated from the freeze-dried intestinal contents of chicks reared in the 'infected' premises at the National Institute for Research in Dairying, Shinfield, Reading, where a growth response to penicillin supplements in the diet occurred regularly. These organisms were identified by the Department of Bacteriology, Walter Reed Army Institute of Research, Washington, D.C.

A strain of *Cl. welchii* type A that produces α, θ and κ toxins isolated from 'infected' chicks (Lev *et al.* 1957) was used. Heat-sealed ampoules containing freeze-dried spores prepared in Ellner's medium (Ellner, 1956) were transferred into the germ-free units through the germicidal trap. Each chick was given 10^4–10^6 freeze-dried spores in 0·5 ml. saline, introduced into the crop by means of a short silicone-rubber tube attached to a 1 ml. syringe. *Lb. lactis* and *Strep. liquefaciens* were grown in micro-inoculum broth (Difco), *E. coli* in nutrient broth (Difco). Cultures of each of these organisms that had been incubated at 37° in 200 ml. medium for 18–24 h were centrifuged, and the cells were suspended in enough saline to provide about 0·5 ml. of a mixture of the organisms for each experimental chick. The suspensions containing *E. coli*, *Lb. lactis* and *Strep. liquefaciens* were sealed in glass ampoules and introduced into the germ-free units through the germicidal trap. In adjacent tanks, containing chicks from the same hatchings, 0·5 ml. of the same bacterial suspension with and without added *Cl. welchii* spores was administered to the chicks immediately after

hatching and before feeding. Vent swabs and faecal specimens were taken 1 day after introduction of the organisms into the chicks, and tested by appropriate procedures to establish the presence of the artificially implanted flora.

At the termination of an experiment, on the 21st day, five chicks in each group were killed, and bacterial counts were made by the method of Miles & Misra (1938) at three sites in the intestine, the duodenum, ileum and caecum, pooled samples of the contents from each site being used. The samples were collected in sterile screw-top jars, diluted with sterile saline and homogenized with glass beads. Samples were then removed for dry-weight determination. *Cl. welchii* were counted on meat-extract agar (Difco) to which 5% of human serum and 10% of egg-yolk had been added, *E. coli* on eosin–methylene blue agar (E.M.B.) (Difco), *Lb. lactis* on lactobacillus-selection medium (Baltimore Biological Laboratory Co.) and *Strep. liquefaciens* on nutrient agar to which 5% of blood had been added. All incubations were made at 37°. Egg-yolk agar plates and blood-agar plates were incubated anaerobically in Brewer's jars, lactobacillus-selection plates in an atmosphere of 90% air and 10% CO_2 and E.M.B. plates aerobically.

RESULTS

The figures in Table 2 show that *Cl. welchii* type A as the sole intestinal flora depressed the growth of chicks on the basal diet compared with that of the germ-free birds. Supplementation of the diet with procaine penicillin (45 mg/kg) had no effect on the

Table 2. *Growth and growth response to dietary procaine penicillin G (45 mg/kg diet) of germ-free chicks and chicks with an intestinal flora composed of* Clostridium welchii *type A only*

(Fourteen chicks/group from two replicate experiments)

Intestinal flora	Mean weight (g) of chicks on 21st day, given: No supplement (*a*)	Penicillin (*b*)	Difference (*b* − *a*) (g)	*t*	*P*
None (germ-free chicks)	257	254	− 3	1	N.S.
Cl. welchii	213*	243	+30	2·66	<0·05

N.S., not significant.

* Significance of difference between germ-free chicks and those with *Cl. welchii*: $t = 3{\cdot}69$; $P < 0{\cdot}01$.

growth of germ-free chicks, but counteracted the growth depression in the chicks infected with *Cl. welchii* only. An intestinal flora composed of *E. coli*, *Lb. lactis* and *Strep. liquefaciens* had no effect on the growth rate. However, when besides these three organisms *Cl. welchii* was also present, the growth of chicks harbouring them was depressed compared with that of germ-free chicks. Penicillin in the diet had no effect on the growth rate of the chicks with an intestinal flora composed of *E. coli*, *Lb. lactis* and *Strep. liquefaciens*, but it did appear to counteract the growth depression when *Cl. welchii* was present as well (Table 3). The apparent increase in weight was not significant ($P = 0{\cdot}1$), but the results showed a trend similar to that obtained when *Cl. welchii* was given alone (Table 2).

The relief by penicillin of the growth depression in the chicks with an intestinal flora composed of *Cl. welchii* only is explained by bacterial counts on the intestinal content. Penicillin in the diet virtually eliminated *Cl. welchii* from the intestine (Table 4). In the chicks with a flora composed of *E. coli*, *Lb. lactis*, *Strep. liquefaciens*

Table 3. *Growth and growth response to dietary procaine penicillin G* (45 *mg/kg diet*) *of germ-free chicks and chicks with a defined intestinal flora*

(Eighteen to twenty chicks/group from two replicate experiments)

Intestinal flora	Mean weight (g) of chicks on 21st day, given: No supplement (*a*)	Penicillin (*b*)	Difference (*b* − *a*) (g)	*t*	*P*
None (germ-free chicks)	240	241	+ 1	1	N.S.
Three species* of bacteria	240	232	− 8	1	N.S.
The three species* with *Cl. welchii*	213†	228	+15	1·72	0·1

N.S., not significant.

* *Escherichia coli*, *Lactobacillus lactis* and *Streptococcus liquefaciens*.

† Significance of difference between chicks with three species of bacteria and chicks with the three species and *Cl. welchii*: $t = 2{\cdot}8$; $P < 0{\cdot}01$.

Table 4. *Number* of* Clostridium welchii ($\times 10^{-6}$/*g dry weight*) *in intestinal contents of chicks, with a flora of* Cl. welchii *only, fed on the basal diet with and without a supplement of procaine penicillin G* (45 *mg/kg diet*)

Intestinal segment	No supplement	Penicillin
Duodenum	9·7	< 0·001
Ileum	3·7	< 0·001
Caecum	2700	< 0·001

* Counts performed on pooled samples of intestinal contents from five chicks.

Table 5. *Number* of bacteria* ($\times 10^{-6}$/*g dry weight*) *in intestinal contents of chicks with a defined intestinal flora* (*see p.* 79) *fed on the basal diet with and without a supplement of procaine penicillin G* (45 *mg/kg diet*)

Exp.	Intestinal segment	*Cl. welchii* U.	*Cl. welchii* P.	*E. coli* U.	*E. coli* P.	*Lb. lactis* U.	*Lb. lactis* P.	*Strep. liquefaciens* U.	*Strep. liquefaciens* P.
a	Duodenum	0·029	< 0·001	4·3	3·4	87	9·1	Not counted	Not counted
	Ileum	0·95	< 0·001	95	1,400	100	240	Not counted	Not counted
	Caecum	14	< 0·001	1,400	11,000	470	330	Not counted	Not counted
b	Duodenum	33	2·7†	42	8·6	28	120	28	240
	Ileum	2·7	9·1†	19	37	140	912	149	839
	Caecum	15	4·6†	25,000	11,000	580	560	8,700	6,700

U., unsupplemented; P., procaine penicillin.

* Counts performed on pooled samples of intestinal contents from five chicks.

† These *Cl. welchii* showed no lecithinase activity on first isolation on the meat-extract–serum–egg-yolk plates. Upon subculture they showed as much lecithinase activity as the clostridia from the chicks on the unsupplemented diet.

and *Cl. welchii*, the clostridia were fewer than when they were the only bacteria present. Penicillin in the diet of the chicks virtually suppressed the clostridia and had little effect on the three other organisms (Table 5, Exp. *a*). In one experiment, however, bacterial counts showed that penicillin did not reduce the number of *Cl. welchii* (Table 5, Exp. *b*), but the clostridia did not show their usual characteristic lecithinase activity on egg-yolk agar when first isolated. The colonies of clostridia could readily be counted and recognized, and upon subculture they produced as much lecithinase as those from the chicks on the non-supplemented diet.

Except for a slower rate of weight increase, the chicks infected with *Cl. welchii* showed no abnormalities in gross appearance during life or at autopsy.

DISCUSSION

These experiments represent the first attempt to establish in previously germ-free animals a simplified model of the intestinal bacterial flora occurring under natural conditions. The bacterial counts showed that *E. coli* and *Lb. lactis* reached numbers similar to those found in normal conventional chicks (Lev & Briggs, 1956*b*). *Cl. welchii* when implanted alone had a predilection for the caecum and, in the presence of *Lb. lactis*, *Strep. liquefaciens* and *E. coli*, were considerably reduced in number. Under certain conditions, not yet defined, penicillin in the diet eliminated the clostridia from the intestine, but under other conditions it reduced their toxigenicity. In either event penicillin in the diet counteracted the growth depression due to the presence of clostridia in the intestine. This reduction of toxigenicity of clostridia seemed to have the same effect on the host as the elimination of the organisms, already shown in conventional chicks (Lev *et al.* 1957).

The mechanism of the growth-depressing activity of the clostridia on the chicks is not known. Impaired lecithinase production may be only a sign of the effect of penicillin on the organisms. In a preliminary experiment (Forbes, Park & Lev, 1959) a laboratory strain of *Clostridium bifermentans* that also produces a lecithinase did not depress growth when established in otherwise germ-free chicks. This species of bacterium is, however, not usually found in the chick intestine.

Our results suggest that *Cl. welchii* type A may be a factor in the unidentified 'infection' whose growth-depressing effect is counteracted by penicillin supplements in the diet.

SUMMARY

1. Germ-free chicks were reared in Reyniers germ-free units and their response to dietary penicillin was compared with that of similar chicks whose gut had been intentionally populated with certain bacteria.

2. Strains of some predominant bacterial species from the intestinal flora of chicks (*Escherichia coli*, *Lactobacillus lactis* and *Streptococcus liquefaciens*) had no effect on growth when implanted together in chicks free of all other bacteria.

3. *Clostridium welchii* type A, either as sole bacterial species or in the presence of the other three bacteria from the intestinal flora, caused a depression of the growth rate of chicks compared with that of germ-free chicks.

4. Procaine penicillin G added to the diet (45 mg/kg) had no effect on the growth of germ-free chicks or of chicks with a flora composed of bacteria that itself did not affect growth, but did counteract the growth depression due to the presence in chicks of *Cl. welchii* alone or together with a limited defined intestinal flora.

5. Penicillin either eliminated the clostridia from the intestine or reduced their toxigenicity.

6. *Cl. welchii* may therefore be a factor in the growth-depressing 'infection' that is counteracted by penicillin supplements in the diet.

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