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THE USE OF LABELLED VITAMIN B_{12} IN THE MEASUREMENT OF GLOMERULAR FILTRATION RATE

By R. P. EKINS, F. S. NASHAT, R. W. PORTAL AND A. M. SGHERZI

From the Institute of Nuclear Medicine and the Department of Physiology, The Middlesex Hospital Medical School, London, W.1

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SUMMARY

1. Two preparations of radioactive vitamin B_{12} labelled respectively with ^{57}Co and ^{58}Co have been employed in the estimation of the glomerular filtration rate (G.F.R.) in dogs. The intravenous injection of these preparations at an interval of 20 min resulted in a changing ratio of their activities in plasma and urine and so permitted a relation in time to be established between the urine and the plasma from which it was filtered.

2. When urine and plasma were sampled at short intervals an effective renal delay or transit time could be derived from their isotope ratio curves and used as a correction in the clearance equation. This allowed serial clearances of either isotope to be calculated despite a falling plasma concentration.

3. This method was applied to the study of the G.F.R. in fourteen dogs using short urine collections of 2 or 4 min for periods up to 80 min. The results under stable conditions and with deliberately induced changes in the G.F.R. are illustrated and discussed.

4. Application of the technique in four patients undergoing differential renal function studies gave results comparable with those in the experimental work.

5. The plasma binding of administered vitamin B_{12} in serial blood samples in dogs and man was investigated.

INTRODUCTION

The direct measurement of the (G.F.R.) is impossible and a continuous record of filtration rate by indirect means has yet to be devised. The nearest approach to this is the calculation of consecutive clearances at very frequent intervals. This requires the collection of numerous urine samples of sufficient volume for analysis of the clearance substance with reasonable accuracy. Radioactive vitamin B_{12}, recently introduced as a means of

measuring the G.F.R. (Nelp, Wagner & Reba, 1964), appears to be well suited to this purpose. Its estimation is simple, highly sensitive over a wide range of dilution, and requires very small volumes of urine.

In the present experiments the use of consecutive B_{12} clearances has been investigated as a method of providing a minute to minute record of the G.F.R. To determine the renal delay time, a problem common to all clearance methods based on urine collection, separate preparations of B_{12} labelled respectively with two different isotopes of cobalt have been employed. When these were injected at an interval their changing ratio served as a means of relating the urine to the plasma from which it had been formed, and thereby permitted the use of a single injection technique with a falling plasma concentration. Simultaneous clearances have also been calculated employing a constant infusion of one isotope and a single injection of the other.

The present studies seem relevant in view of the interest lately shown in the measurement of the G.F.R. by vitamin B_{12} (*Lancet*, 1965) and its introduction for this purpose into clinical practice (Nelp *et al.* 1964; Cutler & Glatte, 1965; Slapak & Hume, 1965; Breckenridge & Metcalfe-Gibson, 1965).

The fall in plasma radioactivity following the rapid injection of labelled vitamin B_{12} reflects the removal of the vitamin from the plasma by at least two processes: (*a*) renal excretion and (*b*) diffusion into the body B_{12} space. Each process is governed by at least one rate constant and the curve relating concentration to time after injection is therefore of a complex form described by an expression containing a minimum of two exponential terms. It follows that the observed fractional rate of fall in concentration is a time dependent variable, in contrast with the situation obtaining when a single exponential term governs the time/concentration curve.

If vitamin B_{12} is injected in two successive shots separated by a suitable time interval (in practice 20–30 min) and if each shot is labelled by a different isotope of cobalt, then the fractional rates of fall of the two radioactivities are different, and the ratio of the isotopes in the plasma progressively changes. Any sample of blood taken from the subject after the second injection may thus be characterized by the ratio of isotopes in it, and the time at which it was withdrawn established by reference to the curve relating isotope ratio to time after injection. The rate of change in the ratio depends solely on the rates of diffusion of the isotopes over the B_{12} space, and is unaffected by renal excretion or changes in circulatory volume.

An isotope ratio curve may similarly be drawn for the urine. Since the urine is derived from the plasma, this curve will have the same configuration as the plasma curve, but, owing to the time taken in transit from the

glomerular blood to the site of urine sampling, it will be displaced to the right on the time scale. Simultaneous plasma and urine samples will thus differ in isotopic ratio, but the ratio observed in the urine will correspond with that observed in plasma at a time (Δt) earlier. This time may be termed the 'effective' or 'mean' renal delay or transit time. Its determination permits the urine concentration of either form of labelled B_{12} to be related unequivocally to a point on the plasma concentration curve of the same isotope, even though this curve is falling steeply. It thus becomes possible to 'pair' a given urine sample with the plasma from which it was derived.

The relation may be amplified by the following analysis:

The clearance of B_{12} is given by the classical clearance equation

$$C = \frac{UV}{P},$$

where U = urine concentration of B_{12}, V = minute volume of urine, P = plasma concentration of B_{12} assuming that B_{12} is infused continuously to maintain P constant.

Following a single shot of B_{12} the clearance is given by

$$C = \frac{U_{(T+\Delta t)}V}{P_{(T)}},$$

where $U_{(T+\Delta t)}$ = urine concentration at time $(T + \Delta t)$ after the injection, $P_{(T)}$ = plasma concentration at time T after the injection, and Δt = the effective transit time from glomeruli to site of urine sampling.

Following the administration of successive shots of [^{57}Co] B_{12} and [^{58}Co] B_{12} we may write

$$C = \frac{{}^{57}U_{(T+\Delta t57)}V}{{}^{57}P_{(T)}} = \frac{{}^{58}U_{(T+\Delta t58)}V}{{}^{58}P_{(T)}}$$

assuming no isotopic discrimination by the glomeruli, whence

$$\frac{{}^{57}U_{(T+\Delta t57)}}{{}^{58}U_{(T+\Delta t58)}} = \frac{{}^{57}P_{(T)}}{{}^{58}P_{(T)}},$$

where the superscripts refer to the concentrations of [^{57}Co] B_{12} and [^{58}Co] B_{12} in plasma and urine and the subscripts Δt_{57} and Δt_{58} are the effective delay times for the two isotopes. Provided Δt_{57} and Δt_{58} are equal this equation reduces to

$$\frac{{}^{57}U}{{}^{58}U}(T + \Delta t) = \frac{{}^{57}P}{{}^{58}P}(T)$$

from which it follows that the delay time appropriate to the calculation of B_{12} clearance is that given by equality of isotope ratios in plasma and urine.

The concept of 'effective' delay or transit time is one of some complexity owing to the fact that the renal tubules are of unequal length and diameter

and of inconstant capacity. The sampled urine thus represents a mixture of discrete elements of filtrate each of which has been subjected to a different time delay in its passage from glomerulus to point of collection. In these circumstances and in the context of this study the appropriate 'mean' transit time is a parameter the value of which will depend on the function relating plasma concentration of B_{12} to time. Since the curves relating these variables are necessarily of a different form for the two isotopes at any given moment (because of the different times of injection) the assumption of an identical transit time for the two isotopes is not strictly valid. In practice the error involved in making this assumption is likely to be small, and does not affect the main argument, nor, we believe, detract from the usefulness of this method of evaluating the renal delay time.

Finally, we would emphasize that the effective delay time is best regarded as a mathematical device for use in clearance calculations, and not as an absolute mechanical or physiological entity. Its value will of necessity increase as the urine collection periods are lengthened, and comparisons of delay times in a single subject or between different subjects are not valid unless a standard period is employed over the time of measurement.

METHODS

Dogs weighing 10–30 kg were anaesthetized with pentobarbitone (30 mg/kg). A jugular or forearm vein was cannulated for the infusion of fluids and a femoral artery for blood sampling. The other femoral artery was cannulated for blood pressure recording with mercury or electro-manometer. The ureters were approached through low abdominal incision and polythene catheters advanced a variable distance up them.

Dextrose in 5 % solution was given intravenously to promote diuresis, and mannitol in appropriate amounts if a greater urine flow was desired.

At the start of the experiment a minimum of 50 μg/kg of stable vitamin B_{12} was given intravenously to saturate the plasma and tissue binding sites. Between 5 and 20 min later 2–3 μc of [^{57}Co]B_{12} with 500 μg of stable B_{12} in 10 ml. of dextrose solution were injected intravenously. Twenty minutes later a similar dose of [^{58}Co]B_{12} was given. The injection of the second isotope was taken as zero time, and marked the commencement of sampling. In four experiments the single injection of [^{57}Co]B_{12} was replaced by a constant infusion. In these animals a priming dose of 1 μc of the isotope was followed by the delivery with a Sigmamotor slow injection pump of a solution containing 1 μc of [^{57}Co]B_{12} and 250 μg of stable B_{12}/100 ml. at a rate of 1·0–1·5 ml./min. The single injection of [^{58}Co]B_{12} was given in the usual way after an equilibration period of 30 min.

Arterial blood samples were taken every 2 min for 10 min, then every 4 or 6 min. Urine collections were made every 2 min for 10 min, then every 2 or 4 min. Volumes of urine were measured gravimetrically, no correction being made for specific gravity.

In four patients studied a saturating dose of 4 mg of stable B_{12} was given intravenously. This was followed by the rapid intravenous injection of the two isotopes, 3μc of each, at an interval of 20–30 min. Blood samples were obtained every 2–5 min from a polythene catheter inserted percutaneously into a brachial artery. Urine was obtained by ureteric catherization, with collection periods of 2–5 min.

Separation of free and bound B_{12} fractions in plasma

The separation of free and bound B_{12} in plasma was effected using activated charcoal (Norit OL) added either as a 5 % suspension in 1 % albumen (200 μl. to 1 ml. of plasma) or as a weighed amount (10 mg. to 1 ml. of plasma) of the dry powder. Similar techniques have been in use in our laboratory for some years in the estimation of serum B_{12} by the saturation assay method (Ekins & Sgherzi, 1965). However, to confirm the efficacy of the separation, preliminary *in vitro* experiments were conducted to check the effect on the tracer distribution of:

1. varying levels of initially added inactive B_{12} (12·5–400 ng/ml.).
2. varying initial levels of heparin in the blood specimen,
3. varying periods of storage (4° C) before the addition of charcoal (stable and labelled B_{12} having been added to the blood *in vivo*),
4. varying durations of incubation of stable and active B_{12} with plasma (37° C).

The results of these studies may be briefly summarized as follows:

1. 10 mg of charcoal adsorbs not less than 95 % of the free B_{12} from 1 ml. of plasma in amounts ranging from 12·5 to 400 ng/ml.
2. Not less than 95 % of radioactive B_{12} added *in vitro* to and incubated with plasma pre-incubated with amounts of stable B_{12} ranging from 12·5 to 400 ng/ml. is in the free form.
3. No increase in the amount of bound activity occurs following incubation at 37° C for periods up to 8 hr. of radioactive B_{12} with plasma loaded with 100 ng/ml. of stable B_{12}.
4. The binding of B_{12} by heparin in plasma containing 100 ng/ml. of stable B_{12} is negligible.
5. The storage of plasma at 4° C for periods ranging from 10 min to 24 hr does not alter the distribution of tracer between free and bound fractions.

Counting of specimens

All specimens of urine and plasma were counted using two channels of a Packard Auto-Gamma spectrometer incorporating a 3 in. (7·62 cm) well-type crystal assembly. The counting efficiency of the system with the channel setting used was approximately 80 % for ^{57}Co and 20 % for ^{58}Co with channel backgrounds in the region of 90 and 55 counts/min respectively. ^{58}Co counts falling in the ^{57}Co channel were roughly 20 % of those occurring in the ^{58}Co channel. Conversely, negligible ^{57}Co counts fell within the ^{58}Co channel. Appropriate corrections were made for background and volume.

All calculations of corrected sample activities for the two isotopes were carried out on an Elliot 803 computer programmed (source language Algol) to solve the pairs of simultaneous equations derived from the counter results and to calculate the standard errors of the corrected counts.

The concentrations of the two isotopes expressed as counts/ml. min, and their ratios in plasma and urine, were then plotted. Serial G.F.R.s were next calculated, using the mid points of the urine collection periods and plasma values read off the plasma curves. Clearances were obtained both from simultaneous plasma and urine values and after applying a correction for the delay time indicated by the ratio curves.

RESULTS

Protein binding of vitamin B_{12}

We have examined the amount of protein binding of vitamin B_{12} in serial arterial blood samples of six dogs following the injection of stable and labelled vitamin as described above. The results may be summarized as follows:

1. The initial fraction of bound $[^{58}Co]B_{12}$ measured within 5 min of

injection varied between 4 and 16 %. The fraction of bound [^{57}Co]B_{12}, injected 20–30 min before the start of sampling, was always higher than the simultaneous fraction of bound ^{58}Co, and varied between 11 and 26 %.

2. With a falling plasma concentration of either isotope the fraction of bound activity increased with time, though the rate of increase varied from one experiment to another. A bound fraction as high as 20–30 % at the end of 1 hr after injection was sometimes seen.

3. An increase in the bound fraction with time sometimes occurred even when the total plasma level of activity was kept constant by a continuous infusion of the isotope. This process could be suppressed by adding a large amount of stable B_{12} to the solution containing the infused isotope (delivering 20–30 μg/min).

4. The absolute amount of bound activity decreased with time when a single injection was given, but sometimes increased when a constant infusion was given.

An essentially similar pattern was observed in four patients to whom labelled vitamin B_{12} was administered in the course of differential renal function studies. Despite a saturating dose of 4 mg of stable B_{12} the fraction of bound activity ranged between 7 and 30 % in serial blood samples taken up to 90 min after administration of the isotopes. The pattern observed in an experimental subject is shown in Fig. 1. Here a saturating dose of 4 mg of stable B_{12} was followed 10 min later by injection of 3 μc of [^{57}Co]B_{12}. The fall in plasma concentration of both free and bound fractions over 12 hr is well demonstrated.

The above findings indicate that calculations based on the total plasma B_{12} activity will give erroneously low clearances and the increase of the bound fraction with time will result in a spurious fall in apparent clearances as the experiment proceeds. Differences in the bound fraction of the two isotopes at any given time may moreover give rise to discrepancies between their clearances. Though the resulting errors may not be large and will not obscure rapid changes in clearance, they are readily avoided by charcoal fractionation of the plasma and use of the free activity in all calculations. This is our present practice.

Isotope ratio curves and delay time

When B_{12} activity expressed as counts/ml. min is plotted semilogarithmically as a function of time a relation of the form shown in Fig. 2*A* is produced. In the plasma the initial steep part of the curve largely represents the spread of the injected isotope into its volume of distribution. As equilibration proceeds the curve approaches a single exponential relation which is essentially a function of excretion of the vitamin by the kidneys. The shape of the urine concentration curve approximates to that of the

plasma, but is subject to modifications resulting from changes in the G.F.R. and in urine flow rate.

In Fig. 2 the ^{58}Co was injected at zero time. The ^{57}Co had been given 20 min earlier so that the flatter portion of its curve coincides with the initial steep portion of the ^{58}Co curve. The ratio $^{58}Co/^{57}Co$ changes therefore rapidly at first, and then more slowly as the two curves assume a similar slope. This ratio plotted against time gives the curve shown in Fig. 2*B*. A similar curve can be drawn for the urine isotope ratio. This has the same

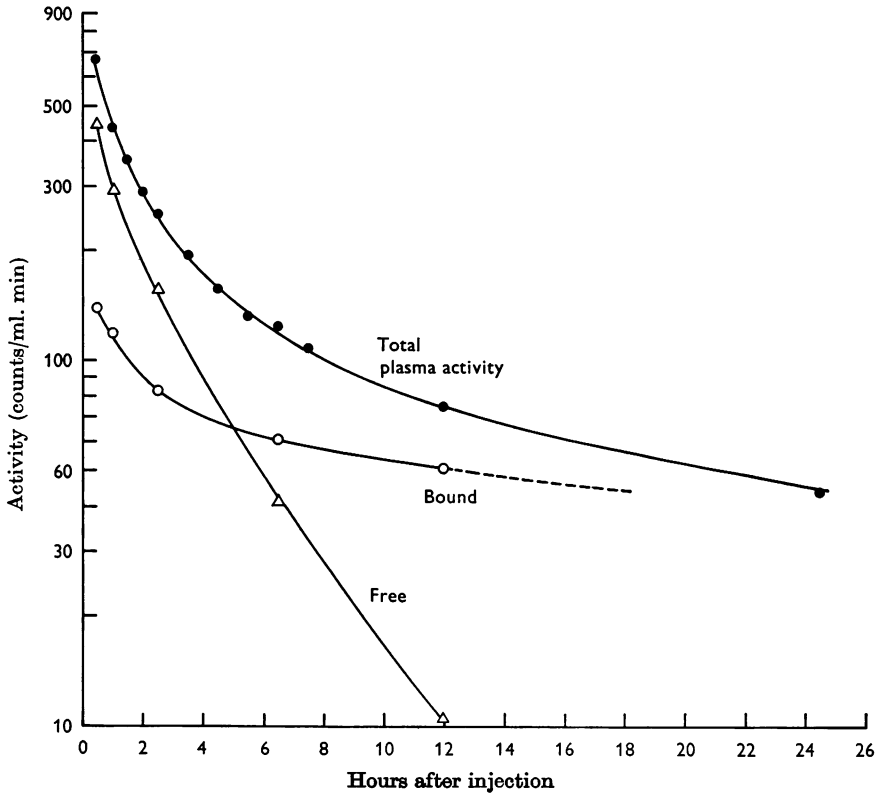

Fig. 1. Plasma curves of total, free and bound fractions following the intravenous injection of labelled vitamin B_{12} in a normal subject. A saturating dose of 4 mg of stable vitamin was given 10 min before the injection. Note the difference between the free and bound fractions, and the failure of the total plasma activity curve to become exponential at any time.

form as the plasma curve but is displaced to the right on the time scale. The horizontal distance between points of identical ratio on the two curves represents the effective renal delay time as described above. In Fig. 2*B* the interval is 2·5 min. As the rate of change of the plasma isotope ratio decreases with time the urine ratio progressively converges on the plasma

value and after some 10 min the delay time is no longer accurately measurable. Failure of the ratio curves to follow the predicted pattern was seen in a number of our early experiments and may have been related to excessive plasma binding of the isotopes. Any deviation from the normal pattern renders the clearance values suspect, and such experiments have been discounted.

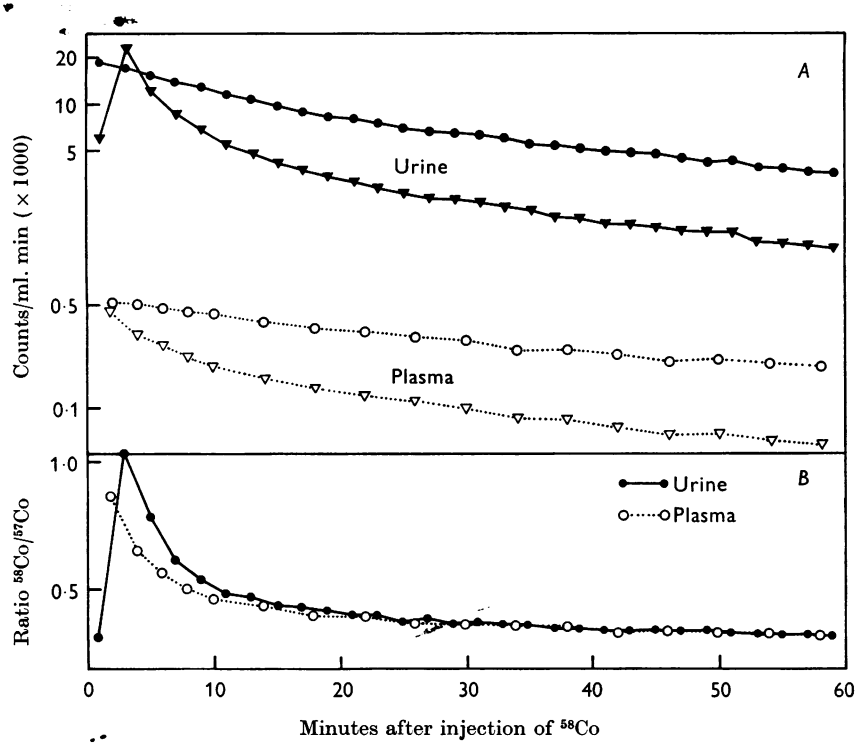

Fig. 2. (*A*) Urine and plasma concentration curves plotted on logarithmic scale: circles, ^{57}Co; trangles, ^{58}Co, and (*B*) isotope ratio curves, following the intravenous injection of labelled vitamin B_{12} in a dog. The [^{57}Co]B_{12} was given 20 min before the start of sampling and the [^{58}Co] at zero time. The effective renal delay time read from the ratio curves is 2·5 min.

The effective delay times in seventeen experiments based on urine collection periods of 2 min are presented in Table 1. They varied between 2 and 5 min at urine flow rates between 0·5 and 6·9 ml./min. In Expts. 8 and 19 increasing the urine flow rate decreased the delay time, though not proportionally. This relation was not always reproducible in a given animal and was not apparent in the group as a whole. The delay time at a given flow rate was not related to the size of the animal nor to the G.F.R.

In three further experiments the flow from one ureter was artificially increased by irrigation of the renal pelvis on the same side with warm

saline. This procedure failed to modify the delay time consistently as is shown in Table 2. The findings are compatible with the view that the greater part of the delay time represents passage of the urine through the renal tubules.

TABLE 1. Urine flow rates and renal delay times

Dog	Weight (kg)	Urine flow (ml./min)	Delay time (min)
8	12·0	(*A*) 0·6	4·0
		(*B*) 4·7	2·0
13	28·0	2·4	3·0
14	26·5	1·6	4·0
16	10·5	1·7	2·5
17	16·0	1·0	5·0
18	22·0	6·9	3·0
19	14·0	(*A*) 1·4	4·0
		(*B*) 6·0	3·0
21	12·0	2·1	3·0
22	13·3	0·5	4·0
23	12·6	0·7	4·0
24	29·4	0·9	4·5
25	20·0	1·5	2·5
26	18·0	(*A*) 2·6	2·0
		(*B*) 1·5	2·0
27	14·5	1·8	2·5

(*A*) and (*B*) refer to consecutive experiments on the same animal. The delay times are given to the nearest half minute.

TABLE 2. Delay times with irrigation of the right renal pelvis

Dog	Weight (kg)	Urine flow (ml./min)		Delay time (min)	
		Right	Left	Right	Left
10	26	8·7	2·5	4	4
11	27	10·5	0·8	3	4
12	25	7·9	1·9	3	4

The urine 'flow' on the right includes the irrigating saline.

The application of the double isotope method in three patients undergoing ureteric catheterization for differential renal function studies gave strikingly similar results both as to the form of the activity and isotope ratio curves and as to the effective delay time. In a fourth patient with unilateral pyelonephritis the isotope ratio in urine from the affected kidney lay consistently below that of the plasma, a finding possibly related to the renal damage.

Serial clearances

'Steady State' experiments. Serial clearances of the two isotopes were calculated on 2 or 4 min urine samples, applying the delay time obtained from the isotope ratio curves. Figure 3 shows the results from a steady state experiment with and without correction for delay. It is seen that the

error introduced by ignoring the correction is small except when the plasma concentration of the isotope is changing rapidly. In five steady state experiments in which ^{57}Co was given 20 min before the start of sampling the difference in the mean clearance of the isotope with and without correction for delay lay between 2 and 8 %.

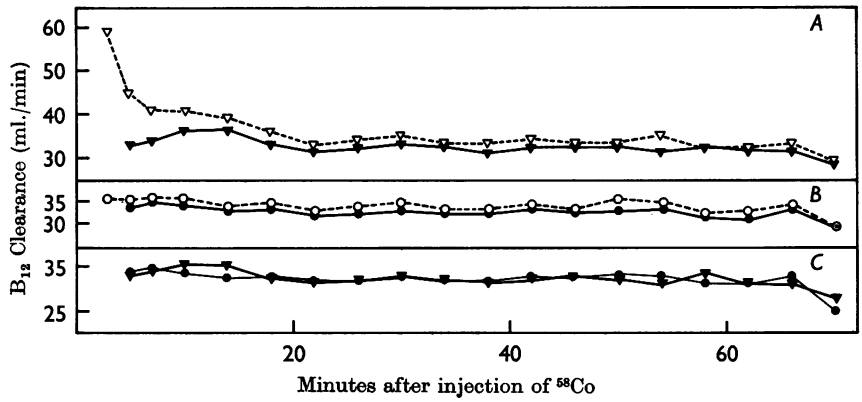

Fig. 3. Serial clearances of vitamin B_{12} in a typical steady-state experiment. (*A*) Values for ^{58}Co clearance uncorrected (interrupted line) and corrected (continuous line) for delay time of 5 min. (*B*) Similar values for ^{57}Co. (*C*) Comparison of values for the two isotopes, both corrected for delay time. (^{58}Co, triangles; ^{57}Co, circles).

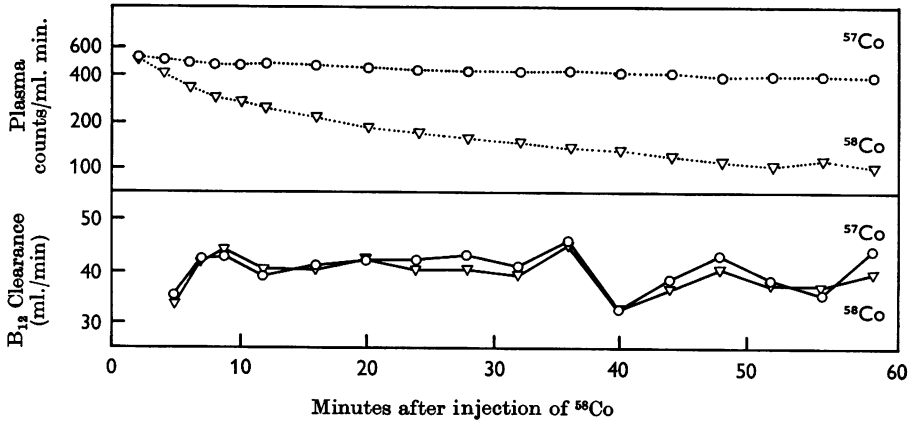

Fig. 4. Plasma concentrations and serial clearances of vitamin B_{12}. The ^{58}Co was given as a single injection during a constant infusion of ^{57}Co. The ^{58}Co clearances are calculated with a delay time of 4 min while the ^{57}Co clearances are based on simultaneous plasma and urine values.

Figure 4 shows the serial clearances based on a constant infusion of ^{57}Co and a single injection of ^{58}Co. In the calculation of the ^{57}Co clearances simultaneous plasma and urine concentrations were employed; for the

^{58}Co clearances the appropriate delay time of 4 min was applied. The good agreement between the two sets of values serves as an empirical proof of the validity of the delay time correction and speaks against the need for a constant infusion.

Variations in urine volume arising from extra-renal mechanical factors such as pelvic or ureteric peristalsis or respiration are unavoidable and may cause fluctuations in the apparent clearance, especially at low flow rates. The recognition of such artifacts may not always be possible but the distortion produced in the serial clearance pattern under steady conditions is small and has not in our experience interfered with the interpretation of genuine changes in the G.F.R.

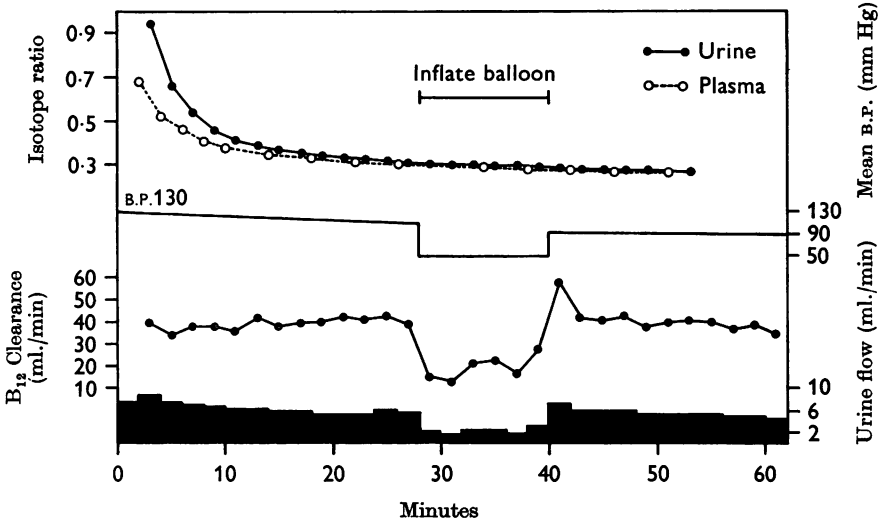

Fig. 5. The effect on the G.F.R. of inflating a balloon in the thoracic aorta. From above downwards the record shows: (1) the urine and plasma isotope ratio curves (undisturbed by the procedure); (2) mean blood pressure recorded from the femoral artery; (3) serial clearances of [^{57}Co]B_{12} and (4) urine flow rate. The period of balloon inflation is indicated by the sudden reduction in blood pressure. Note the maintenance of the G.F.R. after inflation despite a fall-off in pressure. The apparent overshoot of clearance on release of the balloon is discussed in the text.

Changing G.F.R. To determine how rapidly deliberately induced changes in the G.F.R. could be detected, renal arterial pressure was reduced by inflating a balloon in the lower thoracic aorta. The result of such an experiment is shown in Fig. 5. On inflation of the balloon an immediate fall in urine flow is accompanied by an immediate fall in the 'apparent' G.F.R. Urine volume and G.F.R. remain depressed during the period of inflation

and recover promptly on release, when an overshoot in clearance occurs well above the pre-inflation value. The question arises whether the clearance attributed to each period is indeed related to the vitamin B_{12} actually filtered during that time.

TABLE 3. Data from balloon inflation experiment (cf. Fig. 5)

Mid-point of urine period (min)	Urine activity (cts./ml. min)	Urine volume (ml./min)	Plasma activity (cts./ml. (min)	Activity ratio U/P	Clearance (ml./min)
21	5571	5·53	722	77	42·5
23	5403	5·41	700	77·5	41·5
25	5121	5·75	682	75	43·5
27	4963	5·22	660	75	39·0
		Inflate balloon			
29	5072	1·87	647	78·5	14·7
31	5764	1·40	630	91·5	12·8
33	6032	2·09	610	99·0	20·6
35	6084	2·13	600	101	21·6
37	6007	1·53	581	103	15·8
39	5889	2·75	580	102	27·8
		Release balloon			
41	5014	6·72	577	87	58·5
43	4166	5·89	562	74	42·0
45	4090	5·49	547	75	41·0
47	4088	5·63	535	76·5	43·0

The urine and plasma counts shown refer to $[^{57}Co]B_{12}$.

Smith (1951) commented on the apparent increase in clearance that accompanied a sudden increase in urine volume and, conversely, the apparent fall in clearance when urine flow was suddenly reduced. He suggested that this might be related to a change in the functional dead space, but did not commit himself to any more concrete interpretation. The problem is best illustrated by reference to Table 3 which gives the relevant data covering the balloon inflation shown in Fig. 5. The figures refer to the six 2 min periods during inflation and the four periods before and after it. In the first period after release of the balloon the urine volume increases 2·5 times while the apparent clearance is doubled. This increased volume necessarily results from changes occurring at the time, but the B_{12} content in at least some of that volume must have been filtered earlier. The difficulty thus arises from a dissociation in time between the volume of urine and its B_{12} content. This cannot be wholly overcome by manipulation of the renal delay time, but only by collecting urine at its site of filtration, which is impossible. However, if the urine/plasma concentration ratio were to remain constant over the period of inflation (indicating a change in water reabsorption proportional to the change in clearance) then the dissociation between volume and content would be immaterial and the observed increase in clearance on release of the balloon would be real. On

the other hand, if the urine/plasma ratio increases (owing to a relative increase in water reabsorption) as is seen to occur in Table 3, then the observed clearance will exceed the true value for the relevant period. Similar arguments apply to situations in which the urine/plasma ratio or the urine flow are diminished. The governing principle is that the greater the change in the urine/plasma ratio, the greater will be the discrepancy between the observed and the true clearance resulting from a change in urine flow rate.

The validity of the clearance formula $C = UV/P$ thus depends on a constant urine volume, and this applies equally whether the plasma level of the clearance substance is steady or falling. Knowledge of the delay time allows a correct relation to be established between the plasma and urine concentrations, but not between urine concentration and urine volume. In practice, then, the fluctuations of clearance coinciding with sudden changes in urine flow cannot be regarded as necessarily representative of changes in filtration. Once the urine flow has stabilized, however, the actual clearance is again shown. These considerations, though important, do not detract from the value of a running record of the G.F.R., provided the pattern of the G.F.R. is studied with reference to the urine flow and the urine/plasma concentration ratio of the clearance substance. A consistent change or trend in clearance will be demonstrated in two or more consecutive periods and will not be obscured by the above phenomenon.

In the experiment shown in Fig. 5 the delay time was assumed to remain constant at 3 min despite the changed conditions. Reference to the isotope ratio curves shows that once similarity between plasma and urine ratios has been approached, as indicated by the virtual merger of the curves, a very large change in delay time would be required before it became detectable—larger indeed than the range of variation shown in Table 1. An alteration of delay time after the period of equilibration can be detected by a further injection of one or other isotope in order to reproduce the rapidly changing isotope ratio, and this process may theoretically be repeated as often as required. The gain in accuracy of clearances that might be so obtained has not, however, seemed to justify such a procedure, and we have remained content to apply the initial correction throughout the experimental period.

DISCUSSION

Nelp *et al.* (1964) provided convincing evidence that the free fraction of vitamin B_{12} in the plasma of dog and man was excreted by glomerular filtration only. For routine purposes they considered fractionation of the plasma unnecessary and for the clinical estimation of the G.F.R. proposed

the formula [^{57}Co]B_{12} clearance = 0·89 × inulin clearance. Breckenridge & Metcalfe-Gibson (1965) reported a mean vitamin B_{12}/inulin ratio of 0·99 in ten patients using the total plasma activity of infused [^{58}Co]B_{12}. Cutler & Glatte (1965) found a mean free [^{57}Co]B_{12}/inulin ratio of 0·99 (S.D. = 0·09) in thirteen subjects and a mean fraction of bound plasma activity of 15%. Our finding of an increase in the bound fraction with time both in man and dog, always with a falling and sometimes with a constant level of total activity, prompted us to perform routine fractionation of plasma samples, at least until further information was available on the distribution of large doses of administered B_{12}. The pattern of protein binding as revealed by serial blood samples reflects the difference in turnover characteristics of the free and bound fractions in plasma. Though familiar with the observations of Nelp and colleagues we were surprised to find that such a large fraction of injected vitamin was bound. Our present evidence suggests that conclusions based on *in vitro* studies of the binding capacity of plasma significantly underestimate the binding potential available in the living organism to meet a large B_{12} load. In the foregoing experiments the initial concentration of stable B_{12} was in the region of 300 ng/ml. and fell to about 150 ng/ml. in the course of 1 hr. Since the fraction of bound activity following injection of labelled B_{12} was sometimes in the region of 15% it follows that at least 45 ng/ml. must have been bound immediately after the saturating injection.

The interpretation of clearances based on a falling plasma concentration without urine collection ('slope' method) as described by Slapak & Hume (1965) would appear particularly difficult in the light of the above findings, for the initial volume of distribution of the injected isotope is obtained by extrapolation from a curve which represents an increasing percentage of bound activity. Although fractionation of the plasma by adsorption or dialysis enables the free B_{12} disappearance curve to be derived, the volume of distribution is not easily calculated without making assumptions of uncertain validity. Slapak & Hume claimed to have demonstrated that the B_{12} disappearance curve 20–50 min after intravenous injection was linear (when plotted on a logarithmic scale) and reflected the clearance of the substance by glomerular filtration. This view has already been challenged by Aurell (1965) who failed to demonstrate a linear slope until some 2 hr after injection. Our own data confirm this view, as illustrated in Fig. 1. After this interval the error introduced by changes in the bound fraction and by possible sequestration of B_{12} in tissue storage sites is likely to be exaggerated. The misuse by Slapak & Hume of logarithms to the base 10 instead of to the base e in the calculation of their slopes (Ekins, Nashat & Portal, 1966) resulted in an apparent clearance 2·3 times less than the proper value. The agreement they obtained between B_{12} clear-

ances so calculated and simultaneous standard inulin clearances must be coincidental and cannot be explained on a mathematical basis.

We have not carried out a formal comparison of vitamin B_{12} and inulin clearances, but in thirteen anaesthetized dogs in which surgery was confined to the catheterization of ureters and blood vessels the mean initial B_{12} clearance over a period of 16–20 min was 3·8 ml./kg body weight (S.D. = 0·5, range 3·1–4·7). These figures are in accord with the inulin clearances in thirty-two anaesthetized dogs given by Asheim, Persson & Persson (1961) who found a mean value of 3·77 ml./kg (S.D. = 0·84, range 1·74–5·86).

The problems inherent in clearance calculations based on a falling plasma concentration of the clearance substance have been discussed by Smith (1951) and Michie & Michie (1951). The major difficulty has been the uncertainty of the renal delay time. This has usually been equated with the first appearance time—the interval elapsing between injection of a substance and its detection in the urine. In man with intravenous injection and bladder sampling a commonly accepted value is 2·5 min (Smith, Goldring & Chasis, 1938), and in the dog about 100 sec (Morales, Crowder, Fishman, Maxwell & Gomez, 1950). The use of this correction in the clearance equation has not been universally accepted and a longer interval of 5–8 min in man was suggested by Brun, Hilden & Raaschou (1949). Further difficulty arose from evidence that the functional dead space was not a constant volume, but expanded in response to a diuresis (Bojesen, 1949; Morales *et al.* 1950)—a view with which we agree.

The use of the isotope ratio curves allows a more certain value to be ascribed to the effective delay time than has been possible hitherto. Its validity is borne out in practice by the agreement observed between corrected clearances based on a rapidly changing plasma concentration and values obtained from a constant infusion. For experimental work of the sort described, where arterial sampling is no problem, a single injection method yields results as reliable as the conventional infusion technique.

The use of short clearance periods permits the study of renal function on a larger scale than is customary. The resulting magnification of events calls for careful interpretation but is capable, we believe, of producing much interesting information. The concurrent use of the double isotope technique provides a new relation between plasma and urine, which in addition to throwing light on the renal delay time serves to monitor the experimental technique and the validity of clearance results. The principle may be applied to any clearance substance capable of being labelled with two different isotopes, provided that the radiations contributed by each are distinguishable.

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