ACTA CHEMICA SCANDINAVICA 16 (1962) 1999–2004

The Effect of Gamma Radiation on Cyanocobalamin

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Solutions of cyanocobalamin have been irradiated with various doses of γ-rays from a ^{60}Co-source. A number of breakdown fragments that could replace the vitamin as growth factor for *E. coli* 113—3 were detected. Some of these fragments seem to possess activity also towards *Ochromonas malhamensis*. The types of biologically active breakdown products formed and the degree of loss of microbiological activity of irradiated solutions were found to depend on a number of conditions, *e.g.* the dose given, the ionic strength, pH, presence of reducing substances and so forth. In a 0.1 M phosphate buffer of pH 5.0 containing 5 mg cyanocobalamin per ml and given a dose of 2 Mrad at least five compounds are formed that are growth factors for *E. coli* 113—3.

The radiosensitivity of cyanocobalamin in distilled water and raw milk was first established by Markakis *et al.* [1] in 1951. After several years of study, Lester Smith published in 1959 a paper [2] on the instability of vitamin B_{12} containing radioactive cobalt isotopes. This self-degradation has later been confirmed by other authors [3–5]. Burtseva *et al.* [4] have shown that labelled crystalline vitamin B_{12} prepared by neutron radiation disintegrates, when dissolved in water, giving three different coloured zones on a chromatogram. The work presented here has been mainly directed to a search for microbiologically active fragments formed on exposure of the vitamin to γ-rays. Fragments with such properties have been described in preliminary communications from this laboratory [6,7] and have also been observed by Barlow and Sanderson [3]. The latter investigators have found in self-degradated ^{60}Co-vitamin B_{12} a monocarboxylic acid derivate which was about one third as active in a microbiological test system as cyanocobalamin.

In this paper we will present a more detailed description of our experiments. Only model systems with pure cyanocobalamin in buffer solutions have so far been studied.

EXPERIMENTAL

Vitamin solutions. The vitamin cyanocobalamin (Merck, Sharp & Dohme, Haarlem, Netherland N.V.) was irradiated in buffer solutions. In most cases, 5 mg/ml was dissolved in 0.1 M phosphate buffer of pH 5.0. When other buffers and concentrations were used, this will be pointed out when the results are described.

Irradiation. The irradiations were performed in ^{60}Co gamma sources at Conservatom, Lyon, France, at the Danish Atomic Energy Commission, Risö, Roskilde, Denmark, at the University of Stockholm, and at the Swedish Institute for Food Preservation Research, Gothenburg. The vitamin solutions were subjected to various doses. These will be specified in the part of the paper in which the results of the separate experiments are presented. The effect of doses ranging from 0.01—15 Mrad was investigated but in most cases a dose of about 2 Mrad was employed.

Microbiological test systems. In order to determine the residual microbiological activity of the irradiated solutions of cyanocobalamin, tube assays with *Escherichia coli 113—3* ATCC 11105 and *Ochromonas malhamensis* ATCC 11532 were performed.

In a preliminary tube assay with *E. coli* 113—3, a simple medium — a modification of that of Diding [8] — was compared with a more complicated one used by Burkholder [9]. As the two media gave identical result, the simpler one was chosen for all subsequent tube assays. It had the following composition: Glucose 10 g, sodium-citrate·$2H_2O$ 0.5 g, KH_2PO_4 3 g, K_2HPO_4 7 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, thiomalic acid 100 mg, KCN 1 mg, H_2O up to 600 ml, pH 6.8. To each test tube were added 5 ml medium and 5 ml test solution. The tubes were autoclaved for 5 min at 115°C, cooled and inoculated with two drops of a bacteria suspension. This was obtained by growing the bacteria overnight in a Bacto Micro Inoculum Broth (Difco), harvesting the cells by centrifugation, washing them twice with sterile saline, suspending them in saline to a transmittance reading of 70 % and diluting this suspension 1:50 with saline. The tubes were incubated at 37°C and the growth after 20 h was determined by turbidimetric measurements at 640 mμ in a Coleman Model 11 Universal spectrophotometer. A standard curve was obtained by means of a suitable series of dilutions of cyanocobalamin. The test solutions from irradiated vitamin B_{12} were diluted to give a growth falling within the range of the standard curve.

The *O. malhamensis* tests were in the main performed by means of Ford's method [10]. To each tube were added 5 ml test solution and 5 ml single strength medium prepared according to Ford [11]. The tubes were autoclaved for 10 min at 115°C, cooled and inoculated with 1 drop of an undiluted 4 day culture maintained as described in reference [11]. The tubes were shaken in darkness at 29°C and the growth was measured turbidimetrically at 24 h intervals from the second to the seventh day after the inoculation.

Separation methods. Paper chromatography of the irradiated vitamin B_{12} solutions was initially carried out in a solvent system consisting of *sec.* butanol:H_2O:HAc (75:24:1) supplemented with 1 ml of a 0.01 % KCN solution. Since no good separation could be attained even when the chromatograms were run for 48 h we were forced to try modified solvent systems. Friedrich *et al.*[12] have found that some salts (called "chromatotropic salts"), when added to the developer, had a positive effect on the separation of vitamins of the B_{12} group. We tried some of these and got the best separation in the shortest time with the following developer: *sec.* butanol:H_2O:KCN (0.01 % solution) (71:28:1) + 200 mg sodium tetraphenylboron. Whatman No. 4 filter paper and the descending technique were used. The papers were run for about 17 h at 21°C and dried at room temperature.

Electrophoresis of the irradiated vitamin B_{12} solutions was performed with an LKB paper electrophoresis apparatus. In most cases a buffer of 0.2 M HAc, pH 2.8, current of 0.2 mA, tension of 350 V and electrophoresis time of 16—17 h were used. The apparatus was operated in a room with a temperature of 2°C. The papers were dried at room temperature.

Location of spots. When large quantities of the irradiated solutions were applied to the chromatography and electrophoresis papers, different coloured spots could be observed after the separation. Since our main interest was to look for biologically active fragments, we applied however microbiological methods in order to reveal the positions of the substances. The papers were placed on agar plates seeded with *E. coli* 113—3. The plates were prepared as described by Bolinder and Larsen [13] of this laboratory and contained (per 150 ml medium) 5 ml of a bacteria suspension having a transmittance reading of 35—40 %. The same medium as that for the tube assay with *E. coli* was used. After incubation of the plates overnight at 37°C, the papers were removed and the growth zones recorded.

RESULTS AND DISCUSSION

The destructive effect of γ-rays on cyanocobalamin was first established in solutions containing 100 μg cyanocobalamin per ml of a 0.1 M phosphate buffer of pH 5.0 which had been given doses from 0.01 to 1.0 Mrad. A number of breakdown products were detected. Since it was desirable for the subsequent tests to obtain large amounts of irradiated material, the vitamin concentration of the solution was increased to 5 mg/ml. Consequently the doses were also increased so that comparable effects could be obtained. In Fig. 1, residual microbiological activity relative to radiation dose is recorded. The microbiological activity was calculated from a tube assay with *E. coli* 113—3. These activity values are not direct measures of residual cyanocobalamin because a number of new factors with growth-promoting effects on *E. coli* 113—3 are

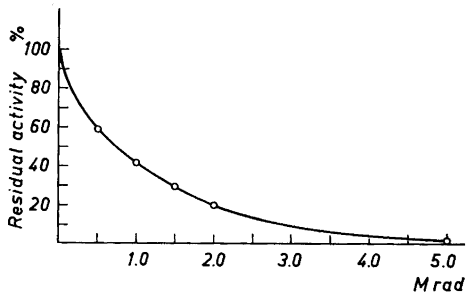

Fig. 1. Residual microbiological activity in solutions of cyanocobalamin (5 mg/ml) irradiated in 0.1 M phosphate buffer of pH 5.0. The values have been calculated from a tube assay with *E. coli* 113—3.

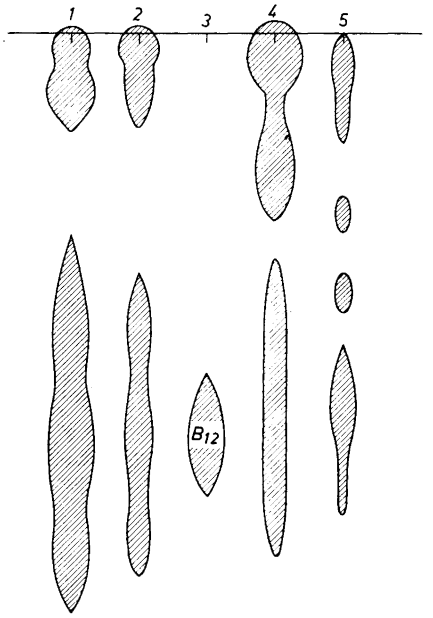

Fig. 2. Bioautography with *E. coli* 113—3 of cyanocobalamin irradiated in 0.1 M phosphate buffer of pH 5.0.

1. 1 μl of a cyanocobalamin solution (5 mg/ml) irradiated with 2 Mrad, diluted 1:100.
2. 1 μl of the same solution irradiated with 7 Mrad, diluted 1:20.
3. 1 μl of a standard solution containing 1 μg/ml cyanocobalamin.
4. 1 μl of the cyanocobalamin solution irradiated with 10 Mrad, undiluted.
5. 10 μl of the cyanocobalamin solution irradiated with 15 Mrad, undiluted.

Fig. 3. Bioautography with *E. coli* 113—3 of 1 μl of a cyanocobalamin solution, 5 mg/ml in 0.1 M phosphate buffer of pH 5.0, irradiated with 2 Mrad, diluted 1:250. Growth zone No. 5 from the top corresponds to cyanocobalamin.

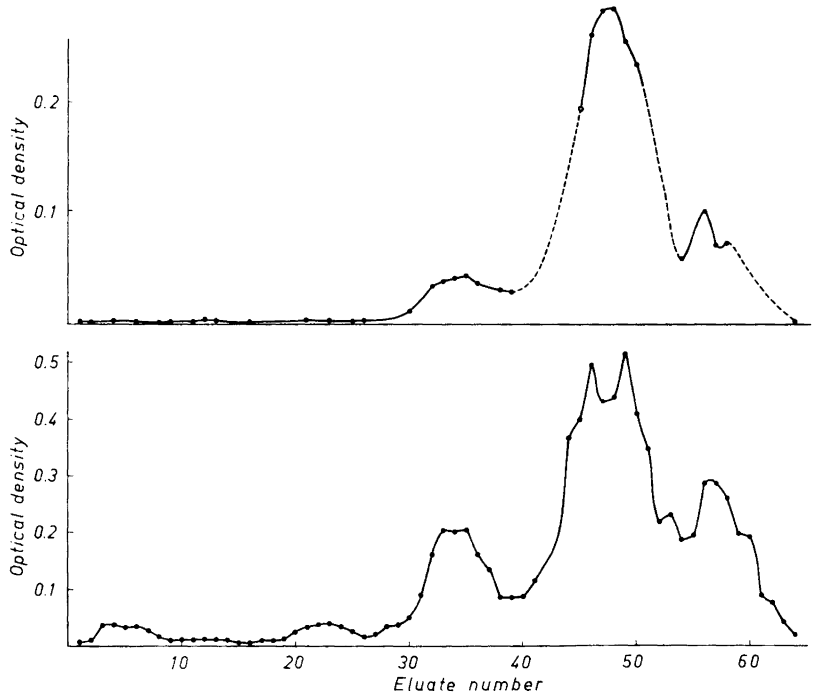

Fig. 4. Growth response to eluates from a paper chromatogram of cyanocobalamin, 5 mg/ml in 0.1 M phosphate buffer of pH 5.0, irradiated with 2 Mrad.
Upper curve: *O. malhamensis.*
Lower curve: *E. coli* 113—3.
The *O. malhamensis* curve is extrapolated in the parts marked with small dashes.

formed during the irradiation. This is shown in Fig. 2 which illustrates a bioautogram of cyanocobalamin solutions irradiated with doses from 2 to 15 Mrad. As can be noticed, the type of factors formed depends to some extent on the dose. Fig. 3 shows another bioautogram of the solution subjected to 2 Mrad. In this case, the chromatogram has been run for a longer time so that the separation is more complete. Six different growth zones can be clearly distinguished. A chromatogram identical to this was run with large quantities (applied as a band across the paper) of the same solution of cyanocobalamin irradiated with 2 Mrad. After drying, the chromatogram was cut in 5 mm broad strips parallel to the line of application. These strips were eluted with water. The eluates were tested by tube assays both with *E. coli* 113—3 and *O. malhamensis*. Optical density, as a measure of the growth in the tubes, is plotted for each eluate in two curves, one for *E. coli* 113—3 and one for *O. malhamensis* (Fig. 4). The growth in eluates 40—54 is probably caused by unchanged cyanocobalamin. It is seen that at least two breakdown fragments — in eluates 28—39 and 54—64 — have growth promoting effects on both the organisms. As is well known, *O. malhamensis* is considered to have a B_{12} requirement similar to that of higher animals.

Besides the paper chromatographic separations, paper electrophoresis in combination with bioautography was performed on the cyanocobalamin solution irradiated with 2 Mrad. In this test, at least four *E. coli* 113—3 active substances could be observed. Both positively and negatively charged compounds were formed.

In order to make it possible to examine the isolated breakdown fragments, we have tried using preparative separation methods on solutions irradiated with 2 Mrad. For instance, the application of column electrophoresis (apparatus of type LKB 3340) in a sucrose density gradient was investigated. In spite of the fact that visual inspection indicated a fairly complete separation — five distinct, coloured zones could be seen — none of the zones contained a single, isolated breakdown fragment. Cellulose ion exchangers have been used with good results by Pawelkiewicz *et al.* [14] and Menke [15] for the separation of cobalamins but application of this technique to our irradiated solutions has not yet given entirely satisfactory results. However, with some further modifications of, for example column length, eluate solvent etc., it might be possible to isolate at least some of the biologically active fragments. Work is being continued to this purpose. Preliminary separation attempts using an LKB 3500 ChroMax pressurized paper chromatography column have also given relatively promising results.

Since none of the microbiologically active fragments has been obtained in the pure state, it has not yet been possible to examine the chemical changes in the cyanocobalamin molecule caused by the γ-rays. One of the fragments might be the monocarboxylic acid derivate found in self-degradated ^{60}Co-cyanocobalamin by Barlow and Sanderson [3].

Some separate experiments with different buffers, buffer strengths and additions of sulphite and ascorbic acid were performed besides the above related studies in which cyanocobalamin was irradiated in a 0.1 M phosphate buffer of pH 5.0. Residual microbiological activity, determined with *E. coli* in a tube assay, was found to depend on the pH of the buffer, *e.g.* for 3 Mrad

it was 20 % for cyanocobalamin (5 mg/ml) irradiated in a 0.05 M acetate buffer of pH 3.0, 10 % in a 0.05 M phosphate buffer of pH 5.0 and 5 % in a 0.05 M phosphate buffer of pH 7.0. A lowering of the buffer strength had, as well as a decreased destruction effect, a tendency to change the proportions between the breakdown products formed. Ascorbic acid and sulphite, when added to the buffer before the irradiation, had a definite protective effect on the cyanocobalamin. Thus, when a solution containing 5 mg/ml of cyanocobalamin was irradiated in 0.05 M phosphate buffer of pH 5.0 with 3 Mrad, the residual *E. coli* activity was 25 % when the solution contained 50 mg ascorbic acid/ml (as compared with 10 % in the absence of vitamin C). In a 0.05 M sulphite buffer of pH 5.0, the residual activity was 33 %. In the cyanocobalamin solutions to which ascorbic acid or sulphite were added, breakdown products different from those discussed above were noticed.

We have also noticed that the polypeptide-cyanocobalamin complex isolated by Hedbom [16] is considerably more resistant to the destructive effect of γ-rays than the free vitamin.

The implication of the observations reported here and in the previous paper with regard to the wholesomeness of irradiated food is as yet unknown and must await studies on the effect of the breakdown fragments on higher animals. Furthermore, it remains to be demonstrated that biologically active destruction products are formed from the various vitamins when these are irradiated in food-stuffs.

The authors gratefully acknowledge the receipt of research grants from the *Swedish Technical Research Council* and the *Swedish Natural Science Research Council.*

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Received May 10, 1962.