THE BINDING OF CYANOCOBALAMIN AND ITS NATURALLY OCCURRING ANALOGUES BY CERTAIN BODY FLUIDS AND TISSUE EXTRACTS

MARGARET E. GREGORY AND E. S. HOLDSWORTH*

National Institute for Research in Dairying, Shinfield, Reading (Great Britain)

(Received January 13th, 1960)

SUMMARY

Chicken serum, an extract of chicken proventriculi and crude and purified extracts of pig pyloric mucosa combined with about equal amounts of cyanocobalamin, factor A, pseudovitamin B_{12} or factor B. In contrast, normal human gastric juice bound factor B significantly less. Preferential binding of cyanocobalamin from mixtures with its naturally occurring analogues was demonstrated with human gastric juice and preparations from pig stomach mucosa. The mechanism whereby animals absorb and retain cyanocobalamin in their body tissues in preference to the other naturally occurring analogues is discussed.

INTRODUCTION

The naturally occurring analogues of vitamin B_{12} are, with the exception of vitamin B_{12III}, inactive in curing pernicious anaemia in man, and are unable to replace vitamin B_{12} as animal growth factors[1]. These analogues are known to be present in appreciable quantities in fermented materials and to arise in the rumen or intestine by microbial synthesis.

It is well established that in man the absorption of vitamin B_{12} depends on the presence of an "intrinsic factor" in the gastric juice and there is evidence that combination with specific glycoproteins plays an important part in the absorption and retention of vitamin B_{12} in man and other animals[2]. We have already shown that the vitamin B_{12} analogues—factor A, pseudovitamin B_{12} and factor B—are bound to the same extent as cyanocobalamin** by concentrates prepared from sow's milk and from desiccated pig stomach[3]. Since body tissues contain predominantly vitamin B_{12} with only a trace of the other factors present, it is evident that animals either have some mechanism for preferentially absorbing vitamin B_{12} from mixtures with its analogues, or some mechanism whereby vitamin B_{12} and its analogues are all absorbed from the intestine but only vitamin B_{12} is retained[4,5].

In this paper some experiments are described which show that the binding

* Present address: Department of Biochemistry, University of Adelaide.

** The term cyanocobalamin denotes a particular compound whereas vitamin B_{12} refers to the naturally occurring vitamin, which is not necessarily in the cyano-form.

components from pig stomach and human gastric juice combine preferentially with cyanocobalamin in the presence of its naturally occurring analogues. This work was briefly referred to by KON[5]. We have also extended our previous studies on the binding of factor A, pseudovitamin B_{12} and factor B by concentrates from sow's milk and pig stomach[3] to include human serum and gastric juice, chicken serum and a chicken proventriculus extract (*i.e.* an extract prepared from the glandular stomach of the chicken).

MATERIALS

A bulk sample of human serum from pernicious anaemia patients, and a sample of neutralized normal human gastric juice were kindly given to us by Dr. D. L. MOLLIN (Postgraduate Medical School, London). The chicken serum was obtained by exsanguination of freshly killed animals. The chicken proventriculus extract was prepared as described by GREGORY AND HOLDSWORTH[2]. Bendogen is a commercial preparation of "intrinsic factor" from pig pyloric mucosa (G.E.A., Copenhagen). Two purified intrinsic factor concentrates were prepared from it. Preparation 1 was obtained by fractionation on a DEAE cellulose ion exchange column as described by HOLDSWORTH[6] and preparation 2 by treatment with acid and papain as described below. Extomak is a commercial brand of desiccated hog stomach prepared by Benger's Ltd., Holmes Chapel, Cheshire (Great Britain). The concentrate was prepared from it as described elsewhere[3]. [^{60}Co]cyanocobalamin of specific activity 42 μC/mg, and [^{60}Co]factor B of specific activity, 200 μC/mg and unlabelled cyanocobalamin (Cytamen) were kindly supplied by Glaxo Laboratories Ltd., Greenford, Middlesex (Great Britain). Factor A, pseudovitamin B_{12} and factor B were purified, and standard solutions prepared as described by GREGORY AND HOLDSWORTH[3]. [^{60}Co]-pseudovitamin B_{12} was prepared by the method of PFIFFNER, DION AND CALKINS[7]. Vitamin B_{12III} was kindly given by Professor K. BERNHAUER, Aschaffenburger Zellstoffwerke, A.G., Stockstadt-am-Main (Germany).

Purified intrinsic factor

Preparation 2. 20 g of Bendogen were suspended in 200 ml of 10% (v/v)HCl. The insoluble material was removed by centrifuging and the clear supernatant liquid immediately dialysed to remove the HCl. The dialysed solution was incubated at pH 4.5 for 6 h at 40° with the addition of 100 mg of papain (British Drug Houses Ltd) activated with cyanide. The digest was then dialysed against running water for 48 h and then against several changes of distilled water before freeze-drying. The 2.5 g of dry material obtained bound 1.23 μg of cyanocobalamin/mg as measured by the ultrafiltration method[8]. Dr. D. L. MOLLIN (Post graduate Medical School, London) very kindly tested the preparation in a patient with pernicious anaemia and found that it still had intrinsic factor activity.

METHODS

Binding of the analogues of vitamin B_{12}

The binding of cyanocobalamin, factor A, pseudovitamin B_{12} and factor B by the various test materials was measured by the ultrafiltration method of GREGORY

AND HOLDSWORTH[8]. The ultrafiltrates containing the unbound cyanocobalamin, factor A or pseudovitamin B_{12} were assayed with both *Escherichia coli* mutant 113-3 and *Lactobacillus leichmannii* ATCC 4797 as the test organisms[9]. Since factor B is inactive for *L. leichmannii*, the ultrafiltrates containing it were assayed only with *E. coli*. The analogues of vitamin B_{12} each differ in their growth promoting activity for these two test microorganisms and therefore separate standards containing known amounts of the analogues were included in each test. The amounts of the analogues bound by the test materials was calculated in the same way as for cyanocobalamin[8], but by reference to the standard of the particular analogue under test.

With certain of the test materials the binding activities were measured with [^{60}Co]cyanocobalamin, [^{60}Co]pseudovitamin B_{12} and [^{60}Co]factor B as described by GREGORY AND HOLDSWORTH[10].

Preferential binding of cyanocobalamin in the presence of its analogues

Measurement by a bioautograph technique: An aqueous solution of the concentrate from Extomak, sufficient to bind 1.0 μg of cyanocobalamin, was added to mixtures containing 0.8, 0.7, 0.6, 0.5 or 0.4 μg each of cyanocobalamin, factor A, pseudovitamin B_{12} and factor B. The resulting solutions, at final volumes of 10 ml in 0.9 % (w/v) saline, were ultrafiltered. Any unbound factors passed into the ultrafiltrates. Their presence was then detected by taking 10 μl of each ultrafiltrate and subjecting it to ionophoresis on paper in 1 *N* acetic acid[11]. By the bioautograph technique of FORD AND HOLDSWORTH[12], with *E. coli* as the test organism and 2,3,5-triphenyltetrazolium chloride included in the agar medium, the positions of the separated factors were revealed as red zones of growth on a colourless background. In addition to acting as a visual aid in detecting the areas of growth on the plate, the red formazan pigment, formed by reduction of the 2,3,5-triphenyltetrazolium chloride, can be used for the quantitative estimation of the amount of factor present on the plate. The pigment from each zone was eluted with tetrahydrofuran and the amount of colour compared with that obtained on the same plate with spots containing known amounts of the different factors.

Measurement with [^{60}Co]cyanocobalamin: An aqueous solution of the purified papain-treated Bendogen, sufficient to bind about 1.3 μg of cyanocobalamin, was mixed with a solution containing 4 μg of [^{60}Co]cyanobocalamin either alone or with 4 or 20 μg of unlabelled factor A, pseudovitamin B_{12}, factor B, or vitamin B_{12III}. The volumes were made to 10 ml in 0.9 % saline and the solutions were ultrafiltered. Free cyanocobalamin was determined from the amount of radioactivity present in the ultrafiltrate[10].

RESULTS

Binding of the analogues of vitamin B_{12}

The amounts of cyanocobalamin, factor A, pseudovitamin B_{12} and factor B bound by normal human gastric juice, human serum and chicken serum and by a chicken proventriculus extract and crude and purified extracts from pig pyloric mucosa are shown in Table I. The figures in this table, for results of microbiological tests used to measure the unlabelled analogues, are mean values from at least three assays. However, the amounts of radioactive compounds available at the time were sufficient for duplicate estimations in only one test with each binding factor.

TABLE I

MEAN VALUES FOR THE AMOUNTS OF CYANOCOBALAMIN, FACTOR A, PSEUDOVITAMIN B_{12} AND FACTOR B BOUND BY CERTAIN BODY FLUIDS AND TISSUE EXTRACTS

		Activity in the ultrafiltrate measured by:	*Cyanocobalamin bound µg/ml*	*Factor A bound µg/ml*	*Pseudovitamin B_{12} bound µg/ml*	*Factor B bound µg/ml*
Human gastric juice (normal)		*E. coli*	0.14	0.12	0.08	0.04
		L. leichmannii	0.15	0.12	0.11	—
		Radioactivity	0.12	—	—	—
Human serum (Pernicious anaemia)	Sample 1	*E. coli*	0.0031	0.0046	0.0080	approx. 0.012
		L. leichmannii	0.0018	0.0033	0.0039	—
	Sample 2	Radioactivity	0.0020	—	—	0.0009
Chicken serum	Sample 1	*E. coli*	0.50	0.54	0.62	0.70
		L. leichmannii	0.62	0.67	0.62	—
	Sample 2	*L. leichmannii*	1.10	1.01	1.01	—
		Radioactivity	1.10	—	—	1.00
			µg/g	*µg/g*	*µg/g*	*µg/g*
Chicken proventriculus extract		*E. coli*	27	33	36	33
		L. leichmannii	33	41	37	—
		Radioactivity	33	—	—	24
Pig pyloric mucosa extract (Bendogen)		*E. coli*	210	220	240	210
		L. leichmannii	204	220	220	—
		Radioactivity	200	—	240	260
Purified intrinsic factor	Preparation 1	*L. leichmannii*	9,600	10,000	9,500	—
		Radioactivity	10,000	—	—	11,800
	Preparation 2	*E. coli*	1,230	—	—	—
		Radioactivity	1,300	—	1,400	—

The two samples of chicken serum, the proventriculus extract, and the crude and purified pig pyloric mucosa extracts combined with about equal amounts of cyanocobalamin and its analogues when these were tested individually. This finding confirms our previous work with sow's milk and a pig stomach extract, when a different technique for measuring binding activity was used[3].

The human gastric juice and serum showed different patterns of behaviour. The gastric juice bound slightly less factor A and pseudovitamin B_{12} than cyanocobalamin. The binding of factor B was significantly less than for cyanocobalamin. The opposite effect was observed with the human serum, which appeared to combine with more factor B than cyanocobalamin. However, it was observed that all the values for binding activity obtained when *E. coli* was used to test the vitamin activity of the serum ultrafiltrates were higher than when *L. leichmannii* was the test organism. *L. leichmannii* requires lower concentrations of cyanocobalamin, factor A and pseudovitamin B_{12} for growth, and therefore the ultrafiltrates can be tested at a more dilute concentration. We have often noticed that *E. coli* does not grow as well with factor B as with cyanocobalamin. When known amounts of factor B were added to an ultrafiltrate of human serum, 70 % of it was available to *E. coli* and when cyanocobalamin was added to the ultrafiltrate, a full recovery was obtained with both *L. leichmannii* and *E. coli*. Thus, serum may contain factors which slightly inhibit the growth of *E. coli* in the presence of factor B, but not in the presence of cyanocobalamin. However, an inhibition of some 30 % of the growth activity of factor B does not entirely account for the very low activities measured in these ultrafiltrates. The human serum and gastric juice were both tested at the same time as the Bendogen, so that it was certain that the different results were not due to some error in the technique of measurement of the binding activity. The one test done with [^{60}Co]-factor B on a different sample of serum indicated that less factor B was bound than cyanocobalamin.

Preferential binding of cyanocobalamin in a mixture of analogues

When a mixture containing 0.8 μg each of cyanocobalamin, factor A, pseudo vitamin B_{12} and factor B was added to a pig stomach preparation, which could bind only 1.0 μg of vitamin B_{12}-like factors, about equal amounts of each factor appeared in the ultrafiltrate. This finding is shown in Table II and indicates that under these

TABLE II

PREFERENTIAL BINDING BY A PIG STOMACH PREPARATION OF CYANOCOBALAMIN IN THE PRESENCE OF EQUAL AMOUNTS OF FACTOR A, PSEUDOVITAMIN B_{12} AND FACTOR B

10 mg of Extomak concentrate bind 1 μg of vitamin B_{12}-like factors.

Amount of each factor added (μg)	*Amount of each factor bound expressed as a percentage of the amount added*			
	Cyanocobalamin	*Factor A*	*Pseudovitamin B_{12}*	*Factor B*
0.8	31	29	31	26
0.7	34	28	31	27
0.6	38	28	33	27
0.5	53	38	46	37
0.4	93	58	69	48

conditions no one of the factors was preferentially bound. As the proportion of cyanocobalamin and its analogues to binding factor was reduced, so the amount of cyanocobalamin bound increased. Finally when 0.4 μg of each factor were present, almost all the cyanocobalamin had become bound, while substantial amounts of the other factors still appeared in the ultrafiltrate. Similar results were obtained with normal human gastric juice, which was tested with 0.8 μg or 0.4 μg of each factor. All factors appeared in the ultrafiltrate at the higher level of addition, but the cyanocobalamin did not at the lower level of addition.

In a further series of experiments the combination of [^{60}Co]cyanocobalamin with the purified pig pyloric mucosa extract was measured in the presence of different compounds structurally related to cyanocobalamin. The results are given in Table III. They show that when equal amounts of cyanocobalamin and of one other structurally related compound competed for a limited amount of binding factor, cyanocobalamin was preferentially bound. Even in the presence of a five-fold excess of the competing compound, substantial amounts of cyanocobalamin were still bound.

TABLE III

COMPETITION BETWEEN [^{60}Co]CYANOCOBALAMIN AND ITS ANALOGUES FOR THE BINDING SITES IN PURIFIED PAPAIN-TREATED BENDOGEN

Analogue added	*μg[^{60}Co]cyanocobalamin bound/mg of Bendogen*	
	4 μg analogue present	*20 μg analogue present*
None	1.3	1.3
Factor A	0.9	0.8
Pseudovitamin B_{12}	0.9	0.5
Factor B	0.8	0.7
Vitamin B_{12III}	0.8	0.5

DISCUSSION

The *in vitro* experiments, described in this paper, have shown that there is a marked difference in the behaviour of the binding factors of human origin and those from the pig and chicken. When three of the naturally occurring analogues of cyanocobalamin —factor A, pseudovitamin B_{12} and factor B—were tested individually with chicken serum or extracts of chicken proventriculi or of pig pyloric mucosa, each of them was bound to the same extent as cyanocobalamin. The human serum also bound each of the analogues, but possibly more so than cyanocobalamin. In contrast to these findings, the human gastric juice bound much less factor B and probably less factor A and pseudovitamin B_{12} than cyanocobalamin.

We have previously shown that cells of the protozoan *Ochromonas malhamensis*, grown with limiting cyanocobalamin, were able to take up about equal amounts of factor A, pseudovitamin B_{12} or cyanocobalamin when these were added individually to the medium[13]. Very little, if any, factor B was taken up. We have since found that extracts of disrupted *Ochromonas* cells can combine with cyanocobalamin, factor A and pseudovitamin B_{12} and render them non-ultrafiltrable. Factor B was not bound.

Thus the uptake of the analogues of cyanocobalamin by *Ochromonas* cells and cell extracts resembles the binding of these analogues by human gastric juice.

Our results with normal human gastric juice point to a different type of binding mechanism from the one present in human serum and in extracts of pig pyloric mucosa. BUNGE, SCHLOESSER AND SCHILLING[14] came to a similar conclusion for human gastric juice and serum. In *in vitro* experiments they showed that pseudovitamin B_{12} did not interfere with the binding of [^{60}Co]cyanocobalamin by gastric juice, whereas it competed with [^{60}Co]cyanocobalamin for the binding sites in human serum. Furthermore, an oral dose of 50 μg of pseudovitamin B_{12} had no effect on the absorption of 1 μg [^{60}Co]cyanocobalamin administered with 50 ml of normal human gastric juice to a pernicious anaemia patient. COATES, DAVIES, DAWSON, HARRISON, HOLDSWORTH, KON AND PORTER[15] have shown that for the chick, large amounts of pseudovitamin B_{12}, given orally, antagonized the growth promoting effect of cyanocobalamin, although if both were given by injection there was no adverse effect. They concluded that its antagonistic effect was due to competition with cyanocobalamin for the absorption mechanism. If one can compare chick growth tests with clinical tests, it would seem that the mechanism of absorption of vitamin B_{12} differs in the chicken and in man, the one being antagonized by pseudovitamin B_{12}, whereas the other is not. An antagonistic effect of pseudovitamin B_{12} on the growth of *Ochromonas* has been reported by FORD[16]. In this respect therefore the absorption mechanism in *Ochromonas* resembles that in the chicken. However, the two differ in that factor A is an inhibitor and factor B inactive for *Ochromonas* whereas factor A is inactive and factor B an inhibitor of growth for the chick[15].

Our *in vitro* experiments also show that under certain conditions cyanocobalamin can be preferentially bound in the presence of its analogues. One of the binding factors used for these tests was a crude preparation from desiccated pig stomach (Extomak), which is a potent source of both "intrinsic factor" and cyanocobalamin-binding activity. Cyanocobalamin-binding activity is not synonymous with "intrinsic factor" activity, and indeed HOLDSWORTH[6] has shown that the pig pyloric mucosa preparation (Bendogen) can be purified by fractionation on a DEAE cellulose column and two similar substances with binding activity obtained only one of which has "intrinsic factor" activity. We show here that such a purified "intrinsic factor" preparation from Bendogen bound equal amounts of each of the analogues when they were tested individually. Unfortunately this preparation was not tested for preferential binding of cyanocobalamin in the presence of its analogues, but a sample of normal human gastric juice with intrinsic factor activity showed the same preferential uptake of cyanocobalamin as the crude pig stomach preparation.

Our *in vitro* experiments were limited by the fact that we were able to study only those tissue extracts or body fluids that have high binding activities. However, our results do show that the absence of factor A and pseudovitamin B_{12} from body tissues is not due to their inability to enter into combination with the binding factors in the intestinal tract. It is more likely that these binding factors, or the receptor sites on the intestinal wall, or the body tissues themselves, have a higher affinity for cyanocobalamin than for its analogues and that the analogues, if absorbed, are quickly excreted. The high proportion of the factor A, pseudovitamin B_{12} and factor B in cow's urine[5] supports this theory.

REFERENCES

[1] M. E. COATES AND S. K. KON, *Vitamin B_{12} und Intrinsic Factor*, Hamburg, 1956, p. 72.
[2] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 72 (1959) 549.
[3] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 55 (1953) 830.
[4] J. E. FORD, E. S. HOLDSWORTH AND J. W. G. PORTER, *Proc. Nutrition Soc. (Engl. and Scot.)*, 12 (1953) xi.
[5] S. K. KON, *Biochem. Soc. Symposia (Cambridge, Engl.)*, 13 (1955) 17.
[6] E. S. HOLDSWORTH, *Biochem. J.*, 66 (1957) 59P.
[7] J. J. PFIFFNER, H. W. DION AND D. G. CALKINS, *Federation Proc.*, 11 (1952) 269.
[8] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 59 (1955) 329.
[9] M. E. GREGORY, *Brit. J. Nutrition*, 8 (1954) 340.
[10] M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 66 (1957) 456.
[11] E. S. HOLDSWORTH, *Nature*, 171 (1953) 148.
[12] J. E. FORD AND E. S. HOLDSWORTH, *Biochem. J.*, 53 (1953) xxii.
[13] J. E. FORD, M. E. GREGORY AND E. S. HOLDSWORTH, *Biochem. J.*, 61 (1955) xxiii.
[14] M. B. BUNGE, L. L. SCHLOESSER AND R. F. SCHILLING, *J. Lab. Clin. Med.*, 48 (1956) 735.
[15] M. E. COATES, M. K. DAVIES, R. DAWSON, G. F. HARRISON, E. S. HOLDSWORTH, S. K. KON AND J. W. G. PORTER, *Biochem. J.*, 64 (1956) 682.
[16] J. E. FORD, *J. Gen. Microbiol.*, 19 (1958) 161.

Biochim. Biophys. Acta, 42 (1960) 462–469

METAL BINDING PROPERTIES OF CHONDROITIN SULPHATE

I. G. F. GILBERT AND N. A. MYERS

Physics Department, Institute of Cancer Research, Royal Cancer Hospital, London (Great Britain)

(Received January 14th, 1960)

SUMMARY

The interactions of H^+, Ca^{2+}, Y^{3+}, La^{3+}, with chondroitin sulphate of cartilage have been studied using a micropotentiometric titration technique. These ions are bound to the carboxyl groups of chondroitin sulphate by the formation of 1:1 complexes, with intrinsic association constants in KCl solution (with the ionic strength in parentheses); H^+ log $K = 3.06 \pm 0.02$ (0.1); 2.92 ± 0.02 (1.0); Ca^{2+} log $K = 0.42 \pm 0.09$ (1.0); Y^{3+} log $K = 0.61 \pm 0.05$ (1.0); La^{3+} log $K = 0.79 \pm 0.03$ (1.0). The electrostatic contribution to the binding constant, due to the charge of the polymeric molecule, accords with the "nearest-neighbour interaction" description of the titration curve. The influence of the sulphate groups on these results is discussed and is considered small.

INTRODUCTION

Chondroitin sulphate is a poly-β-D-glucopyranosyluronic acid 2-deoxy-2-acetylamino-galactose sulphate[1], which occurs in bone, and as a major constituent of cartilage, probably in the form of chondromucoprotein, in which it is bound to a noncollagenous protein[2]. There appear to be two closely related forms, the A and C isomers, in which the ester sulphate group is in position 4 and 6 of the galactose ring respectively[3].