IMPROVED URINARY EXCRETION TEST FOR ASSAY OF VITAMIN B-12 TAGGED WITH COBALT-60

DETERMINAÇÃO DE VITAMINA B-12 MARCADA COM Co-60 EM URINA

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INTRODUCTION

Vitamin B-12 tagged with cobalt-60 has been used in different propedeutic proves in the diagnosis of pernicious anemia (1,2), as well as an assay for intrinsic factor (3,4). The most used test which has given the best results is the one of Schilling in which 0.5 to 1.0 microcuries of vitamin B-12 tagged with cobalt-60 is given to the patient followed by a flushing dose of vitamin B-12 not tagged. The urinary excretion is then measured for radioactivity, and the total excreted in 24 hours compared with the amount taken.

Usually, the detection of the cobalt-60 in the urinary excretion is carried out using a large volume of urine. The urine is placed in a beaker or in a plastic container, with a flat bottom, and the container is placed directly on a scintillation counter. The activity is calculated by comparison with standard solutions whose geometry and volumes are identical to the sample.

This procedure presents some very serious short-comings. Since human beings are used as experimental subjects it is necessary to use little doses of tagged vitamin B-12; consequently, it is necessary to use a large volume of urine as well as a large container. Since the dimensions of the sample are large, the shielding for the counter must be correspondently large (7). The standard is a homogeneous solution but the urine has an appreciable amount of material which settles during counting time and a fraction of the active material is entrained with it. A previous agitation of the urine is not helpful since the counting time is, usually, quite long and sedimentation takes place during counting. To avoid these sources of errors a small sample (about 3 milliliters) and a scintillation well counter could be used; the reproducibility is quite good but since the activity per milliliter is low the counting time must be long in order to get reliable results.

Ellenbogen and Williams devised a special type of container for counting (5). This container surrounds the top and the sides of the scintillation counter and there is a part of it that fits into the well. However, the same inconvenience of large volumes and sedimentation is still present.

In all systems in which urine is counted directly, a problem arises concerning the lack of proportionality between the activity and the volume, from a certain volume on. After the volume has reached a corresponding height of about two or three centimeters above the face of the scintillation counter, the addition of more urine will not give a proportional increase in the activity.

The present work was carried out with the aim of finding a counting procedure that would avoid the short-coming of the usual counting methods, specially to reduce the counting time and to eliminate the errors consequent of a poor geometry.

To reach this aim a way should be found to concentrate in a small volume the tagged vitamin B-12, or its products of degradation which might have cobalt-60. Various experiments were made until satisfactory results could be found. Some of these experiments will be briefly described since they gave some indication of a disruption of the molecule of vitamin B-12 excreted in the urine. Techniques such as liophilization and crioconcentration were not tried because of the difficulty for operation. Liophilization would contaminate the equipment and the smell of urine would be an inconvenience; crioconcentration would take a long period of time and about ten percent of material would always be retained with the supernatant.

EXPERIMENTAL METHODS TRIED FOR EXTRACTION OF VITAMIN B-12 FROM URINE

A - Precipitation methods

1. - Precipitation of the CO_2O_3.

Carrier of cobalt was added to 50 milliliters of urine in which tagged vitamin B-12 was present. The cobalt was precipitated with sodium hydroxide and hydrogen peroxide; the precipitation was carried out at boiling. The precipitation was filtered, dissolved with hydrochloric acid; the solution was concentrated, placed in a plastic vial that would fit on the well of the scintilometer and counted. Some activity was present on the cobalt oxide precipitate, but the precipitation of cobalt was not complete, because of the high concentration of ammonium salts and the filtrate was still active. The hot precipitation is an unpleasant operation because of the smell, specially when old urine is used. The material does not filtrate easily.

2. - Precipitation with Zinc-mercuri-thiocyanate.

Carrier of cobalt was added to 50 milliliters of urine and a cold precipitation was carried out with a concentrated solution of zinc-mercuri-thiocyanate. Cobalt is coprecipitated with zinc; the precipitate is a crystalline and easily filterable. However, the activity present in the precipitate is quite small and this method has shown to be less efficient than the previous one.

B - Solvent extraction

1. -

Ammonium Thiocyanate and carrier of cobalt were added to 50 milliliters of urine and the cobalt thiocyanate formed was extracted with a mixture of ether and amyl alcohol. The carrier cobalt was extracted completely but no activity was present in it, even when the time of agitation of urine and solvent was 72 hours.

C - Use of ion exchange

Kinmory "et al." (8) developed a method in which the cobalt-60 originating from the labelled vitamin B-12 is precipitated as cobalt sulphide, with hydrogen sulphide, and the radioactivity of the precipitate is measured. However, the operation is unpleasant because of the smell of urine and hydrogen sulphide and it has to be carried out twice. The filtration is difficult, and it is necessary to add ammonium chloride (twice 300 grams) for a volume corresponding to 24 hours excretion to avoid the formation of colloidal cobalt sulphide.

In order to get a better way of precipitating cobalt sulphide, experiments were carried out with an anionic resin in sulphide form which was put in this form by treating a chloride resin, Nalcite SAR, with a fresh solution of ammonium sulphide. The resin was then washed with deionized water up to complete elimination of sulphide anion.

Urine, with pH equal to 4, was passed through this sulphide resin. Cobalt is retained, mixed with the resin, as cobalt sulphide, and the activity removed from the urine is dependent of the amount of carrier. A small

Experiments in which urine was treated with activated carbon, 50 to 250 ml of urine were agitated with 300 mg of carbon for 20 minutes. After this time, another portion of 300 mg of carbon was added and the agitation was carried out for 15 minutes. The pH of the urine was ascertained previously in between one and two. After the agitation the mixture was left to settle and then the carbon was filtered through a Whatman 40 paper. The filter paper was placed in a small plastic vial which was put into the well of the scintillation crystal.

RESULTS

In order to compare results obtained with the techniques of adsorption on activated carbon and the ones obtained with the liquid placed in an ordinary beaker or on the Ellenbogen and Williams recipient placed directly on the scintillation crystal, the data are plotted on Figure 1.

This graph shows taht in the case of the Ellenbogen - Williams recipient or ordinary beaker there is a lack of proportionality between the various volumes of urine added and the counts per minute obtained. In the case of activated carbon in which the adsorption was carried out with technique previously described, the count per minute is proportional to the amount of labelled vitamin B-12. The curves have the same shape when pure solution of labelled vitamin B-12 or urine are used for experiments.

It is known that the gamma ray efficiency of a sodium iodite-thallium scintillation counter for gamma ray of cobalt-60 is about 22% (9), for a scintillation crystal of 1 1/2 by 2 inches and a well of 1/2 inches by 1 5/8 inches, suposing a geometry of 100%. Evidently the geometry efficiency in the case of an Ellebogen-Williams beaker or an ordinary one, is far from being 100%.

column that would fit in the well of the scintilometer will have resin to treat at most 50 milliliters of urine with 10 miligrams of carrier cobalt.

D - Adsorption of vitamin B-12 in a cationic resin

Since vitamin B-12 has a slight alkaline character it was tried to retain the vitamin in a cationic resin in hydrogen form. Using urine with pH from six to seven, it was possible to remove a good part of its activity with a cationic resin, without the necessity of adding isotopic carrier to the urine. The determination of activity was carried out directly on the resin, - which was placed on a small plastic column that could fit on the well of the scintillation crystal. However, the resin is satured with other cations present in the urine and this small amount of vitamin B-12, as compared to the concentration of other salts present on the urine, is not retained quantitatively by resin. One gram of strong cationic resin, Nalcite HCR, will be satured with 10 to 15 milliliters of urine.

Marsh and Kuzel (6) observed that, in the case of purification of vitamin B-12, it is possible to elute the vitamin with sodium hydroxide 0.1N. However, in the case of urine, elution with sodium hydroxide 0.1N were not effective in removing the activity, even when elution was carried out with a hot solution.

Elution with hydrochloric acid 0.1N and 1.0N in 60% acetone gives slightly best results than with pure hydrochloric acid. The same type of experimentswere made using an anionic resin but the difficulties concerning elution of the activity still remain.

E - Adsorption of the vitamin B-12 from urine with activated carbon

By using the techniques of adsorption of activity in activated carbon, and placing the filter paper with the carbon into the bottom of a vial that fits into the well of the scintillater, it is possible to get a geometry efficiency quite close to 100%. In order to show that a maximum of total counting efficiency is obtained using the adsorption techniques on activated carbon, we give on Table I the results for experiences obtained with an Ellenbogen-Williams beaker, a common one, and adsorption on carbon. The total efficiency of counting is defined as the ratio of counting per minute to desintegrations per minute, For these experiences a solution of labelled vitamin B-12 with specific activity of 7.73 x 10^{-4} microcuries per milliliter was used.

TABLE I

Efficiency of counting solution of tagged vitamin B-12

		Ellenbongen-Williams beaker		Common Beaker		Adsorption on carbon	
ml	d.p.m.[+]	c.p.m.[++]	effic.%	c.p.m.	effic.%	c.p.m.	effic.%
50	$8.6x10^4$	3,975	4.68	2,558	3.01	19,441	22.9
100	17.1	5,297	3.06	4,892	2.88	37,722	22.2
150	25.7	7,321	2.88	6,902	2.71	-	-
200	34.2	9,213	2.72	8,662	2.53	73,556	21.7
250	42.8	10,689	2.52	10,083	2.38		
300	51.4	11,960	2.34	11,568	2.28		
350	60.0	13,559	2.27	12,870	2.18		
400	68.5	15,299	2.25	13,876	2.06		
450	77.1	17,003	2.22	14,971	1.96		
500	85.7	18,603	2.16	15,770	1.86	167,239	20.3
550	94.3	19,493	2.06	16,503	1.77		
600	102.8	20,765	2.04	17,078	1.67		
650	111.4	21,559	1.95	17,844	1.61		
700	112.0	22,610	1.90	18,341	1.54		
750	128.5	23,197	1.82	18,699	1.46		
800	137.1	23,959	1.76	19,315	1.42		

[+] d.p.m. = desintegration per minute

[++] c.p.m. = counts per minute

On Table II is given the total counting efficiency obtained with the activated carbon techniques for a urine with specific activity of 1.25 x 10^{-4} microcuries.

TABLE II

Efficiency of counting tagged vitamin B-12 in urine previous adsorption on activated carbon:

Volume ml	d.p.m.	c.p.m.	% effic.
2	$5.7x10^2$	129	23.3
48	133.2	3,061	23.1
100	277.5	6,558	23.6
150	416.3	9,376	22.7
200	555.0	12,153	22.1
248	688.2	14,028	20.6

Solutions of vitamin were agitated with carbon and samples of the supernatant were taken from time to time and their activities measured in order to verify the required time for a complete adsorption of the labelled vitamin B-12 on carbon (see Fig. 2). As it can be seen from the graph, the activity remaining in the solution after fifteen minutes of agitation is very small. By a second adsorption of the supernatant the remaining activity is removed in a few minutes.

DISCUSSION

The proposed method is very simple and can be carried out rapidly. The filtration of the carbon is very easy and only when old urine was used some difficulty was experienced on the filtration; however, by adding some drops of "separan"(Dow Chemical Co.)the carbon was sttled and the filtration could be carried out easily. Activated carbon is less expensive than ion exchangers, and just by incinerating the carbon it is possible to recover the cobalt-60 which can be used as tracer for other chemical experiments. To incinerate the carbon it is better to treat it with some drops of a dilute solution saccharose and some milligrams of calcium oxide; the carbon is then dried and incinerated.

The use of carbon will avoid the stocking of large volumes of urine since the adsorption can be carried out when the material is collected and the urine can disposed off. By counting the whole volume of urine the counting time should be from 30 to 60 minutes since the specific activity of the urine is very low and a long counting time is necessary to reduce statistical counting errors. The counting time is shortened by using the adsorption techniquesto about ten minutes, and activities as low as 0,5 cpm/ml could be easily determined. The counting of the same urine directly, was completely unsucessful.

Some very interesting inferences could be drawn from the various experiments carried out before the final procedure with activated carbon had been stablished.

The solvent extraction with ether and amyl alcohol of the cobalt thiocyanate has shown that no exchange of the type

$$B_{12} - Co^{*} + Co^{++} \rightleftarrows B_{12} - Co^{++} + Co^{*}$$

had taken place. This indicates also that there was no degradation of the molecule of vitamin B-12, giving ionic cobalt. Otherwise the free cobalt-60 would get mixed with the cobalt added as carrier and be extracted by the organic solvent.

That the molecule of vitamin B-12 is not broken during the metabolic pathway was also shown by the difficulty of eluting cobalt-60 from a cationic resin where the vitamin had been absorbed. It is well known that vitamin B-12 is absorbed by a cationic resin (6) and that elution is possible by using sodium hydroxide 0.1N which elutes the vitamin as a whole and not the cobalt cation that might had been formed if the molecule of B-12 had been degrated to ionic cobalt. If there was ionic cobalt-60 absorbed on the resin, and which had originated from the molecule of B-12 it would be possible to elute this ionic cobalt-60 with hydrochloric acid or by a strong complexing agent for cobalt such as ethylenediaminetetraacetic acid. However, such was not the case and none of those two eluting agents could take the cobalt-60 from the resin, indicating that the cobalt was not in an ionic form and that the molecule of vitamin B-12 had not, probably, been broken.

The precipitation of cobalt with zinc-mercuri-thiocyanate is a typical ionic process. The failure to precipitate completely the cobalt present in the urine shows that the cianocobalamine (vitamin B-12) is a very strong complexed compound. The small amount of cobalt-60 precipitated with this reagent shows that only a very small number of vitamin B-12 molecules are decomposed on the metabolic pathway.

On conclusion these experiences show that no degradation of vitamin B-12 occurs or if such a degradation exists it is in a very small amount.

SUMARY

In this paper the various methods available to analyze vitamin B-12 tagged with cobalt-60 are experimentaly examined. A very simple and efficient method is proposed by the authors. The vitamin present in urine, or in aqueous solutions, is adsorbed by activated carbon and the carbon is counted in a gamma scintilometer. The techniques of the proposed method are described, and its advantages over classical methods of direct counting, precipitations, solvent extraction and absorption in ionic resins are presented.

SUMARIO

Estudam-se, nêste trabalho, experimentalmente, vários métodos para análise quantitativa de vitamina B-12, marcada por cobalto-60 por meio da determinação da atividade dêste cobalto. É feita a crítica de vários métodos e propõe-se então um método novo no qual a vitamina é adsorvida em carvão ativo,e êste é contado diretamente no cintilômetro gama. Descrevem-se as técnicas do método em questão e faz-se uma comparação com os métodos clássicos existentes de contagem direta das soluções de vitamina B-12, contagem do cobalto precipitado, do cobalto extraido por solventes orgânicos e do cobalto absorvido em resina iônica.

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2. Activity of a vit. B_{12} solution measured with an Ellenbogen-Williams container. The specific activity of the solution 1.0×10^{-4} uc/ml, is the same as that of the patient's urine. Successive points correspond to the addition of some portions of solution into the container.

1. Similar measurements made with a common beaker of diameter height and material equal to that of the Ellenbogen-Williams container.

3. Acitivities absorbed by 0-5 g portions of carbon from 50, 100, 250 and 400 ml respectively of the same solution.

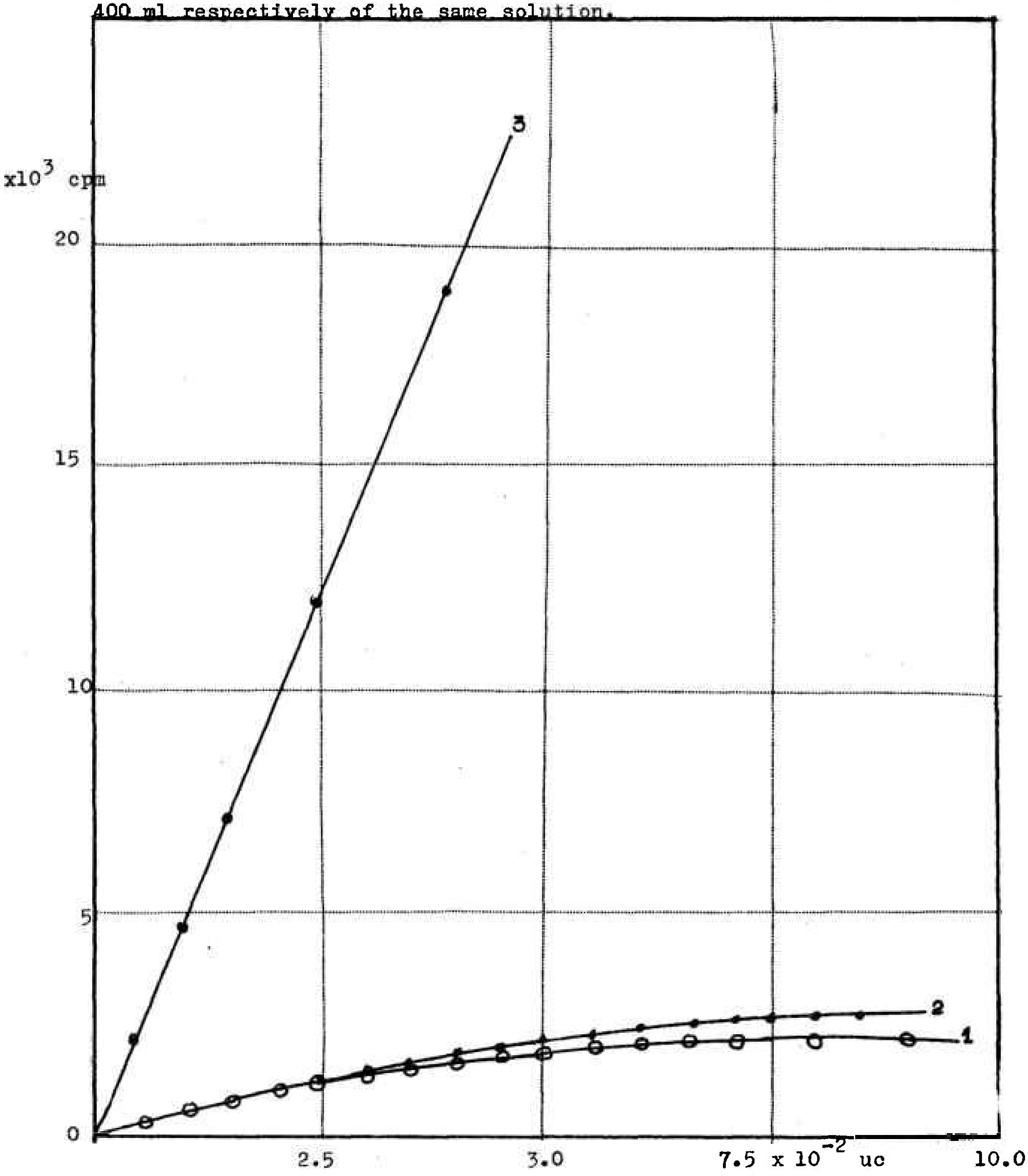

FIGURE I

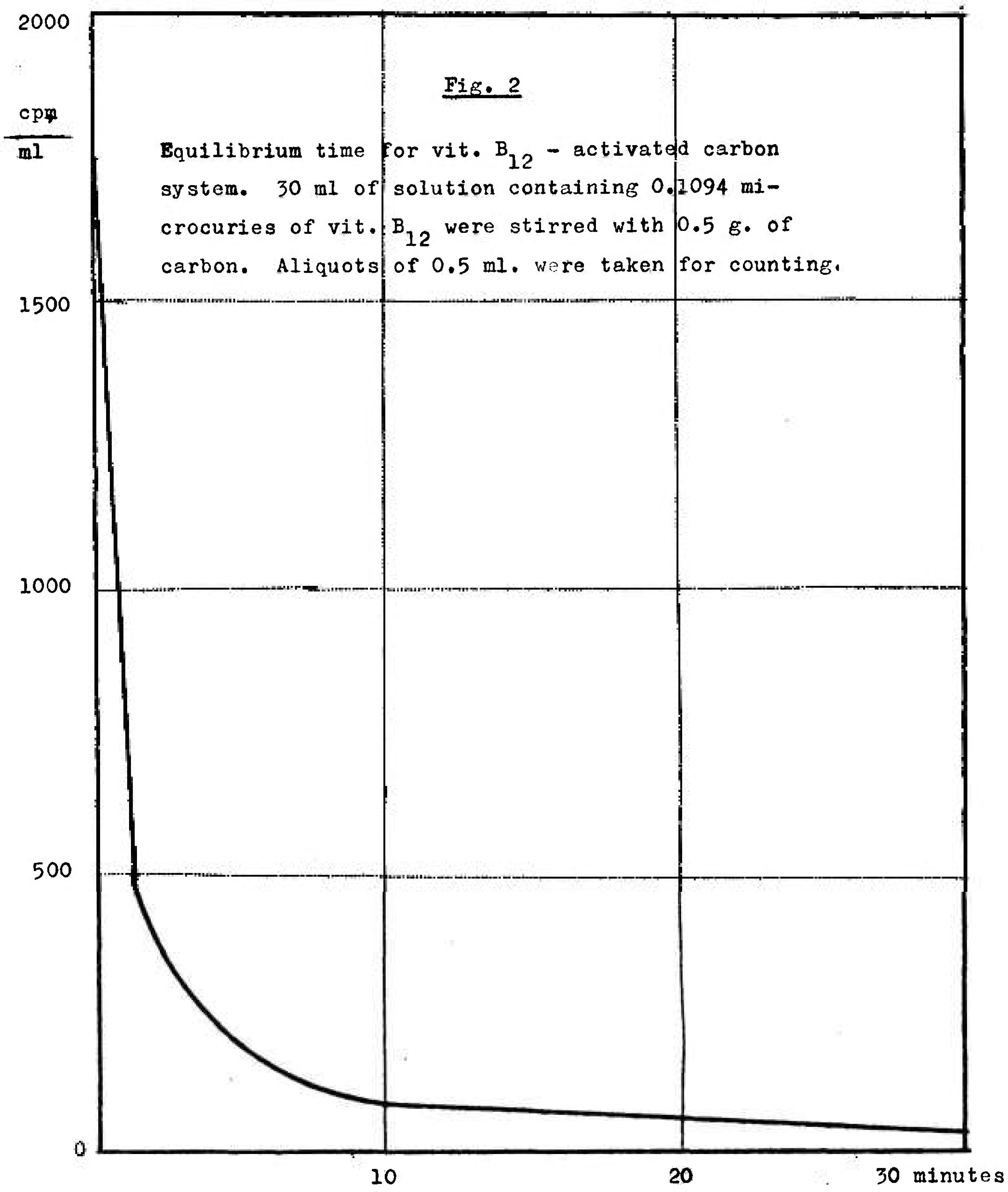

Fig. 2

Equilibrium time for vit. B_{12} - activated carbon system. 30 ml of solution containing 0.1094 microcuries of vit. B_{12} were stirred with 0.5 g. of carbon. Aliquots of 0.5 ml. were taken for counting.