ings, it would be appropriate to designate these materials as hemosidera.

Summary. 1. A method is outlined for isolating hemosiderin from horse spleen by sedimentation from salt solutions of different specific gravities. 2. The iron, nitrogen, phosphorus and ash concentration varied considerably in hemosiderin samples obtained from different spleens. Fractionation of these preparations by sedimentation in organic liquids showed that fractions could be obtained which varied in their iron concentrations from about 25 to 41% iron. 3. Hemosiderin has been found to contain hexoseamine, galactose, mannose and fucose.

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Fractionation of Radioactive B_{12}-Complex In Kidney Homogenates.* (23273)

CHARLES H. BARROWS, JR.† AND BACON F. CHOW
The Johns Hopkins University, Department of Biochemistry, Baltimore, Md.

Rosenblum, *et al.*(1), demonstrated that among the various target organs of rats injected with radioactive B_{12} (referred to hereafter as B_{12}*) kidneys contained initially the greatest amount of radioactivity per gram of the wet organ. However, a major portion of its radioactivity disappeared within 21 days, Harte, *et al.*(2). In contrast, the amount of radioactivity initially retained by the liver and pancreas of these animals decreased only little even after as long as 90 days after injection. Furthermore, administration of nonradioactive B_{12} to rats previously injected with B_{12}* resulted in a decrease in radioactivity in liver and pancreas but an increase in kidneys. These unique properties of kidney prompted us to study the manner in which B_{12} is retained by this organ. To this end, the ability of the vitamin to combine with components of kidney tissue has been examined by various chemical fractionation procedures and differential centrifugation. The results of such studies are reported in this communication.

Methods. Preparation of Homogenates. Adult female rats from our stock colony were injected subcutaneously with 1.0 μg Co^{60}-

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† Present address: Baltimore City Hospital, Dept. of Gerontology.

TABLE I. Equilibrium Dialysis of Kidney Homogenates.

	Radioactivity in cpm			
	Inside	Outside	Bound	% bound
Homogenate from rat: 1	507	142	365	56
2	485	112	373	63
3	436	94	343	64
B_{12} alone	244	224	20	

All kidneys were homogenized with 10 times their wt of distilled water. Five ml of these homogenates were used for each exp.

labeled B_{12}* with a specific activity of 40 μc mg. Forty-eight hours later, the animals were sacrificed and their kidneys were removed, trimmed of fat, and homogenized in a Potter Elvehjem Homogenizer with 10 times their weight of cold distilled water. *Analysis of Radioactivity.* Unless otherwise stated all samples for radiometric measurements were prepared by wet ashing with a mixture of nitric and sulfuric acid. To each sample was added 1 mg of cobaltous chloride which served as a carrier. After evaporation of the excess acids, the residue was transferred quantitatively to a planchet and dried. The radioactivity was then measured with a thin window Geiger Mueller counter.

Results. Determination of Bound B_{12}. (A) *By equilibrium dialysis.* Five ml aliquots of homogenates from kidneys of 3 rats were dialyzed with continuous shaking at 4°C for 48 hours against an equal volume of distilled water. As a control, 5 ml of a solution with a known amount of radioactive B_{12} was dialyzed simultaneously in a like manner. After 48 hours of equilibration, the fluids inside and outside the cellophane bags were analyzed for radioactivity. The results of a typical experiment (Table I) established satisfactory equilibration since radioactivity on either side of the bag containing the control sample was essentially the same. On the other hand, the inside fluids with the homogenate contained a greater amount of radioactivity than the corresponding outside fluids. The difference between these values may be considered as B_{12}* bound to kidney tissues and is approximately two-thirds of the total radioactivity. The total radioactivity in the undialyzed kidney homogenates which differed somewhat with various rats, was also measured. Recovery of radioactivity in the fluids inside and outside the membrane was approximately 90% or greater.

(B) *By exhaustive dialysis.* Another means of measuring the bound B_{12}* in the homogenate is to remove completely the free B_{12}* by prolonged dialysis. Thus, 5 ml of the pooled kidney homogenate from several rats were dialyzed with gentle shaking against an equal volume of distilled water in a cold room with frequent changes of water. However, approximately 72 hours afterwards the dialysis bag was allowed to equilibrate with an equal volume of distilled water for an additional 48 hours. This was done to show that main bulk of the dialyzable B_{12} has been removed. Our findings showed that total radioactivity in the original homogenate was 123 μg but was reduced to approximately one-half (67 μg). The unbound B_{12}* was almost completely removed since the total radioactivity in the last dialysate amounted to only 6 μg. The union between the vitamin and kidney tissue thus is not readily dissociated by dialysis.

(C) *Extraction with butanol.* Two other procedures were employed to determine the amount of the bound B_{12}* in kidney tissue. The first was an attempt to extract the free vitamin in the kidney homogenate with butanol. Thus, 2 ml of a solution containing 50 μg of unlabeled B_{12} and saturated with ammonium sulphate was added to 2 ml of the kidney homogenate. This mixture was then extracted 5 times with 8 ml of butanol saturated with water. The radioactivity in the combined butanol extracts was measured after evaporation to dryness, and found to be 19 μg or 33% of the radioactivty in the whole homogenate (57 μg).

(D) *Precipitation of B_{12}*-protein complex with acetone.* To 2 ml of the kidney homogenate was added 10 ml of redistilled acetone. After centrifugation radioactivity in the precipitate and the supernatant fluid was measured, and found to contain 35 μg and 20 μg respectively. All 4 independent methods demonstrate that two-thirds of the total radioactivity in the kidney was bound.

Differential Centrifugation. Differential

TABLE II. Distribution of Radioactivity among Various Cellular Fractions.

	Groups	Avg total cpm/pair of kidneys	% of whole homogenate			
			Nuclear (N)	Mitochondria (M)	Soluble (S)	Recovery
Exp. A	1	3404 ± 160*	39 ± 1	12 ± 1	40 ± 1	92
	2	1200 ± 131	39 ± 1	15 ± 1	38 ± 1	92
B	3 (Control)	1714 ± 168	38 ± 4	12 ± 1	44 ± 1	94
	4 (Post-inj.)	1998 ± 140	42 ± 1	9 ± 1	41 ± 3	92
	5 (Pre-inj.)	2860 ± 108	42 ± 1	10 ± 1	39 ± 3	91

* Stand. error of mean.

centrifugation is useful in the separation of particulates. It is of interest to determine distribution of the injected B_{12}* in the various fractions of kidney homogenates. In the first experiment 5 adult female rats were injected subcutaneously with 1 μg of B_{12}* administered in 4 equal doses during a 48-hour period. Forty-eight hours after the last injection the animals were sacrificed. The kidneys of each animal were trimmed of fat and homogenized with 10 times its weight of cold 30% sucrose solution employing a Potter Elvehjem Homogenizer. The homogenate was centrifuged at 2°C for 10 minutes at 800 g. The precipitate (nuclear fraction) was resuspended and recentrifuged twice in a 10 ml of cold 30% sucrose solution. The washings were combined and added to the original supernatant fluid and centrifuged for 30 minutes at 25,000 g. This precipitate is referred to as the mitochondrial fraction and the supernatant fluid as the soluble fraction. All isolated fractions as well as 2 aliquots of the original homogenate were assayed for radioactivity as previously described. The results demonstrate that approximately 38 ± 4% of the total radioactivity of the whole homogenate is found in the nuclear fractions, 12 ± 1% in the mitochondrial fraction and 44 ± 2% in the soluble fraction. Since in contrast to other target organs the kidney of rats loses the major portion of radioactivity within 3 weeks after parenteral administration of B_{12}*, it was of interest to ascertain whether loss of radioactivity from any one of these isolated fractions was responsible for this phenomenon. Eight adult female rats were injected with 2 μg of B_{12}* as previously described. Four of the animals were sacrificed 2 days (Group 1) and the remaining rats 16 days (Group 2) following the last injection. The kidneys of all animals were fractionated by the differential centrifugation method. Microscopic examination showed the presences of only a few intact cells and some mitochondria in the nuclear fraction. The supernatant fraction contained no mitochondria and the mitochondrial fraction was found to be free from microsomes by dark field microscopy. These results (Table II Exp. A) demonstrate that in spite of a marked loss of radioactivity 16 days following administration of B_{12}*, distribution of radioactivity among the various fractions remains unchanged. This finding led to an examination of distribution of radioactivity in kidney homogenates under other experimental conditions known to affect amount of radioactivity of kidney homogenates. Fifteen adult female rats were employed in this experiment. Five animals were injected with 50 μg of unlabeled B_{12} per day for one week preceding administration of one μg of B_{12}* to all animals. Forty-eight hours following the last injection of B_{12}* the 5 treated animals (pre-injection Group 5) as well as 5 additional ones (control Group 3) were sacrificed. The remaining rats (post-injection Group 4) were then injected with 50 μg of unlabeled vitamin B_{12} per day for one week. Twenty-four hours following the last injection these animals were sacrificed. The kidney homogenates of all animals were fractionated immediately following sacrifice. The results (Table II Exp. B) again demonstrate that distribution of radioactivity among the various isolated fractions is the same regardless of total amount of radioactivity or experimental conditions imposed upon the animals.

Discussion. Numerous reports (Ross(3),

Rosenthal(4) and Pitney(5)), have demonstrated that serum contains mostly bound and a little free vit. B_{12}. More recently it has been shown that the capacity of serum to bind this vitamin may vary according to diseases, (Beard(6) and Davis(7)). The ability of an organ to perform certain functions may be decided by the amount of vit. B_{12} retained by the organ. Therefore, it seems likely that information of the capacity of various fractions of tissue to combine with vit. B_{12} is important. Although only radioactivity of Co^{60} is measured in this study, we believe this measurement represents vitamin B_{12}, *per se,* since it has been shown that the vit. B_{12}, given by injection, is not altered in the body and is excreted in the urine in its original form(1,9). Our results indicate that approximately two-thirds of the radioactivity found in the kidneys of rats following parenteral administration of B_{12}* is bound to tissue components. Furthermore, the union between the vitamin and kidney tissue is not readily dissociated by dialysis, nor can the radioactive B_{12} bound to kidneys be dissociated by subsequent injection of unlabeled B_{12}. The firmness in the binding is further demonstrated by our inability to remove, *in vitro,* the bound radioactive B_{12} in kidney homogenate after the addition of a massive amount of unlabeled B_{12} either by dialysis or extraction with butanol. The results obtained by the fractionation of kidney tissue by differential centrifugation indicate a relatively high concentration of vit. B_{12} in the nuclear and soluble fractions and a low concentration in the mitochondrial fraction. It is of interest to point out that these findings differ from those of Swenseid, Bethell and Ackerman. who employed(8) a microbial assay for the vitamin, demonstrated a high concentration in the mitochondrial fraction of mouse liver. This difference in results may be due to the different technics used, or to the innate characteristic of the organs or to the difference in the species of animals. Finally distribution of radioactivity among the various isolated fractions was found to be the same regardless of total amount of radioactivity or experimental conditions imposed upon the animals.

Summary. The amount of radioactive B_{12} bound by substances in kidneys of rats preinjected with this radiovitamin was found to be approximately one-half to two-thirds of the total radioactivity by 4 independent procedures. The amounts of radioactivity in the 3 fractions obtained by differential centrifugation were also determined with only a small portion in the mitochondria. Relative percentages of the radioactivity in these 3 components remained essentially unchanged as a result of pre-injection of unlabeled B_{12}.

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