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Grain-fed pigeons revisited: a pioneer test for vitamin B_{12}

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Preface. Janet Vaughan was born on 18 October 1899. She wanted to be a doctor, studied at Somerville College Oxford and then qualified from University College London in 1924. Her career was mainly in research, particularly on diseases of the blood and in 1939 she set up one of the first blood transfusion depots. She subsequently became Principal of Sommerville College Oxford in 1945 and was a member of several government committees. She was awarded the OBE in 1944, the DBE in 1957 and was elected FRS in 1979. In the following paper, Dr Castle points out that she published her pioneer work on 'substances effective in pernicius anaemia' in *The British Journal of Experimental Pathology* in 1930. *The Editor*

Introduction

In retrospect it appears that the first successful animal test for the anti-pernicious anaemia principle of liver was described in *The British Journal of Experimental Pathology* by Vaughan, Muller and Zetzel as early as 1930. Minot & Murphy (1926) had reported the consistent therapeutic effectiveness in pernicious anaemia of a special diet 'rich in calf's or beef liver'. By 1928 when George Minot became Professor of Medicine at Harvard and Director of the Thorndike Memorial Laboratory at the Boston City Hospital a nearly protein-free extract of liver for oral use had been prepared by Harvard's Professor of Physical Chemistry, Edwin Cohn, and was shown by Minot in clinical trials to provide a 25-fold concentration of the native liver's erythropoietic activity (Cohn *et al.* 1930). However, it was to require 20 more years of international scientific endeavor, chemical and clinical, before the elusive active principle of liver could be identified (Rickes *et al.* 1948; Smith & Parker 1948) as crystalline vitamin B_{12}, a cobalamin.

In 1929–1930 Janet Vaughan, then a junior clinical pathologist at University College Hospital, was on leave of absence and working on a Rockefeller Travelling Fellowship in Minot's laboratory. Her colleagues in research there were Dr Gulli Lindh Muller, an Assistant Physician on the Thorndike Laboratory staff and a Harvard medical student, Mr Louis Zetzel. A prospect of success had come from earlier observations (Muller 1927) that in grain-fed pigeons erythropoiesis in the femoral bone marrow as seen in stained tissue sections was 'megaloblastic' and so resembled that in untreated pernicious anaemia. At the conclusion of their experiments, when several liver extracts active in pernicious anaemia had been found to evoke new red cell production (reticulocyte responses) and sometimes gains in weight in healthy pigeons, Vaughan and her colleagues (1930) concluded that 'the grain-fed pigeon may perhaps suffer from a mild dietary deficiency ... permitting its bone marrow to respond to certain substances'. That supposition can now be confirmed specifically for vitamin B_{12}, by a

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retrospective review of relevant trends in nutritional research and their final conclusions.

Original description of the research with pigeons

The results of the most significant experiments reported by Vaughan *et al.* can be summarized as follows. Twelve pigeons showed positive reticulocyte responses to the oral administration of a commercial preparation, liver extract no. 343 NNR (effective in pernicious anaemia) in total doses of 1.5 to 20 g. Of five other pigeons given a relatively unrefined liver extract fraction (132 E) of established clinical potency, prepared by Cohn and McMeekin, four responded positively to total intravenous doses of 150 to 1600 m, while the fifth bird had no reticulocyte response to 40 mg. (Fig. 1). Five different, more refined but clinically active experimental liver extracts, were given intravenously to another group of 17 birds. Of these, 14 clearly responded positively, while three pigeons given two liver extracts in less than half of the standard dose of 150 mg. showed little or no increase of reticulocytes (Table 1). Beef-steak, fed to four birds, 'gave a reticulocyte rise in every way similar to that obtained with effective liver preparations'. Over all, the gains in weight appeared in relatively few birds; however, this would be expected in seemingly adult pigeons except in those still capable of further growth.

Substances known to be erythropoietically inactive in pernicious anaemia, namely autoclaved liver extract no. 343 NNR, NaCl,

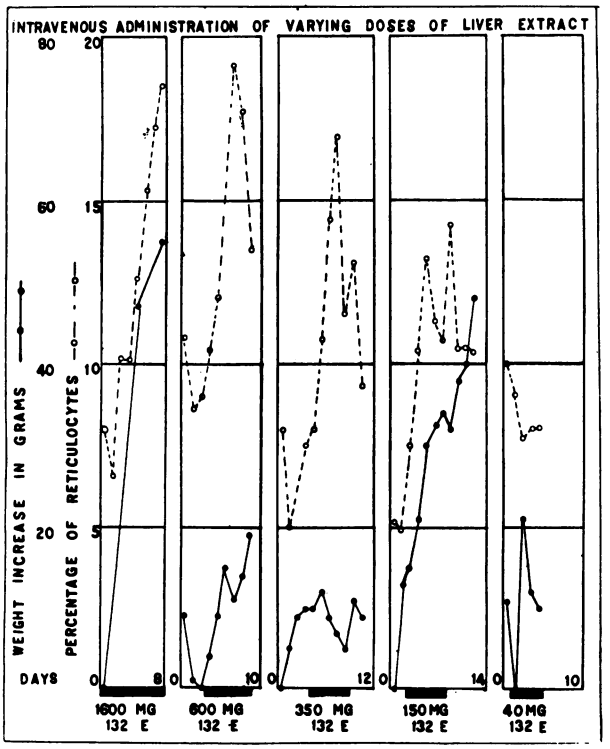

FIG. 1.—The response of the reticulocytes and the weight of healthy grain-fed pigeons to the intravenous administration of varying doses of a liver preparation effective in pernicious anæmia.

Reproduction of Fig. 1 from Vaughan et al. (1930).

TABLE I.—*Response in Healthy Grain-fed Pigeons to Intravenous Administration of Liver Preparations Effective in Pernicious Anæmia.*

Pigeon number.	Preparation number.	Dose in mgm.	Percentage of reticulocytes. Average in control period.	Maximum response.	Body-weight gain in gm.
182	132 E	2400	10·0	17·0	38
181	132 E	2200	11·2	20·1	38
175	132 E	1600	8·0	18·5	55
174	132 E	1400	10·0	20·9	41
189	132 E	600	5·7	18·8	15
188	132 E	350	7·1	16·9	18
190	132 E	150	6·0	14·2	64
191	132 E	40	9·7	12·5	0
208	134 ECDB	150	9·3	18·7	0
213	134 ECDB	60	8·7	20·1	0
244	134 Z	20	10·9	15·9	0
228	134 ZHB	60	11·0	21·0	0
227	134 ZHB	60	9·7	22·1	0
220	134 ZHA2	50	9·8	13·8	0
206	134 ZHA2	60	11·0	11·0	0
242	134 ZH2A	20	11·2	14·6	0
240	135 ZHA	20	10·4	20·2	0

Reproduction of Table I from Vaughan et al. (1930).

casein, histamine, and somewhat concentrated preparations of vitamin B_1 and B_2, proved inactive in control experiments. In addition, a refined liver extract fraction that had proved ineffective in pernicious anaemia was completely negative on intravenous injection in twice the standard dose, 300 mg, in a pigeon that later gave a positive response to a clinically active extract fraction. Finally, a single discrepancy was found with a clinically inert liver extract fraction, apparently composed of leucine. As will be discussed below, commercial preparations of leucine and lysine, though inactive in pernicious anaemia patients, subsequently gave typical reticulocyte responses in pigeons, but caused different morphological changes in their bone marrows from those produced by liver extracts.

Only healthy pigeons were used, that in a control period had shown a steady body weight and a reticulocyte count 'not above 12%' after being fed for some weeks on the exclusively grain diet. During the experimental periods each bird was weighed and bled minimally every day to provide blood films for determining the percentage of reticulocytes in at least 500 red cells. To ensure complete staining of the reticulocytes of the pigeons's nucleated red cells two cover slips (not one) with a thick dried film of brilliant cresyl-blue were employed in making the blood smears. Intravenous injections were made into a leg vein using a volume of 2 ml.

The priority of Vaughan's apparently successful methodology now seems unchallenged. Earlier classical expe~iments by Whipple & Robscheit-Robbins (1925) with haemoglobin regeneration as enhanced by liver feeding in chronically bled dogs are now known to have been due principally to the liver's iron and copper content (Frost *et al.* 1940), as were, presumably, other bleeding experiments performed by Jeney (1927). Other early techniques such as animals rendered anaemic by infection or by toxic or haemolytic substances, were non-specific and gave uncertain results, as summarized by Jacobson (1935). Barlow (1930) reported that the anaemia of pigeons on a polished

rice or mixed grain diet was not improved by daily oral doses of Lilly liver extract 343, an extract potent in anti-pernicious anaemia activity which, however, was shown to be active in Vaughan's pigeons that same year.

Attempts at confirmation by others

Thus, because at the time of the publication by Vaughan *et al.* (1930) no acceptable method of evaluation of the anti-pernicious anaemia activity of liver extracts existed other than by clinical trial, their report stimulated attempts at repetition. Edmunds *et al.* (1933) and Peabody & Neale (1933) confirmed the method with several clinically active liver extracts; the latter found that increases in red cells followed positive reticulocyte responses, but saw none with leucine. On the other hand, Wills (1932), Heimann *et al.* (1934) and Gurd (1935) found such extreme fluctuations in reticulocyte counts in their pigeons that any evidence of liver extract activity was obscured.

Further comment on experimental details

In an attempt to explain the discrepancies, co-author Muller (1935) called special attention to certain potentially important experimental details of the 1930 report. She wrote that pigeons showing considerable variations in reticulocytes or weight during the pre-experimental period were discarded. All birds were kept in small individual cages 'with space sufficient only for movement of the wings'. The floor of the cages was of wire screen (J.M. Vaughan, personal communication). Great care was taken to avoid undue excitement or significant blood loss in connection with reticulocyte preparations. In response to Gurd's (1935) suggestion that variations in staining technique and counting of reticulocytes could affect results, Muller (1935) referred to the heavy staining with brilliant cresyl-blue, as described in the 1930 report. She emphasized that only the newest young red cells of birds' blood show heavy reticulation and that only such cells and an evenly progressive rise in their percentage were accepted as a positive reticulocyte response.

The term 'megaloblastic' to describe the hyperplastic erythropoietic morphology of the pigeon's femoral bone marrow as seen in tissue sections in Boston at the time was that used by Doan *et al.* (1925). This is reflected by Muller's description of its erythroid cellularity as 'megaloblastic or early erythroblastic stage'. During the subsequent year, Jones (1936), using 'touch preparations', rather than tissue sections, clearly demonstrated that the pigeon's bone marrow was 'normoblastic', rather than of the special 'megaloblastic' character of the marrow in pernicious anaemia. This change in terminology did not affect the distinction between the general character of the marrow response to active liver extracts and a quite different morphological response to clinically inert leucine and lysine.

Both liver extract and these two nutritionally essential amino acids caused similar reticulocyte responses. As described by Muller (1935), however, and illustrated with photomicrographs, the response to liver extract involved a diminution of erythropoietic centers in the femoral marrow with loss of early erythroblastic forms. On the other hand, the reticulocyte responses to leucine and lysine were followed by retention of early erythroblasts in the femoral marrow and by striking erythroid hyperplasia of the normally fatty radial bone marrow. Further support for the original report of Vaughan *et al.* (1930) came when Wakerlin *et al.* (1936), counting only heavily reticulated red cells, as advised by Muller, observed somewhat dose-related maximum reticulocyte responses in grain-fed pigeons to four different clinically active liver extracts.

Pigeons and chickens both require dietary folic acid

By 1940 attention focused on the specific dietary requirements of pigeons and chickens in studies employing simplified diets

based on casein as the only source of protein, as had been used in the classical nutritional experiments with rats. Hogan *et al.* (1940) reported that on such a ration pigeons grew slowly and many developed anaemia. This was not prevented by additions of thiamin, riboflavin, nicotinic acid and wheat germ oil. A similar anaemia was observed in chicks and the unrecognized vitamins required were shown by Richardson *et al.* (1942) to be present in a Fuller's earth adsorbate of an acidified water extract of liver. Finally, Pfiffner *et al.* (1943) reported the isolation of the anti-anaemia factor for chicks in crystalline form (vitamin B_c) from an alkaline eluate of the Fuller's earth adsorbate of liver. During the subsequent year, Street (1944) reached a similar conclusion for the pigeon, thus indicating a nutritional requirement common to both species.

Hens and chicks both require dietary vitamin B_{12}

These nutritional experiments, based on casein as the source of protein, differed significantly from the diet of Vaughan's 'healthy' and exclusively grain-fed pigeons, but a closer dietary approximation was, perforce, soon to develop. With the advent of World War II the livestock industry in America, including the commercial poultry farmers, was faced with the severe problem of increasing food production without the supplements empirically found essential to sound and economical feeding programmes (The Farmer's wartime feeding problem. *Nutrit. Rev.* (1942–43) 1, 248–250). Of these additives, the most severe shortage was of conventional sources of animal protein, such as fish meal, meat scrap and dried milk (Emergency livestock nutrition. *Nutrit. Rev.* (1942–43) 1, 273–274). This persisted despite a tremendous increase in the production and use of processed soybean protein. Chickens and other animals failed to grow at the expected rate and, though hens on a purely vegetable diet laid eggs, many did not hatch. This was attributed to the poor quality of vegetable compared to animal protein.

This practical feeding problem only became the subject of scientific study with the end of the war and the recognition that the so-called 'animal protein factor', not the animal protein itself, was the unknown substance lacking in corn- and soybean-based chick feeds (Patton *et al.* 1946). It was noticed that in summer free-range hens with access to food particles contaminated by their droppings did not require the animal protein factor (Smith 1965) for good egg hatchability, but they did in the winter, when kept 'hygienically' in a laying battery on an all-plant-protein diet. Whitson *et al.* (1945) showed that 3% sardine fish meal and 8% dried cow manure were equally effective in improving the growth of chicks fed a diet containing 35% soybean oil meal and no animal protein. A year later Rubin & Bird (1946*a*) found that a chick growth factor in solubilized liver and cow manure was distinct from previously defined factors, including synthetic folic acid. It was obvious that a new micronutrient was involved when Rubin & Bird (1946*b*) prepared concentrates of cow manure factor that, when added at 3.75 to 7.5 mg per 100 g of a diet free of animal protein, supported optimal chick growth. The factor was also transmitted to their chicks through the eggs of laying hens kept on that diet.

Almost at once came other observations. Similarities were drawn between a 'factor X' present in concentrated anti-pernicious anaemia liver (Cary *et al.* 1946), the animal protein factor (Patton *et al.* 1946), and the cow manure factor (Rubin & Bird 1946*a*). Welch *et al.* (1946) and Stokstad & Jukes (1946) found very little *L. casei* factor (folic acid) in concentrated injectable anti-pernicious anaemia liver extract. Two years later Stokstad *et al.* (1948) discovered that animal protein factor was formed by an organism isolated from hen droppings when grown in a simplified medium. Concentrates of the medium when injected into chicks grown on experimental diets based on either soybean

meal protein or vitamin-free casein and supplemented with all the then known vitamin B complex factors enhanced their growth comparably to an injectable liver extract clinically evaluated at 10 USP units per millilitre.

The relevance of all this to pernicious anaemia was directly established when a suitable concentrated preparation made from the growth medium was administered parenterally to a severely anaemic pernicious anaemia patient (Stokstad *et al.* 1948). It caused a prompt, typical reticulocyte response followed by a gain in red cells, haemoglobin and health. Also in 1948 crystalline vitamin B_{12} was identified as the factor in purified liver extract erythropoietically active in pernicious anaemia (West 1948); and Ott *et al.* (1948) showed that, in amounts as small as 6 μg/kg crystalline vitamin B_{12} would promote growth in day-old chicks obtained from the eggs of hens fed the all-vegetable-protein diet of Rubin & Bird (1946*a*). The chicks were fed the same simplified diet as the hens or a diet based on vitamin-free casein supplemented with all the previously known essential vitamins.

Discussion and interpretation

Apparently others have not attempted to detect reticulocyte responses in grain-fed pigeons using vitamin B_{12} instead of the anti-pernicious anaemia liver extract fractions employed by Vaughan *et al.* (1930). However, the similar dietary requirement of pigeons and chickens for folic acid, when fed on special diets based on casein, has been described above, as well as that of hens and chickens for vitamin B_{12} when raised on simplified diets with protein supplied only by vegetable sources or by vitamin-free casein. Moreover, because, according to several references cited by Cartwright *et al.* (1951), mice, rats, chicks and pigs kept on diets containing only vegetable protein have shown growth responses to vitamin B_{12}, it would seem reasonable to assume that such closely related species as pigeons and chickens would share a similar need.

Johnson *et al.* (1950) studied baby pigs fed a soybean protein-based diet deficient in vitamin B_{12} and deprived of folic acid synthesized in the intestinal tract by sulphathalidine added to the feed (experiment II). They showed that, without supplementation with vitamin B_{12}, growth was retarded and that a marked reticulocyte response occurred when vitamin B_{12} was given and was followed by a second when folic acid was administered. Cartwright *et al.* (1951), using a similar vitamin B_{12}-deficient diet, but without an inhibitor of biosynthesis in the intestine, found growth to be much slower in young pigs than in others on the diet supplemented with vitamin B_{12}. Moreover, addition of vitamin B_{12} to the unsupplemented diet caused definite and sometimes marked reticulocyte increases without necessarily correcting the mild to moderate anaemia when present. The bone marrow was not megaloblastic and showed only a slight increase in normoblasts. Cartwright *et al.* (1951) stated that they did not supplement the vitamin B_{12}-deficient diet of their pigs with folic acid 'since it seems possible that . . . this vitamin in large amounts might protect the animals from the development of anaemia'. Accordingly, the probable dietary basis for the reticulocyte responses to the anti-pernicious anaemia liver extracts by Vaughan's exclusively grain-fed pigeons, also with normoblastic marrows, would seem to be due to some degree of vitamin B_{12} deficiency. They were receiving small amounts of folic acid in the grain (about 60 μg/100 g—United States–Canadian Tables of Feed Composition, 1982, Washington DC, National Academy Press), but no vitamin B_{12}—except in the liver extracts and beef-steak when given experimentally.

Coates (1962), an experienced British poultry scientist, has stated: 'If hens are given diets devoid of animal protein and kept on screens out of reach of their own droppings, after a few weeks the eggs they produce contain very little vitamin B_{12}.' The

gradual decline in reticulocyte levels of Vaughan's pigeons, while kept for 'some weeks' under similar dietary and housing conditions prior to selection for the experimental trials, may have a similar explanation. At any rate, four pigeons then showed typical reticulocyte responses to beef-steak feeding, a recognized source of vitamin B_{12} (Gardner *et al.* 1949), but a poor source of folate: about 5 μg/100 g (Chen *et al.* 1983). In the liver extracts studied by Vaughan *et al.* (1930) and by Muller (1935) there were, of course, both folic acid and vitamin B_{12}. Analysis of commercial liver extracts containing 15 USP injectable units per ml has shown that seven extracts averaged 0.19 μg of folate per USP unit (Clark 1945) and seven others averaged 0.34 μg of vitamin B_{12} per unit (Rickes *et al.* 1948). The pigeons were already receiving folic acid in the grain, but no vitamin B_{12}. In addition, a response to the folic acid in the liver extracts, rather than to the vitamin B_{12}, seems unlikely because in human vitamin B_{12} deficiency (pernicious anaemia) a detectable reticulocyte response to folic acid given intramuscularly requires 0.4 mg daily (Marshall & Jandl 1960), whereas a comparable reticulocyte response to intramuscular vitamin B_{12} requires less than 1 μg daily (Gardner *et al.* 1949), an amount about 1000 times as small.

A reasonable conclusion

The exclusively grain-fed pigeons in the pioneer study of their reticulocyte responses to liver extracts active in pernicious anaemia, as described by Vaughan, Muller and Zetzel in *The British Journal of Experimental Pathology* in 1930, were responding to vitamin B_{12}.

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