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## Intracellular Distribution of Vit. $B_{12}$-$Co^{60}$ in Liver and Kidney of $B_{12}$ Deficient and Normal Rats.*† (25127)

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Results of microbiological assays of fractions of mouse liver(1) indicated that Vit. $B_{12}$ is concentrated in mitochondria. Kidneys of rats(2) contained most radioactivity 96 hours after parenteral administration of $B_{12}$-$Co^{60}$. At 48 hours(3) most radioactivity was present in supernatant fraction of kidneys in bound form, and measurements at various time intervals after administration of $B_{12}$-$Co^{60}$ (4) showed that kidneys progressively lose radioactivity. Intracellular distribution of radioactivity of administered $B_{12}$-$Co^{60}$ in livers of rats(3,5,6) and of $cobalt^{60}$ in organs and intracellular organelles of mice(7) was studied. The relationships of amounts of Vit. $B_{12}$ in fractions of kidneys and livers of normal and deficient animals at different time intervals after administration of vitamin has not been studied. We studied the intracellular distribution of $B_{12}$-$Co^{60}$ and $cobalt^{60}$ in kidneys and livers of normal and $B_{12}$ deficient rats at intervals after the last injection of radioactive compounds.

*Methods.* Rats of the St. Louis University colony were fed soybean meal diet of Hogan *et al.*(8) for production of deficiency of $B_{12}$ and soybean meal diet with 90 $\mu$g Vit. $B_{12}$/kg of diet or Purina lab chow for control animals. Rats fed these diets for 8 to 10 weeks received 1 or 5 injections (1 $\mu$c each injection at 24 hour intervals) of $Co^{60}Cl_2$ or high specific activity $B_{12}$-$Co^{60}$.‡ At intervals after last injection, animals were sacrificed and samples of liver and kidney excised, weighed, homogenized in cold 0.88 M sucrose, and cellular components separated by differential centrifugation(9). Microsomal fractions were prepared by centrifuging 1 hour at 100,000 x g in a Spinco Model L centrifuge. Each sedimented fraction was washed twice with sucrose and the sediment suspended in 5 ml of sucrose for each gram-equivalent of original tissue used. There was good agreement between cts/min§ of minced tissue, homogenates and the sum of cts/min of the fractions. Radioactivity recovered in fractions was 88% to 102% of that in homogenates. Nitrogen was determined by micro-Kjeldahl procedure.

*Results.* Table I shows distribution of nitrogen in fractions of liver and kidney of normal and deficient animals. Normal group contains data on animals fed laboratory chow (Table II) and soybean meal diet supplemented with 90 $\mu$g of $B_{12}$ (Table III). Separate treatment of data on nitrogen distribution showed no difference between groups so the data were combined. Nuclear and mitochondrial fractions of liver and kidney of deficient animals contain more nitrogen than these fractions of normal animals. Conversely, microsomal fraction of tissue of normal animals

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§ Radioactivity was counted with a Tracerlab SC-57 low background well scintillation counter and an SC-18A scaler. cts/min = counts/min.

TABLE I. Distribution of Nitrogen in Fractions of Liver and Kidney Tissue. Values given are the mean ± standard error in terms of mg N/fraction equivalent to one gram of fresh tissue.

| | No. rats | Homogenate | Nuclei | Mitochondria | Microsomes | Supernatant | Recovery total means |
|---|---|---|---|---|---|---|---|
| Normal liver* | 14 | 37.07 ± 3.12 | 5.87 ± .61 | 6.03 ± .44 | 7.16 ± .52 | 17.54 ± 1.82 | 36.60 |
| Deficient liver | 22 | 39.87 ± 1.41 | 6.23 ± .36 | 7.38 ± .27 | 5.21 ± .28 | 19.10 ± .71 | 37.92 |
| Normal kidney | 14 | 31.62 ± 1.89 | 5.38 ± .46 | 5.26 ± .21 | 5.46 ± .47 | 14.98 ± .79 | 31.08 |
| Deficient kidney | 22 | 31.55 ± .91 | 6.06 ± .31 | 5.81 ± .09 | 4.17 ± .31 | 15.13 ± .52 | 31.17 |

* The term normal is applied to animals fed laboratory chow or soybean meal diet supplemented with 90 $\mu$g vit. $B_{12}$/kg and the term deficient is for those animals fed basal soybean meal diet.

contains most nitrogen. There was no marked difference in distribution of nitrogen (8 normal animals) or radioactivity (2 deficient animals injected with $B_{12}$-$Co^{60}$) in fractions obtained from 0.25 M sucrose by the modified procedure of Hogeboom(10) as compared with the data in the tables.

Eight animals fed laboratory chow received 5 injections of $B_{12}$-$Co^{60}$ (5 $\mu$c) and 8 animals fed soybean diet ($B_{12}$ deficient) were given $B_{12}$-$Co^{60}$ (5 $\mu$c). Table II shows distribution of radioactivity in fractions of liver and kidney.

Kidneys concentrated radioactivity the most, but during 30 days, concentration of radioactivity declined. At 12 hours supernatant fraction of kidney tissue of both groups of animals contained the most radioactivity. However, at 30 days mitochondrial fraction of kidneys concentrated the most radioactivity. These data suggest a stronger affinity of mitochondria for radioactivity.

Homogenates of liver of control animals contained less than one-half as much radioactivity as livers of animals fed the $B_{12}$ deficient diet. Mitochondria of liver under all conditions concentrated the most radioactivity. Furthermore, at 30 days radioactivity in mitochondria was 3 times as much as at 12 hours. This marked rise in liver mitochondria is a result of transfer of radioactivity to the liver from extrahepatic sources. Since whole kidney lost radioactivity during this period, it is one possible source of radioactivity.

These studies were repeated with a modified procedure in which 2 to 4 animals were used for each experiment. Animals were fed soybean meal diet or soybean meal diet with 90 $\mu$g of $B_{12}$/kg of diet. Animals consuming $B_{12}$ deficient diet received 1 $\mu$c of $B_{12}$-$C^{60}$ or $Co^{60}$

TABLE II. Intracellular Distribution of Radioactivity at Intervals of Time after Injection of $B_{12}$-$Co^{60}$ (1 $\mu$c Given Daily for 5 Days). Values expressed as counts/min./mg N.

| | Laboratory chow | | | Deficient* | | |
|---|---|---|---|---|---|---|
| | 12 hr 3 rats | 12 day 2 rats | 30 day 3 rats | 12 hr 2 rats | 12 day 2 rats | 30 day 4 rats |
| Liver | | | | | | |
| Homogenate | 211 | 331 | 350 | 486 | 777 | 1,141 |
| Nuclei | 252 | 162 | 249 | 722 | 838 | 1,523 |
| Mitochondria | 364 | 607 | 1,294 | 816 | 1,752 | 2,402 |
| Microsomes | 104 | 146 | 66 | 194 | 275 | 336 |
| Supernatant | 194 | 371 | 382 | 543 | 675 | 782 |
| Kidney | | | | | | |
| Homogenate | 20,616 | 13,363 | 9,208 | 30,840 | 23,967 | 8,600 |
| Nuclei | 15,310 | 11,499 | 3,665 | 16,637 | 18,081 | 4,355 |
| Mitochondria | 8,668 | 11,866 | 12,357 | 30,470 | 19,282 | 10,406 |
| Microsomes | 8,178 | 7,410 | 2,043 | 11,714 | 6,796 | 2,408 |
| Supernatant | 28,086 | 14,645 | 11,227 | 46,103 | 31,856 | 8,873 |

* Basal soybean meal diet deficient in vit. $B_{12}$.

TABLE III. Intracellular Distribution of Radioactivity at Intervals of Time after Injection of $B_{12}$-$Co^{60}$ or $Co^{60}Cl_2$. Values are expressed as counts/min./mg N.

| | Deficient;* 1 $\mu$c $B_{12}$-$Co^{60}$ inj. | | | Deficient + 90 $\mu$g $B_{12}$/kg diet; 1 $\mu$c $B_{12}$-$Co^{60}$ inj. | | | Deficient; 1 $\mu$c $Co^{60}Cl_2$ inj. | | |
|---|---|---|---|---|---|---|---|---|---|
| | 12 hr | 12 day | 30 day | 12 hr | 12 day | 30 day | 12 hr | 12 day | 30 day |
| | 4 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Liver | | | | | | | | | |
| Homogenate | 161 | 401 | 309 | 68 | 117 | 70 | 290 | 159 | 31 |
| Nuclei | 150 | 341 | 288 | 60 | 62 | 63 | 291 | 155 | 98 |
| Mitochondria | 177 | 895 | 735 | 83 | 182 | 168 | 265 | 332 | 73 |
| Microsomes | 172 | 165 | 252 | 68 | 44 | 45 | 321 | 215 | 27 |
| Supernatant | 181 | 339 | 190 | 44 | 72 | 73 | 240 | 103 | 4 |
| Kidney | | | | | | | | | |
| Homogenate | 4,963 | 2,944 | 1,617 | 8,518 | 3,424 | 4,379 | 1,450 | 255 | 72 |
| Nuclei | 3,505 | 1,937 | 979 | 6,100 | 2,613 | 3,844 | 991 | 174 | 35 |
| Mitochondria | 3,115 | 5,715 | 3,174 | 11,667 | 4,066 | 5,906 | 2,240 | 528 | 129 |
| Microsomes | 3,992 | 1,314 | 501 | 1,769 | 909 | 1,262 | 487 | 306 | 70 |
| Supernatant | 6,470 | 2,738 | 1,393 | 9,062 | 5,021 | 4,637 | 1,279 | 150 | 48 |

* Basal soybean meal diet deficient in vit. $B_{12}$.

$Cl_2$ parenterally. Animals fed deficient diet supplemented with Vit. $B_{12}$ received 1 $\mu$c of $B_{12}$-$Co^{60}$.

The results of this experiment are shown in Table III. The fate of radioactivity of $Co^{60}Cl_2$ is markedly different from that of $B_{12}$-$Co^{60}$. Kidney tissue retains much less radioactivity from $Co^{60}Cl_2$ than from $B_{12}$-$Co^{60}$. Furthermore, at 30 days there is little radioactivity from $Co^{60}Cl_2$ in kidney or liver tissue.

*Discussion.* Our results show that liver tissue of Vit. $B_{12}$ deficient rats binds greater quantities of radioactivity of $B_{12}$-$Co^{60}$ than liver of rats fed diets containing the vitamin. Our data at 12 hours and 12 days are in agreement with those of Barrows and Chow(3) taken at 48 hours, which show that supernatant fraction of kidney contains the greatest concentration of radioactivity from parenterally administered $B_{12}$-$Co^{60}$. However, over 30 days, radioactivity of supernatant fraction of kidney decreases more rapidly than that of the mitochondrial fraction.

The increase in concentration of radioactivity in rat liver with time after injection of 5 $\mu$c $B_{12}$-$Co^{60}$ was concomitant with loss of radioactivity by kidney. Most vitamin thus obtained by liver was concentrated in the mitochondrial fraction. Therefore, these data are in agreement with those of Swenseid *et al.*(1), and possibly show interrelationship between kidney and liver. Since tissues of $B_{12}$ deficient rats took up greater quantities of $B_{12}$-$Co^{60}$, use of isotopically labeled vitamin for tracing the fate of Vit. $B_{12}$ may prove a useful tool for studying the metabolic role of the vitamin.

Rosenblum *et al.*(2) reported that most parenterally administered Vit. $B_{12}$ was excreted unchanged in urine of rats within a short period of time. We compared the rate of disappearance of radioactivity of $B_{12}$-$Co^{60}$ and $Co^{60}Cl_2$ from kidneys and livers of rats and found that inorganic cobalt is not retained to the same extent as is cobalt of Vit. $B_{12}$. The data suggest that radioactivity of $B_{12}$-$Co^{60}$, bound by mitochondria of liver and kidney, is deposited in these tissues in a form other than inorganic cobalt (presumably, Vit. $B_{12}$).

*Summary.* 1) In a study of intracellular distribution of radioactivity of $B_{12}$-$Co^{60}$, the mitochondrial fraction of livers of $B_{12}$ deficient rats takes up more radioactivity than does this fraction of normal animals. This increases between 12 hours and 30 days. 2) At 12 hours the supernatant fraction of kidneys of normal and deficient animals is highest in radioactivity, however, at the end of 30 days, radioactivity in the mitochondrial fraction is greatest. 3) There is a transfer of radioactive $B_{12}$ to liver from extrahepatic sources. 4) Radioactivity of $Co^{60}Cl_2$ is not retained by fractions of tissues as is that of $B_{12}$-$Co^{60}$.

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## Distribution of Injected Heparin in Different Blood Fractions.* (25128)

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Although the effect of heparin in inhibiting coagulation of blood has been known for a number of years, almost nothing is known with respect to the mechanism by which this action is exerted. It is most probable that at least one step involves an interaction with one of the plasma proteins since the presence of a normal blood component is a necessary requirement in prevention of blood clotting by heparin. As an initial step experiments were, therefore, conducted to determine in which blood fraction the heparin resides subsequent to intravenous administration. When this is decided further studies could be made on the specific fraction involved. We employed heparin-$S^{35}$ since this can be measured in minute amounts. The present report describes experiments on blood fractionation after intravenous injection of heparin and determination of radioactivity in the different fractions.

*Methods.* Heparin-$S^{35}$ in the form of sodium salt was prepared as described previously(1). This material, having an anticoagulant activity of 152 U.S.P. units/mg, maintained a constant specific activity (8,010 c.p.m./mg) after repeated recrystallization. Other analytical data were as follows: glucosamine 23.22%; nitrogen 2.25%; sulfur 12.02%; sodium 7.88%; $[\alpha]_D^{25}$ + 51°. Three dogs weighing 8-9 kg were injected intravenously with solution of 30 mg of heparin-$S^{35}$ ($2.4 \times 10^5$ c.p.m.) in physiological saline. Blood samples drawn from animals 15 and 30 minutes after injection were employed in fractionations and subsequent radioactivity assay. First, blood was separated into plasma and cells. A second portion of blood was clotted and serum separated from clotted material was fractionated into components of different densities. After mixing a serum aliquot with aqueous sodium chloride to give a solution with density of 1.006, the mixture was centrifuged at 79,000 g for 20 hours at 4° (Spinco Ultracentrifuge, Model L). The top layer (density, less than 1.006) was removed for radioactivity analysis and the infranate was mixed with salt solution (D, 1.346 to give a final density of 1.063). The latter mixture was centrifuged at 79,000 g for 20 hours and the top layer (D, 1.006-1.063) taken off. The above procedure was repeated with the infranate using potassium bromide as the salt to give top layers with densities of 1.063 - 1.120 and 1.120 - 1.200. The different fractions were then combusted, converted to barium sulfate, and counted in gas flow counter. Another 10 ml of serum was mixed with 90 ml of 5% trichloroacetic acid. The precipitated protein was collected and washed with trichloroacetic acid. The washings were combined with the original supernate. In another experiment serum was mixed with 3 volumes of alcohol and the precipitated material separated from the supernate. Additional experiments were conducted in which plasma was submitted to chemical fractionation by Cohn

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