THE FREE AND BOUND FORMS OF THE SERUM VITAMIN B₁₂ IN VARIOUS ANIMAL SPECIES

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When vitamin B₁₂ (B₁₂) in the serum is determined microbiologically, two forms are distinguishable, of which the "free" or "easily available" form has a growth-promoting activity on the assay organisms without any treatment of the serum, whereas the "bound" form is activated through liberation by heating or enzymatic digestion from the protein with which it is bound (1, 2). The physiological roles of the two forms have not yet been clarified. From the fact that the major part of B₁₂ in normal human serum consists of the bound form, some investigators have assumed that the bound form is physiologically active or hematopoiesis-promoting (3, 4, 5), whereas in another study the free form has been considered to have a direct hematopoiesis-promoting action (6).

While studying the serum B₁₂ in various animal species of mammals, it has been found that the ratio of the free form to the total serum B₁₂ levels varies remarkably according to the animal species. These results are presented in the present report. Some reports have already appeared concerning the B₁₂ activity of the plasma or serum of various animal species (7, 8), without distinguishing the free form from the bound.

MATERIALS AND METHODS

Serum Samples Examined — The sera of the mouse, rat, guinea pig, rabbit, dog, horse, cow and calf were examined. The mice, rats, guinea pigs and rabbits had been kept on natural diets in our laboratory. Adult animals of both sexes were used. The mice used weighed from 20 to 25 g, rats from 200 to 250 g, guinea pigs from 500 to 600 g, and rabbits from 2.5 to 3.0 kg. Mouse serum was separated from the collected blood of five mice. Dog blood was given by the Second Department of Surgery, Nagoya University School of Medicine. Bloods of horses, cattle and calves were obtained from the slaughter house of Nagoya City. Anemic blood was excluded from the test samples by the determination using hematocrits.

Vitamin B₁₂ Determination — Microbiological assay with *Lactobacillus leichmannii* ATCC 4797 was used. The treatment of the sera and the procedure for the assay were described previously (1, 9). The liver B₁₂ levels were

also determined by the same procedures as described in a previous paper (9). The determination of alkali-stable fraction of B₁₂ activity in the serum and liver was neglected due to the reason stated previously (1, 10). Bacto B₁₂ Assay Medium USP "Difco" was used throughout the present study.

RESULTS

The total and free form levels of the serum B₁₂ of the mouse, rat, guinea pig, rabbit, dog, cow and calf are shown in Tables I—VI. The bound form of the serum B₁₂ can be obtained by subtracting the value of the free form from the total. It is seen in the Tables that the total B₁₂ levels of the serum and the ratio of the free form to the total B₁₂ levels varied remarkably according to the animal species. Even in the same animal species the total and free form levels of the serum B₁₂ fluctuated in relatively wide ranges, but the differences of the total B₁₂ and of the ratios of the free form to the total were fairly distinct among the various animal species.

TABLE I
Serum Vitamin B₁₂ Levels of Mice

No. of serum ^a	Free B ₁₂	Total B ₁₂	Free/total
	<i>mμg/ml</i>	<i>mμg/ml</i>	%
1	0 ^b	7.20	0
2	0	6.70	0
3	0	6.65	0
4	0	6.95	0
Mean	0	6.88	0

^a A serum sample was separated from the collected blood of five mice.

^b Less than 0.01 mμg.

TABLE II
Serum Vitamin B₁₂ Levels of Rats

No. of serum	Free B ₁₂	Total B ₁₂	Free/total
	<i>mμg/ml</i>	<i>mμg/ml</i>	%
1	0.22	0.63	35.2
2	0.30	1.05	28.6
3	0.24	0.80	30.0
4	0.20	0.54	37.4
5	0.15	0.51	29.4
6	0.32	0.90	35.5
7	0.35	1.25	28.0
8	0.34	0.44	77.3
9	0.16	0.64	25.0
Mean	0.25	0.75	36.3

The ranges and average values of the total and free form levels of the serum B₁₂ of the various animal species are summarized in Table VII together

TABLE III
Serum Vitamin B₁₂ Levels of Guinea Pigs

No. of serum	Free B ₁₂	Total B ₁₂	Free/total
	<i>mμg/ml</i>	<i>mμg/ml</i>	<i>%</i>
1	1.60	1.89	85.5
2	0.68	6.47	93.9
3	2.56	2.86	89.5
4	1.84	2.27	81.0
5	0.66	0.70	94.3
6	0.65	0.69	94.2
Mean	2.23	2.48	89.7

TABLE IV
Serum Vitamin B₁₂ Levels of Rabbits

No. of serum ^a	Free B ₁₂	Total B ₁₂	Free/total
	<i>mμg/ml</i>	<i>mμg/ml</i>	<i>%</i>
1	19.0	67.0	28.3
2	10.5	49.0	21.4
3	14.4	36.0	40.0
4	29.6	72.0	41.1
5	26.3	49.0	53.6
6	21.8	44.0	49.6
7	12.5	50.0	25.0
8	15.0	50.5	29.7
9	14.8	43.0	34.4
10	9.8	16.0	61.3
Mean	17.4	47.7	38.4

^a The serum samples were the same as in a previous report (9).

TABLE V
Serum Vitamin B₁₂ Levels of Dogs

No. of serum	Free B ₁₂	Total B ₁₂	Free/total
	<i>mμg/ml</i>	<i>mμg/ml</i>	<i>%</i>
1	0.22	0.96	23.9
2	0.21	1.11	19.8
3	0.20	1.14	17.5
4	0.17	0.84	20.2
5	0.20	0.54	37.0
Mean	0.20	0.92	23.7

with the data on human serum cited from a previous report (1). The total B₁₂ levels of the various species could be ranged from high to low as follows: rabbit, mouse, horse, guinea pig, dog, rat, calf, cow and human. Although the differences of the total B₁₂ levels among the last five species were minute, rabbit serum had about 100 times and mouse serum about ten times as high B₁₂ levels as the last five animal species. The ratios of the free

TABLE VI
Serum Vitamin B₁₂ Levels of Horses, Cattle and Calves

No. of serum	Free B ₁₂	Total B ₁₂	Free/total
	<i>μg/ml</i>	<i>μg/ml</i>	%
Horse 1	1.08	2.20	49.1
2	3.12	3.44	90.1
3	1.62	2.84	57.0
Mean	1.94	2.83	65.4
Cow 1	0.04	0.43	9.3
2	0.07	0.64	10.9
Mean	0.06	0.54	10.1
Calf 1	0.21	0.40	52.5
2	0.22	0.72	30.6
Mean	0.22	0.56	41.6

TABLE VII
Serum Vitamin B₁₂ Levels of Various Animal Species

Animal species	Number of animals	Serum B ₁₂ Levels (<i>μg/ml</i>)				Free/total (%)	
		Free		Total			
		Range	Mean	Range	Mean	Range	Mean
Mouse	20	0	0	6.65—7.20	6.88	0	0
Rat	9	0.15—0.34	0.25	0.44—1.25	0.75	25.0—77.3	36.3
Guinea pig	6	0.55—6.08	2.23	0.69—6.47	2.48	81.0—94.3	89.7
Rabbit	10	9.8—29.6	17.4	16.0—72.0	47.7	21.4—61.3	38.4
Dog	5	0.17—0.22	0.20	0.54—1.14	0.92	17.5—37.0	23.7
Horse	3	1.08—3.12	1.94	2.20—3.44	2.83	49.1—90.1	65.4
Calf	2	0.21—0.22	0.22	0.40—0.72	0.56	30.6—52.5	41.6
Cow	2	0.04—0.07	0.06	0.43—0.64	0.54	9.3—10.9	10.1
Human ^a	40	0—0.08	<0.02	0.16—0.81	0.42	0—14.7	<4.8

^a Cited from a previous report of the author (1).

TABLE VIII
Liver Vitamin B₁₂ Levels of Various Animal Species

Species	Number of animals	Liver B ₁₂ levels (<i>μg per g wet tissues</i>)	
		Range	Mean
Mouse	20	0.14—0.73	0.36
Rat	9	0.06—0.62	0.20
Guinea pig	5	0.95—1.20	1.08
Rabbit ^a	12	0.34—1.00	0.61
Dog	5	0.08—0.43	0.28
Human ^b	18	0.48—1.16	0.74

^a Cited from a previous report of the author (9).

^b Cited from a previous report of the author (10).

form to the total in various species could be ranged in a decreasing order as follows: guinea pig, horse, calf, rabbit, rat, dog, cow, human and mouse. There was no parallelism between the total and free form levels of the serum B_{12} among these species. In guinea pigs almost all of the serum B_{12} consisted of the free form, and in horses more than 50 per cent of the mean total B_{12} level was the free form. These are a striking contrast to human and mouse sera in which the free form is hardly found more than a trace.

The liver B_{12} levels of these species except horse, cow and calf were also determined with the results shown in Table VIII. As is seen from the Table, the liver B_{12} levels of these species ranged similarly, and the variation among the various species was not so marked as in the serum B_{12} levels. There was no significant relationship between the B_{12} levels of the serum and of the liver among these species.

DISCUSSION

The total and free form levels of the serum B_{12} of various animal species were determined in the present investigation. The total B_{12} levels of the serum varied remarkably among the species. Comparing the mean values of each animal species, human serum had the lowest vitamin B_{12} level, and cow, calf, rat and dog sera had similar B_{12} levels to human serum. In guinea pig and horse sera the mean total B_{12} level was several times, in mouse serum about ten times and in rabbit serum about one hundred times higher than in human serum. The high B_{12} level of rabbit serum has already been reported by the author (9, 11) and some other workers (7, 8). The liver B_{12} levels per gram of wet tissues of the various species were similar to each other and rabbit liver was not an exception. Because liver is apparently the major storage organ of B_{12} , this fact can not support a simple assumption that the absorption of B_{12} through the intestinal tract is much more active in rabbits than in other species.

The ratios of the free form to the total B_{12} levels of the serum in various species was not constant, but varied considerably among the species. The serum B_{12} of various species was classified according to the mean ratios of the free form to the total into following three groups. (a) Most or more than fifty per cent of the serum B_{12} consists of the free form, such as in the sera of guinea pigs and horses. (b) The free form is present in some degree but the majority of the serum B_{12} consists of the bound form, such as in the sera of rats, rabbits, dogs, calves and cows. (c) Scarcely more than a trace of the free form can be found and the serum B_{12} consists almost entirely of the bound form, such as in the sera of humans and mice. Wilson *et al.* (12) have found by Euglena assay that the total B_{12} levels of monkey serum are lower than those of human serum and more than 50 per cent of the mean total B_{12} level was in the free form. Thus, monkey serum appears to belong to the first group.

It has been reported that the bound form in human serum is bound with

the protein in α -globulin fraction (1, 5), whereas the free form is recovered from β -globulin fraction by paper electrophoresis (1). The free form may not be free in the true sense of the word inasmuch as it seems to be in a loose binding with the protein in β -globulin fraction (1). By the assay organisms this binding may be splitted easily and utilized as the true free B₁₂, in contrast to the binding of the bound form. Although in the serum of other species, especially of guinea pigs, the nature of the free form is not yet confirmed, such binding with protein must be present to prevent loss of the serum B₁₂ by renal excretion. The differences of the property of the serum B₁₂ among various species may be due to the differences in the proportion of the two types of the B₁₂-binding proteins present in these sera. Two types of the B₁₂-binding proteins have been dealt with in the previous papers (1, 2).

Among the results obtained in the present investigation some problems remain for further studies. For example, the exact cause for such a wide variation of the total B₁₂ levels of the serum among various species was not understood, although it may partially account for the difference of the amount or capacity of the B₁₂-binding proteins in these sera. The distinction in the physiological significance in animal body of both the bound and free forms of the serum B₁₂ is still quite obscure.

SUMMARY

Range and average values were determined for the total and free form levels of the serum vitamin B₁₂ of the mouse, rat, guinea pig, rabbit, dog, horse, cow and calf. Liver vitamin B₁₂ levels of these animal species were also determined except the horse, cow and calf. The total B₁₂ levels of the serum had a wide variation among the various animal species. The species with the highest total B₁₂ level of the serum was the rabbit and the level was about one hundred times higher than that of human serum, which was lowest among the sera of the various species examined. The ratio of the free form to the total B₁₂ varied also widely among the species. The serum B₁₂ of the various species was classified by the mean ratios of the free form to the total into three groups. The liver vitamin B₁₂ levels of various species did not show such a remarkable diversity in contrast to the serum B₁₂ levels.

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