SUMMARY

1. The fate in the rabbit of the following compounds has been investigated: benzohydrazide and its *p*-chloro, *p*-methyl and *m*- and *p*-hydroxy derivatives; 1-acetyl-2-*m*- and *p*-hydroxybenzoylhydrazines and their anhydrides; 2-*m*- and *p*-hydroxyphenyl-5-methyl-1-oxa-3:4-diazoles.

2. Benzohydrazide, *p*-chlorobenzohydrazide and *p*-methylbenzohydrazide were lethal to rabbits in oral doses of just over 100 mg./kg. and were metabolized to the corresponding hippuric acids, which were isolated from the urine. Hydroxylation of the benzene ring and acetylation of the hydrazide group were not detected.

3. *p*-Hydroxybenzohydrazide and its acetyl derivative and 1-acetyl-2-*p*-hydroxybenzoylhydrazine were relatively non-toxic and were metabolized by direct conjugation with glucuronic and sulphuric acids.

4. *m*-Hydroxybenzohydrazide was intermediate in toxicity between benzohydrazide and *p*-hydroxybenzohydrazide, and was metabolized mainly by direct conjugation but partly by hydrolysis to *m*-hydroxybenzoic acid.

5. 2-*m*- and *p*-Hydroxyphenyl-5-methyl-1-oxa-3:4-diazoles were relatively non-toxic and were metabolized by direct conjugation mainly with glucuronic acid.

6. It is suggested that the toxicity of benzo-

hydrazide and its *p*-chloro and *p*-methyl derivatives is due to release of hydrazine *in vivo*.

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The Vitamin B₁₂-binding Systems of Isolated Intestine of the Rat

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It has recently been demonstrated (Schilling & Schloesser, 1957) that pseudovitamin B₁₂ does not affect the absorption of vitamin B₁₂ by human beings, nor does it compete to any great extent for binding by human gastric juice. They concluded from this that the vitamin B₁₂-binding property of gastric juice was very specific.

It occurred to us that similar observations might be useful in our studies of the uptake of vitamin B₁₂ by isolated rat intestine from buffered solutions containing the vitamin. We hoped that it might be possible in this way to obtain information concerning different binding systems. These anticipations have been justified since our observations have led to the demonstration that there are at least two vitamin B₁₂-binding systems in the small

intestine of the rat. One of these is susceptible to competition by the analogues we have used, whereas the other seems to be highly specific for cyanocobalamin itself.

MATERIALS AND METHODS

Animals. Black-and-white rats of the Scott-Russ strain were used. The animals were starved overnight before an experiment. They were killed by a blow on the head and the intestine was then removed.

Radioactive vitamin B₁₂ and analogues. Radioactive vitamin B₁₂, containing a mixture of ⁵⁶Co and ⁵⁷Co was dissolved in Krebs's phosphate buffer at pH 7.4 (Umbreit, Burris & Stauffer, 1949) to give a concentration of 5 μmg./ml. Various analogues of vitamin B₁₂, including pseudovitamin B₁₂, a mixture of the ethylamides of vitamin B₁₂

(anti-vitamin B₁₂) and the lactone of vitamin B₁₂ (Smith, 1957), were dissolved at various concentrations in the radioactive-vitamin solution and the mixture of the vitamin and its analogue was used in the test flask. In the control flask there was the buffered solution of the radioactive vitamin. The concentrations of the analogues covered the range 5–1250 μ mg./ml.

Counting procedure. The scintillation counter used contained a sodium iodide crystal, 2 in. diam. $\times$ 1 in. long, protected by lead shielding 3½ in. thick. The material was introduced into the counting chamber in standardized flat-bottomed glass specimen tubes.

The background count for the instrument was of the order of 250 counts/min. All counts were carried out over a period of time such that the true counting rate, i.e. the count less the background count, was obtained with an accuracy of at least $\pm 3\%$. The results were expressed as counts/min./g. wet wt. of tissue. By comparison with the counts obtained with a standard radioactive cyanocobalamin solution the results could be converted into absolute amounts of vitamin.

Tissue preparations

Preparation of intestine. The upper small intestine was used. The gut was ligatured at the pylorus before removal from the animal; it was filled with oxygenated glucose saline from a hypodermic syringe, according to the technique of Wilson & Wiseman (1954). This procedure greatly facilitated the removal of the gut with the minimum of mesentery attached.

Intestinal strips. The intestine was severed just distal to the pylorus and removed from the animal. It was cut into lengths of 3–4 cm. and all superfluous mesentery and fat were removed. Each portion was opened by a cut along the line of mesenteric attachment, washed in 0.9% NaCl and then divided into two equal and comparable halves by a second longitudinal cut. One half was used as test and the other as control. This obviated possible differences in absorptive capacity inherent in the different levels of the gut. Three such halves from the upper 12 cm. of the intestine were used for each observation. The strips were incubated in 5 ml. of Krebs phosphate buffer contained in 50 ml. conical flasks, which were shaken throughout the experiment. The buffer was gassed with O₂ before use and O₂ passed over the medium during the experiment. The incubation temperature was 37° and the experiments lasted 30 min., except where the time course of the reaction was being followed.

At the end of the experiment the pieces of intestine were removed, rapidly washed in water, blotted dry, transferred to screw-capped bottles and weighed. The screw-capped bottles, which were specially adapted to the MSE top-drive microhomogenizer (Measuring and Scientific Equipment Ltd.), were used in order to prevent splashing during homogenization. After the bottles had been reweighed, approximately 5 ml. of water was added and the tissue then macerated for 2.5 min. at top speed. The volume of the homogenate was made up to 10 ml. with water and the radioactivity measured.

Homogenized intestine. Some experiments were carried out with homogenized small intestine. A weighed piece of intestine was macerated in the phosphate buffer for 5 min. at top speed in the microhomogenizer. The material was kept cool by surrounding the container with ice. The final

dilution was adjusted so that the concentration was 0.5 g./ml. The homogenate was divided into two equal portions. One of these was incubated with radioactive vitamin B₁₂ in the usual manner at 37° in O₂ with constant shaking. For the test, pseudovitamin B₁₂ was mixed with the radioactive vitamin B₁₂ and the two were added simultaneously to the other half of the homogenate. At the end of the experiment the uptake of vitamin B₁₂ was determined by ultrafiltration. This was done by determining the radioactivity of the ultrafiltrate and subtracting it from that of the original solution.

Intestinal sacs. One possible criticism of observations made on intestinal strips is that both the mucosal and the serosal surfaces are exposed to the vitamin B₁₂ solution. In order to obviate this we have carried out a number of experiments with everted intestinal sacs (Wilson & Wiseman, 1954), in which only the mucosal surfaces are exposed.

Oxygenated buffer and a bubble of O₂ were introduced into the inside of the sacs; the outer fluid contained the radioactive vitamin with or without the analogue. In one series of experiments the uptake of radioactivity was determined by homogenizing the whole sac at the end of the experiment. In another series the mucosa and sub-mucosal layers were separated before determining the radioactivity; this was done by scraping the surface with a small cataract knife. Small pieces of the scraped intestine were examined histologically. A typical picture is shown in Fig. 1. It will be seen that a very clear separation was obtained at the level of the muscularis mucosae. In one experiment, the radioactivity of the two layers was determined in relation to wet weight, in a second experiment the uptake was related to the dry weight of the tissue. Drying was carried out at 110° overnight. The dried tissue was then dissolved in 1 ml. of conc. H₂SO₄ by heating to

Fig. 1. Section of small intestine (A) before and (B) after scraping off the superficial mucosal layer.

110° for 2 hr. The resultant dark-brown solution was cooled, made up to 10 ml. with water and the radioactivity determined.

RESULTS

Intestinal strips. The results are shown in Table 1. Pseudovitamin B₁₂ depressed the uptake of vitamin B₁₂ up to a concentration of 100 µmg./ml. of analogue. Increased concentrations of the analogue failed to depress uptake of radioactive vitamin B₁₂ further (see Fig. 2). The uptake at this maximum depression we refer to as the plateau level; it was of the order of 36 % in this series of experiments.

Like pseudovitamin B₁₂, anti-vitamin B₁₂ (ethylamide of cyanocobalamin) depressed the uptake of the vitamin by the intestine (see Fig. 2). The maximum depression gave a plateau of about 20 %.

With a mixture of anti-vitamin B₁₂ and pseudovitamin B₁₂, the depression was somewhat greater than that obtained with pseudovitamin B₁₂ alone,

Fig. 2. Individual results of percentage uptake of vitamin B₁₂ at varying concentrations of pseudovitamin B₁₂ (●), vitamin B₁₂ lactone (Δ), anti-vitamin B₁₂ (○) and a mixture of the anti-vitamin and the pseudovitamin (⊙). The curves for pseudovitamin B₁₂ and anti-vitamin B₁₂ are shown as continuous and broken lines respectively.

but no greater than that obtained with anti-vitamin B₁₂ alone.

The vitamin B₁₂ lactone has been tested only at two high levels, namely 100 and 1000 µmg./ml. In each case the depression indicated a plateau level of 38 %, which was very similar to that with pseudovitamin B₁₂.

Rate of uptake by intestinal strips. It seemed possible that the analogues might diminish the rate of transport of vitamin B₁₂ into the tissues. This would give an apparent diminution of uptake at 30 min. An experiment was therefore carried out in which the uptake of vitamin B₁₂ with and without pseudovitamin B₁₂ was studied at 15, 30, 45 and 60 min. respectively. The vitamin B₁₂ was present in the usual concentration and the pseudovitamin at a concentration of 500 µmg./ml. The results of the observations at varying intervals of time indicated that there was no increased uptake with or without pseudovitamin B₁₂ after 30 min. (Fig. 3). The percentage uptake with pseudovitamin B₁₂ was therefore constant after 30 min.

Fig. 3. Uptake of vitamin B₁₂ by intestinal strips at varying time intervals with (○) and without (●) pseudovitamin B₁₂ at a concentration of 100 µmg./ml. Concentration of vitamin B₁₂ was 5 µmg./ml. The uptake is expressed in terms of dry weight.

Table 1. *Uptake with intestinal strips*

Results are expressed in terms of wet weight of tissue. Counts are all corrected to the date of the first observation; 900 counts/min. $\equiv$ 1 µmg. of radioactive B₁₂.

Analogue used	Concn. of analogue (µmg./ml.)	No. of observations	Mean counts/min./g.		Mean uptake (µmg./g.)		Percentage uptake (test/control $\times$ 100)
			Controls	Tests	Controls	Tests	
Pseudovitamin B ₁₂	5	3	2522	2011	2.8	2.2	82
	50	4	2892	1666	3.2	1.9	59
	100	3	3522	1366	3.9	1.5	39
	250	2	3021	1141	3.3	1.3	39
	1250	1	2812	922	3.1	1.0	32
Anti-vitamin B ₁₂	25	1	4056	3012	4.5	3.3	73
	250	1	1896	324	2.1	0.4	19
	1250	1	2784	816	3.1	0.9	29
Anti-vitamin B ₁₂ and pseudovitamin B ₁₂	250 each	2	2134	502	2.4	0.55	23
Lactone	1000	1	3220	1292	3.5	1.4	40
	100	1	6000	2048	6.6	2.3	35

Table 2. *Uptake with homogenized intestine*

Results are expressed in terms of wet weight of tissue. Counts are corrected to the date of the first observation; 900 counts/min. $\equiv$ 1 μ mg. of radioactive B₁₂.

Concn. of pseudovitamin B ₁₂ (μ mg./ml.)	No. of observations	Mean counts/min./g.		Mean uptake (μ mg./g.)		Percentage uptake (test/ control $\times$ 100)
		Control	Tests	Control	Tests	
50	3	4906	2926	5.6	3.3	59
250	3	—	2879	—	3.3	59
1000	3	—	2806	—	3.2	58

Table 3. *Uptake with everted intestinal sacs*

Unless otherwise stated, this is expressed in terms of wet weight of tissue. Counts have been corrected to the date of the first observation; 11 870 counts/min. $\equiv$ 1 μ mg. of radioactive B₁₂.

Material	Concn. of pseudovitamin B ₁₂ (μ mg./ml.)	No. of observations	Mean counts/min./g.		Mean uptake (μ mg./g.)		Percentage uptake (test/ control $\times$ 100)
			Control	Test	Control	Test	
Whole sacs	0	2	39 000	—	3.52	—	100
	50	2	—	21 150	—	1.8	52
	250	2	—	6 220	—	0.58	17
	1000	2	—	6 290	—	0.58	17
Mucosa	0	1	22 400	—	1.9	—	100
	50	1	—	17 400	—	1.5	77
	250	1	—	6 450	—	0.56	29
	1000	1	—	8 890	—	0.76	39
Mucosa (dry wt.)	0	1	136 000	—	11.6	—	100
	50	1	—	43 000	—	3.7	32
	250	1	—	36 000	—	3.0	26
	1000	1	—	39 000	—	3.3	29

Homogenized intestine. The results are shown in Table 2. There was a plateau value of 48 %. The absolute uptake in this system was greater than in the intact isolated intestinal strips (Table 1).

Intestinal sacs. The results with whole sacs are shown in Table 3. In each case a plateau was obtained which was very similar to that with intestinal strips in the previous experiments. Although the results with the submucosal layers are not included, they were essentially similar to those obtained with the mucosa.

DISCUSSION

There is evidence to show that the uptake of vitamin B₁₂ by the isolated rat intestine is in fact a measure of absorption by the tissue. In the first place we have demonstrated that there is an initial build up of the vitamin in intestinal tissue before systemic absorption, in intact animals which were fed on radioactive vitamin B₁₂ and in which the distribution in the gut and liver was studied after various time intervals (Latner & Raine, 1957). Secondly, in the present work, intact intestine which had been incubated with the radioactive vitamin and subsequently homogenized retained the vitamin B₁₂ which had been taken up, thus demonstrating that the substance is not

dissolved in the extracellular fluid in equilibrium with the solution in the flask.

One of the difficulties in this work is the possibility that the vitamin B₁₂ is absorbed into the tissue across the cut edges and not across the mucosa. We consider that any absorption through these cut surfaces must be negligible since their area is so small in comparison with that of the intestinal mucosa. Another difficulty is the fact that the results with the intestinal strips might be influenced by the fact that the serosal surface as well as the mucosa was exposed to the vitamin B₁₂ solution. These difficulties have been adequately dealt with by our observations that everted sacs of small intestine behave in a fashion practically identical with that of the strips. With the sacs, of course, only the mucosal surface was directly exposed to the vitamin B₁₂ solution.

The most likely explanation of the depression of uptake of vitamin B₁₂ by pseudovitamin B₁₂ and the other analogues is that there is competition between the two substances for binding sites within the tissue. Another possible explanation would be that the analogues interfere with the rate of transport of vitamin B₁₂ into the intestinal tissues. The experiment in which observations were made at different time intervals shows that this latter explanation is not applicable, since the uptakes of

the vitamin at 45 min. and 1 hr. in each case indicated that a maximal value had been reached and that this value was lower in the presence of pseudovitamin B₁₂.

Although we have not carried out observations proving that the analogues were actually bound by the tissues, we believe that it is safe to infer that this happened. The results could thus be best explained in terms of a competition between the analogues and vitamin B₁₂ for binding sites within the tissue. This does not apply to all the binding of vitamin B₁₂. The plateau level of uptake of vitamin B₁₂ we consider could be a measure of a binding system highly specific for the vitamin itself; this system we have called 'the non-competitive system'.

It is possible to explain the existence of such a system in terms of a 'dead-space' phenomenon. That is a penetration by vitamin B₁₂ of extra-cellular spaces between villi or between cells. As the volume of this space would be relatively constant it could create an apparently constant specific uptake of the vitamin, since in all the experiments a constant concentration of the vitamin has been used. This dead-space effect, however, could not possibly apply to the experiment in which we used homogenized intestine throughout. Any dead space that existed in the intestinal wall would have been markedly diminished, if not entirely obliterated, by the homogenization procedure. In spite of this, in the presence of the analogue, there was still a binding plateau which can only be regarded as strong evidence for the presence of a non-competitive binding system. It is interesting that the plateau was distinctly higher with the homogenate than with intact strips or sacs. Analysis of the absolute uptakes showed that the increased binding by the homogenate could largely be attributed to increased non-competitive uptake. This might well indicate that the non-competitive binding is in some way related to the intracellular contents.

The non-competitive system is unaffected even in the presence of a concentration of the analogue some 250 times that of the vitamin. The system is not necessarily exactly the same for each analogue. The plateau level for anti-vitamin B₁₂ was approximately 20%, whereas that for pseudovitamin B₁₂ and for the lactone of vitamin B₁₂ was about 36%. The mixture of anti-vitamin B₁₂ and pseudovitamin B₁₂ gave a plateau corresponding to that for anti-vitamin B₁₂ alone, i.e. the lowest which had been obtained.

Although the other vitamin B₁₂-binding system does allow for competition with the analogues, there seems to be a greater affinity for the vitamin itself. The concentration of the analogue must be several times that of the vitamin before there is any marked diminution in uptake of cyanocobalamin.

If vitamin B₁₂ binding is essential to the process of intestinal absorption of the vitamin, it is possible that a study of the two types of binding we have demonstrated in the intestinal wall may shed some light on the actual mechanism of absorption. With this in view we have been carrying out feeding experiments with radioactive vitamin B₁₂ and the analogues in intact rats to see whether or not the analogues depressed absorption of the vitamin (A. L. Latner & L. C. D. P. Raine, unpublished work). We have demonstrated that neither pseudovitamin B₁₂ nor vitamin B₁₂ lactone interferes with the absorption of the vitamin itself. On the other hand, the anti-vitamin B₁₂ does interfere.

Although pseudovitamin B₁₂ does not affect absorption in the intact animal, it is capable of abolishing the competitive type of binding in the isolated intestine. One can only assume that it is unlikely that this binding system is concerned with absorption from the intestinal lumen. It is possible, however, that the non-competitive system may play some part in this process. An analysis of the percentage uptakes at the plateau levels with intestinal strips rather favours this possibility. In Fig. 4, the various plateau percentage uptakes are compared. It will be seen that there is no difference between the values obtained with pseudovitamin B₁₂ or the lactone. On the other hand, when anti-vitamin B₁₂ is present there is a marked diminution. For this comparison the observations with the anti-vitamin B₁₂ alone have been grouped with those obtained with a mixture of the anti-vitamin and

Fig. 4. Individual percentage uptake: (A) with pseudovitamin B₁₂, (B) with B₁₂ lactone, and (C) in the presence of anti-vitamin B₁₂. Each point is the mean of two observations. The analogue concentrations were at the plateau levels of 100 μ g./ml. and above.

pseudovitamin B₁₂. Comparing this group with the combined results obtained with pseudovitamin B₁₂ and the lactone, statistical analysis demonstrates that the lower uptakes in the presence of anti-vitamin B₁₂ are highly significant ($t = 3.1$; $P = 0.003$). In other words, non-competitive binding is appreciably diminished in the presence of the anti-vitamin; this material is also capable of affecting absorption of cyanocobalamin in the intact animal, whereas no such effect has been observed with either pseudovitamin B₁₂ or the lactone.

It is interesting to note that recent work (Bunge, Schloesser & Schilling, 1956) has demonstrated that normal human gastric juice also sometimes possesses two types of vitamin B₁₂-binding activity. One is highly specific for cyanocobalamin and the other allows for competition with pseudovitamin B₁₂.

Other workers have also studied competition phenomena between vitamin B₁₂ and its analogues (Rosenblum *et al.* 1956; Coates *et al.* 1956). In the chick, high dosage of pseudovitamin B₁₂ by mouth does affect absorption of vitamin B₁₂ (Coates *et al.* 1956).

SUMMARY

1. The measurement of uptake of radioactive vitamin B₁₂ by isolated intestine has been described.

2. The effect of pseudovitamin B₁₂, an anti-vitamin B₁₂ and the lactone of vitamin B₁₂ on uptake of radioactive vitamin B₁₂ has been studied.

3. Two vitamin B₁₂-binding systems have been demonstrated, one competitive and the other non-competitive.

4. The implications of the non-competitive binding system in relation to uptake of vitamin B₁₂ are discussed.

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The Proteolytic Activity of the Venom of *Bothrops jararaca*

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The presence of proteolytic activity in the venom of *Bothrops jararaca* seems to have been the first evidence for the occurrence of enzymes in snake venom (Lacerda, 1884). Since then, knowledge of the proteolytic activity of snake venoms has progressed rather slowly (Zeller, 1951). The hydrolysis of casein by the venom of *B. jararaca* was studied by Taborda & Taborda (1940) under various conditions. Rocha e Silva & Andrade (1945) observed that benzoylarginine amide was also hydrolysed by the venom of *B. jararaca*, a fact which seemed to indicate that the substrate

specificity of the proteolytic enzyme contained in the venom was similar to that of trypsin. More recently, Hamberg & Rocha e Silva (1956, 1957) reported in the venom of *B. jararaca* the presence of both heat-labile and heat-stable proteolytic activity. The latter, after heating, retained the power to hydrolyse benzoylarginine methyl ester, while apparently losing all hydrolytic activity towards casein. Holtz & Raudonat (1956) have separated two fractions from the venom of *B. jararaca*, by precipitation with ammonium sulphate, namely 'protease', which had strong proteo-