IX. MAINTENANCE NUTRITION IN THE PIGEON. VITAMIN B₃ CONCENTRATES.

By CYRIL WILLIAM CARTER AND JOHN RICHARD O'BRIEN.

From the Department of Biochemistry, Oxford.

(Received November 30th, 1935.)

VITAMIN B₃ may be defined as a factor required for full weight restoration in the pigeon on a diet which, in addition to vitamins B₁ and B₅, includes an adequate supply of basal dietary constituents such as salts and protein. We have previously shown [Carter and O'Brien, 1935] that one of the factors involved in the failure of pigeons on a rice diet to recover normal weight is a partial deficiency of protein. Further, the weight recovery of birds on a diet adequate as regards vitamins B₁ and B₅ and protein is influenced by the severity of the depletion to which they are subjected on rice. It was observed that, whereas birds subjected to moderate depletion of stored vitamins recovered weight completely on receiving supplements of vitamins B₁ and B₅ and protein, with more drastic depletion many birds failed to regain their maximum weight on this diet. This may explain the differences between the results of Morris [1933] and those of Waterman and Ammerman [1935]. The former was led to question the existence of vitamin B₃ on the ground that normal weight could be attained on a diet of autoclaved wheat and 6–16 times the curative dose of vitamin B₁. The latter workers found that, on the same basal diet, pigeons receiving up to 160 γ of crystalline vitamin B₁ daily attained only 80–90 % of their normal weight and concluded that a further factor was required for full weight recovery. In those birds which fail to regain their maximum weight on a diet with adequate protein we have found that full weight restoration was made on administration of an extract of liver which we consider to supply vitamin B₃.

The present paper gives further evidence of the character of this supplement from liver.

EXPERIMENTAL.

Preparation of vitamin B₃ concentrates.

The liver concentrate was prepared as follows: 2 sheep livers weighing about 2 kg. were freed as far as possible from adherent fat and connective tissue. After mincing, the liver was suspended in 6 l. of 97 % alcohol and kept with occasional stirring for 48 hrs. The alcoholic extract was filtered off and the residue again extracted with an equal volume of alcohol. The combined filtrates containing most of the fat were concentrated *in vacuo* at 60° to a small volume. 200 ml. ether were then added, the whole readily dissolving to give a reddish solution. This was shaken in a separating funnel with 200 ml. of water, the aqueous phase removed and the extraction of the ether repeated twice. During this process a bulky white precipitate appeared in the aqueous phase. The aqueous extracts were combined and reserved. The liver residue was again twice extracted with 4 l. 50 % alcohol and the combined filtrates which possessed a strong yellow-green fluorescence were concentrated *in vacuo* at 60° to about 200 ml. This concentrate was shaken two or three times with ether and added

to the solution reserved from the extraction with 97% alcohol. The turbid solution was treated with an equal volume of acetone and kept overnight. After filtration it was concentrated *in vacuo* to a volume such that 1 ml. was equivalent to 20 g. liver (fraction A).

The marked yellow-green fluorescence of fraction A suggested the presence of hepatoflavin and further fractionation was made following the procedure for the isolation of flavins [Kuhn *et al.*, 1933]. 250 ml. of fraction A were treated with 12.5 ml. conc. HCl and 15 g. of fuller's earth added. The mixture was kept for 12 hours with repeated stirring and was then filtered on a Büchner funnel. The earth was extracted 3 times with 200 ml. of a mixture of pyridine, methyl alcohol and water in the ratio 1 : 1 : 4. The pyridine extracts were concentrated *in vacuo* to a syrup which was taken up in 20 ml. of water. On addition of an equal volume of methyl alcohol and 1-2 drops of acetic acid precipitation of fuller's earth occurred. After filtration the solution was concentrated *in vacuo* nearly to dryness and the residue dissolved in hot water. The solution containing flavin was further purified by adsorption upon lead sulphide [Ellinger and Koschura, 1933]. The sulphide was repeatedly extracted with hot water and the eluate concentrated to a volume such that 1 ml. was equivalent to 20 g. liver (fraction F). This fraction possessed a marked flavin fluorescence with only traces of a blue-fluorescent substance. The filtrate obtained after the treatment with fuller's earth was neutralised to p_H 4.0 with 20% NaOH and concentrated *in vacuo* so that 1 ml. was equivalent to 20 g. liver (fraction B). This preparation showed in the ultraviolet a strong blue fluorescence which persisted in acid and alkaline solutions. The operations described above were conducted with minimum exposure to light.

Results.

The procedure followed was that of Carter and O'Brien [1935], *i.e.* birds were maintained on a rice diet supplemented with 12 doses of a vitamin B₁ concentrate and 2 g. of caseinogen daily. Approximately 60% of the birds failed to regain their maximum weight on this diet, the remainder recovering their initial weight completely. This unfortunately limits the number of birds available as suitable material for testing the potency of vitamin B₃ preparations. The group of birds selected for these tests had maintained for periods of 11-220 days a stationary weight level which varied within the limits of +5 to -16 g. before administration of liver concentrates. Following administration of the liver concentrates an initial daily rise of 1.0-4.0 g. was recorded, although later as maximum weight was approached the rise became more gradual. This comparatively small rise in weight as a result of the administration of vitamin B₃ may be due to the fact that a level of 80-90% of the maximum had already been attained on vitamin B₁ and caseinogen. This may be attributed either to considerable powers of storage of vitamin B₃ in the bird or to the possibility, not yet excluded, that the vitamin B₁ concentrate carries some of the activity present in the liver preparation. The significance of the results lies, in our opinion, in the restoration of maximum weight in the majority of cases.

Three liver preparations were tested in daily doses equivalent to 10-30 g. liver: fractions A, B and F, and fractions B and F combined. In Table I, the response of 6 birds to fraction F is shown. In two cases, birds 247 and 288, there was a rise nearly to maximum weight and no further improvement occurred when they received whole wheat. In three cases the response was negligible. No further increase in weight was observed on doubling the dose of fraction F. From Table I it is also seen that the effect of fraction B is insignificant. A more

Table I. *Effect of liver supplements.*

Bird	Max. wt. g.	Depletion wt. ex- pressed as % of max. wt.	Stationary wt. period on vitamin B ₁ and caseinogen (days)	Wts. before and after administra- tion of liver suppl. (g.)		Duration of suppl. (days)	Gain or loss in wt. g.	Av. daily gain in wt. g.
Fraction F								
247*	428	73	54	390	412	5	22	4.4
288	402	72	222	363	395	19	32	1.7
299*	398	70	120	369	379	15	10	0.6
292	477	71	49	437	440	9	3	0.3
293*	400	56	11	366	362	11	-4	Nil
822*	426	64	100	406	401	9	-5	Nil
Fraction B								
91*	356	56	115	316	323	10	7	0.7
287*	474	72	113	433	434	9	1	Nil
247*	428	73	119	392	389	10	-3	Nil
190*	500	75	41	440	442	10	2	Nil
357*	380	77	11	350	351	7	1	Nil

Note. Compare response of birds denoted by asterisk to combined fractions F and B and to fraction A (Table II).

Table II. *Vitamin B₃ effect of liver fractions B + F, and of fraction A.*

Bird	Max. wt. (g.)	Wts. before and after administra- tion of fractions		Duration of fractions B + F (days)	Gain in wt. (g.)	Av. daily gain in wt. (g.)	Wts. before and after administra- tion of fraction A		Duration of frac- tion A (days)	Gain in wt. (g.)	Av. daily gain in wt. (g.)
		B + F (g.)					(g.)				
A											
293	400	362	384	7	22	3.1	384	395	9	11	1.2
91	356	323	343	9	20	2.2	—	—	—	—	—
299	398	379	382	3	3	1.0	382	388	12	6	0.5
822	426	401	410	9	9	1.0	410	419	14	9	0.6
247	428	389	409	10	20	2.0	409	413	7	4	0.5
190	500	442	479	9	37	4.1	479	497	8	18	2.2
287	474	434	451	5	17	3.4	—	—	—	—	—
289	440	—	—	—	—	—	377	432	20	55	2.7
294	373	—	—	—	—	—	327	361	21	34	1.6
B											
248	500	—	—	—	—	—	475	498	8	23	2.8
296	360	—	—	—	—	—	345	363	4	18	4.5
719	398	—	—	—	—	—	378	399	6	21	3.5
733	500	444	468	7	24	3.4	468	474	26	6	0.2
357	380	351	380	8	29	3.6	—	—	—	—	—

Note. 1 bird failed to respond to liver fractions or subsequently to whole wheat.

definite effect was observed when the combined fractions F and B were given to a group of birds including 7 which had previously received independently one or other of these fractions. The results are recorded in Table II A. In certain cases, after the response to these fractions had been observed, they were substituted by fraction A, the administration of which was continued either until maximum weight was restored or until no further rise was produced. Two birds received fraction A without previous administration of B and F. It is seen that the responses to the combined fractions and to fraction A are similar to that previously recorded with crude liver preparations [Carter and O'Brien, 1935]. Seven birds rose to within 10 g. of their maximum weight. In birds 287 and 91 an initial response was observed but complete restoration occurred neither with

the liver supplements nor with whole wheat. In Table II B the effect of liver concentrates is seen in 5 birds which, having reached maximum weight on a vitamin B₁ concentrate and caseinogen, showed partial failure to maintain this weight when these supplements were continued for 40–60 days. Recovery was observed in four cases on addition of liver concentrates; one bird which did not respond to the supplement showed no recovery on wheat or mixed corn.

DISCUSSION.

The difficulty in securing a sufficient number of birds for testing our vitamin B₃ preparations has limited the data upon which our evidence is based. The results so far obtained however point to the presence in these preparations of one or possibly two factors which may be necessary for pigeon nutrition. No effect has been observed with fraction B alone. With fraction F, the flavin fraction, definite response has been observed in two or three cases, but it is much less definite than the effect of the two fractions combined. These facts may be explained either on the ground that the state of depletion of these factors may vary independently from bird to bird, or that the birds are already receiving a partial supply of the factor present in fraction B from other sources, for instance the vitamin B₁ concentrate. The evidence does not permit us to decide between these alternatives. Moreover, it is premature to suggest any possible relationship between the factors in our liver concentrates and (a) factors in liver described by other workers and (b) other components of the vitamin B complex. With regard to the former the presence in liver of a factor necessary for the normal nutrition of the rat [Mapson, 1932; Seegers and Smith, 1932], and of factors necessary for the chick [Hogan and Boucher, 1933; Elvehjem *et al.*, 1932–33; Kline *et al.*, 1934] have been reported. The last group of workers observed that young chicks on a diet including vitamins B₁ and B₂ failed to grow normally and developed a characteristic leg paralysis accompanied by degeneration of the cerebellum. Normal growth and protection from paralysis resulted from feeding preparations of liver. They concluded that the growing chick requires, in addition to vitamins B₁ and B₂, a growth factor insoluble in water and stable to autoclaving and an antiparalysis factor, soluble in water and destroyed by autoclaving, both being present in liver. Elvehjem and Koehn [1935] found that chick pellagra could be cured by a liver fraction from which flavin had been removed by adsorption on fuller's earth. This observation has been confirmed by Lepkovsky and Jukes [1935]. These workers found that the fuller's earth adsorbate of liver extract containing hepatoflavin markedly stimulated the growth of chicks. There is thus the possibility that the factors present in liver and necessary for the chick, namely a factor adsorbed on fuller's earth (? flavin) and one present in the filtrate after treatment with this reagent, are also required by the adult pigeon. It is possible that the activity of our fraction F may be ascribed to its flavin content but until further purification has been effected this cannot be definitely settled. We have suggested that fraction B makes adequate a supply of a factor present in the impure vitamin B₁ concentrate. This is believed to contain vitamin B₆ and is a source of vitamin B₅. At present, we are unable to define the relation between the factor in fraction B, vitamin B₅ and vitamin B₆. Nevertheless, our results give support to the view that a pigeon receiving a well-balanced basal diet requires in addition to vitamin B₁ at least two other factors.

SUMMARY.

1. A method is described for the preparation of a liver concentrate with vitamin B₃ activity.

2. From this concentrate two fractions have been obtained: (a) one adsorbed on fuller's earth carrying with it flavin and (b) one present in the filtrate after treatment with this reagent, fraction B. The flavin fraction showed some weight restorative potency whereas fraction B given alone was without effect. Greatest activity was shown when the two fractions were given together.

3. The relation of these results to the factors necessary for the nutrition of the chick is discussed.

The authors wish to thank Prof. Peters for his continued encouragement and advice. One of us is indebted to the Medical Research Council for a personal grant.

REFERENCES.

- Carter and O'Brien (1935). *Biochem. J.* **29**, 2746.
Ellinger and Koschara (1933). *Ber. deutsch. chem. Ges.* **66**, 1411.
Elvehjem, Keenan, Kline and Hart (1932-1933). *J. Biol. Chem.* **99**, 309.
— and Koehn (1935). *J. Biol. Chem.* **108**, 709.
Hogan and Boucher (1933). *Miss. Agric. Exp. Sta. Res. Bul.* No. 198.
Kline, Elvehjem, Keenan and Hart (1934). *J. Biol. Chem.* **107**, 10.
Kuhn, György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1034.
Lepkovsky and Jukes (1935). *J. Biol. Chem.* **111**, 119.
Mapson (1932). *Biochem. J.* **26**, 970.
Morris (1933). Dissertation. New York.
Seegers and Smith (1932). *Proc. Soc. Exp. Biol. Med.* **30**, 365.
Waterman and Ammerman (1935). *J. Nutrition*, **10**, 161.