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VITAMIN B_{12}

E. Lester Smith

METHUEN'S MONOGRAPHS ON BIOCHEMICAL SUBJECTS

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General Editors' Foreword

Methuen's Biochemical Monographs are similar in form and aim to the series of Methuen's Monographs on other subjects. The volumes can be regarded as chapters of a large work which records progress in biochemistry in general. Each volume in the series aims to provide an authoritative survey of the present position in a particular field of biochemistry by an expert in that subject, written in such a way that the book can provide an introduction both for the student who is reaching the end of his undergraduate studies and for the research worker who wishes to have an account of a subject cognate with his own. Although these accounts of the subject are intended for those who wish to study biochemistry, in practice the books have proved to be attractive to a much wider group.

The books are intended to be handy and to be rather more than a review but less than a detailed monograph. No attempt has been made to include a complete bibliography, but references are given which should provide a key to the essential relevant literature.

As biochemistry widens its interests so the diversity of the subjects included in this series will grow. They naturally range from some which are primarily chemical in their emphasis to those which are essentially biological in their outlook.

'Vitamin B_{12}' by Dr E. Lester Smith stands in the middle of the range. The manner in which convergent researches

have reached a focus in Vitamin B_{12} provides the basis for a most interesting account of an important phase in the development of Biochemistry. Dr Lester Smith was the leader of one of the two groups which almost simultaneously isolated the pure vitamin so that his account of the earlier work, as well as that of more recent researches, is naturally an authoritative one. This book appears to be the first balanced review of the whole topic of Vitamin B_{12}. It is indeed a most timely addition to the present series of Monographs on Biochemical subjects.

R. A. P.
F. G. Y.

Preface

In the ten years since its isolation in 1948, vitamin B_{12} has been the subject of an immense amount of research, which still continues almost unabated and in many directions. Although the chemical structure has been fully elucidated by a brilliant application of X-ray crystallography techniques, aided by chemical degradation, interest persists in the chemical fields of biogenesis, analogues and derivatives and attempts at synthesis. Work is also actively proceeding on absorption mechanisms and biochemical functions and on other aspects of the subject. Thus a monograph written at this stage can only be a review, or progress report, with many of the important questions left without final answers. The state of knowledge changed even while it was being written and some of the earlier chapters had to be extensively revised. A great deal had to be omitted: the proceedings of just one of the several symposia on vitamin B_{12} occupy a book about six times this size. Apologies are accordingly offered in advance to anyone who may feel slighted because their contributions have necessarily been passed over. Nevertheless this does appear to be the first attempt to compile a balanced review of the whole topic, and it may well prove useful to those who have neither time nor necessity to study it more extensively. Even for the specialist it may serve for some years as a useful reference book, since an effort has been made to include all the more significant papers up to the middle of 1958 in

the bibliographies. Grateful thanks are due to all who have permitted reproduction of tables, and especially to those good friends who have read the manuscript and made constructive suggestions; these include Drs S. K. Kon, E. S. Holdsworth, J. W. G. Porter, Marie Coates, Margaret Gregory, J. E. Ford, and J. Pawelkiewicz: also to Mr A. L. Bacharach for his invaluable help as a semantic sieve rejecting all ambiguities and faults in English usage, redundant words and gaucheness of expression. If any of these remain, they must be in sections written after his editing: and finally to Miss D. M. Mathews who checked the references, and to my tireless secretary Miss Sylvia Gore who typed and retyped the manuscript.

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CHAPTER 1

Convergent Researches

It is possible to trace nine originally independent lines of investigation that all converged upon the treatment of megaloblastic anaemias. Five of them led to vitamin B_{12}, the other four to the functionally related substance, folic acid or pteroylglutamic acid.

Anti-pernicious anaemia factor

Pernicious anaemia is a macrocytic anaemia; the red blood cells are abnormally large, but relatively few in number, 1–3 million per cu.mm. instead of the normal 4·5–6 million. The bone marrow is megaloblastic; the blood-forming cells have become enlarged while still immature. In 1920 Whipple (1) found that feeding liver accelerated the regeneration of red cells in dogs made anaemic by bleeding. Minot and Murphy (2) in 1926 followed up this discovery by trying liver for the treatment of pernicious anaemia, which at that time was incurable and almost always fatal. They found that about half-a-pound a day of raw or lightly cooked liver brought about a remarkable improvement in their patients. They soon felt and looked better in health, and the blood picture slowly returned to normal. An earlier sign was a dramatic rise in the proportion of reticulocytes in the blood cells, so called because these newly formed red cells, when suitably stained, display a net-like appearance under the microscope. The proportion of reticulocytes could rise in a week or two to 10 or even 20% of the total red cells,

and then slowly fall again to the normal one per cent or two.

Clearly liver must contain something, then called the 'liver factor' or the 'anti-pernicious anaemia factor' (A.P.A.F.) that brought about these striking effects. Cohn (3) started in 1928 a long series of investigations into the extraction of this factor from liver. But another 20 years were to elapse before it was isolated in the pure state and called vitamin B_{12}.

The long delay was due largely to the absence of any condition in animals analogous to pernicious anaemia, and the need therefore to use pernicious anaemia cases in relapse for the 'assay' of concentrates. Suitable patients were not available in adequate numbers, and the test lacked precision. The problem might have been solved earlier had it been realised that the liver factor was identical with other factors under investigation towards the end of the period. However, there were other difficulties as well, such as the lack of any chemical characteristics that could be exploited readily in the isolation, and notably the extremely low concentrations of this exceptionally potent substance in natural sources: liver usually contains less than one part per million of vitamin B_{12} activity.

Finally, isolation of the pure crystalline factor was announced by two independent teams within the space of a few weeks (4, 5). In view of the large bulks of starting materials that had to be processed, it is not surprising that this work was done in industrial laboratories, namely, Merck in America and Glaxo Laboratories in England.

Extrinsic and intrinsic factors

Pernicious anaemia is also characterised by achlorhydria, a failure to secrete hydrochloric acid (6), and by partial atrophy of the mucous membrane of the stomach (7).

These observations made over 40 years previously led Castle, in 1928, to a different method of treatment (8). He thought that the atrophied stomach glands might be failing to secrete some essential kind of digestive juice, which he later called 'intrinsic factor'. This was supposed to act upon something present in certain foods, called the 'extrinsic factor', to yield the 'liver factor', as the product of this reaction. The intrinsic factor could be supplied as gastric juice from a normal human stomach, and its administration, along with meat as the source of extrinsic factor, proved an effective treatment for pernicious anaemia. Later, hog stomach, dried at low temperature, was found to be a more convenient source of intrinsic factor. This discovery led inevitably to two more quests. The intrinsic factor, a labile protein, is still not fully characterised. The extrinsic factor was never isolated as such, but when vitamin B_{12} became available it was found itself to have the properties of extrinsic factor. That is to say, the function of intrinsic factor is to promote the intestinal absorption of dietary vitamin B_{12}. Accordingly it has been said that Castle's theory was wrong or required modification. However, this really depends upon what definition one accepts for the 'liver factor'. It is usually taken to mean vitamin B_{12}, but in liver the vitamin is known to be loosely bound to protein, and this complex, or a related protein-bound B_{12}, may well arise from the interaction of the vitamin with intrinsic factor. This problem is still under investigation but the Castle theory may yet be fully vindicated—possibly by Castle himself, who is still working actively in the field. In retrospect, one can see that the identity of extrinsic factor with the factor in liver extracts might have been established much earlier, for already in 1931 Reimann (9) had demonstrated that the efficacy of oral liver extract was enhanced about 30-fold by giving it with intrinsic factor.

Animal protein factor

During the second World War attempts were made to raise pigs and poultry on purely vegetable rations. They were not altogether successful; the animals and birds failed to grow at the expected rates, and although hens laid eggs on such diets, many of them failed to hatch. These conditions could be rectified by including animal or fish waste in the diets, so the term 'animal protein factor' (A.P.F.) was coined for the substance missing from the corn and other vegetable feedstuffs. The Factor X of Cary and Hartman, and the zoopherin of Zucker and Zucker, both discovered by rat growth tests, were later shown to be identical with A.P.F. Bird and his colleagues in Maryland used tests involving the hatchability of eggs in their search for other sources of the factor, and made some progress towards its isolation. Dried cow manure was found to be a rich source. Also, hens on range in the warmer months of the year could manage without A.P.F. in their diet: it was found that they were getting it from food particles fermented by organisms from their own droppings. These two observations pointed to fermentation as a possible means for producing the factor. Accordingly, a product derived from fermenting a simple medium with an organism from hen faeces was tested and found to have A.P.F. activity. Some years earlier, liver extracts used for the treatment of pernicious anaemia, had been shown to contain A.P.F. factor, so the converse experiment was tried of trying this fermentation product in pernicious anaemia (10): it was indeed effective, suggesting that A.P.F. and A.P.A.F. were identical or at least closely related. From subsequent work it appears that although vitamin B_{12} certainly increases growth rates of animals and birds on vegetable protein diets, the crude 'animal protein factor' may contain other unidentified beneficial factors as well.

In any event, these investigations had an importance far greater than could be foreseen at the time, because they pointed the way to economic sources of vitamin B_{12}. Examination of industrial fermentations already established soon showed that two of them were already producing vitamin B_{12}, namely, fermentations with *Streptomyces griseus* and *Streptomyces aureofaciens* for streptomycin and aureomycin respectively. These discoveries enabled vitamin B_{12} to be produced commercially within a year or two of its isolation, at a fraction of the cost of extraction from liver.

L.L.D. factor

The fourth line of investigation that led to vitamin B_{12} was a study of the essential nutrients for certain micro-organisms. Vitamins of the B group are growth factors for most bacteria, as well as for higher organisms. Some bacteria make for themselves all they need, but others rely upon an external supply of one or more vitamins. These requirements have been gradually uncovered by attempts to replace natural media, such as meat infusions, by mixtures of known substances, such as sugars and amino-acids, in suitable proportions. Often little or no growth occurred unless small proportions were added of some complex natural mixture like yeast or liver extract. The way was then open to fractionation of the preparation, leading eventually to isolation of the active principle. Sometimes this would turn out to be a known amino-acid, peptide, purine or pyrimidine, absent from the 'synthetic medium' or present at too low a level. In one instance the missing factor was simply glucose. In others, however, this approach has led to the isolation of new growth factors. Some of these were factors required only by certain micro-organisms; *p*-hydroxybenzoic acid, for example, was tracked down by this approach. Though a known substance, it was not previously