and every cubic centimeter of tumor tissue received more microspheres than each and every cubic centimeter of liver tissue after hepatic artery injection, the overall distribution of microspheres showed a much higher average concentration in tumor tissue than in liver tissue. The converse was true of portal system infusion. Unevenness of microsphere distribution has been observed in other organs and may represent segmental and alternating variations in blood supply with time(5).

Conclusions. 1. Radioactive microspheres reach a concentration in hepatic metastases averaging 4 times the concentration in surrounding liver parenchyma when infused *via* the hepatic artery. 2. When delivered into the portal system radioactive microspheres reach metastases in only an average of one-third their concentration in the surrounding liver. 3. Spill-over of radioactive microspheres 15 μ in diameter from liver to lungs and kidneys is negligible. 4. Arteriovenous shunts in liver and in V2 carcinoma are either few in number or are less than 15 μ in diameter.

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Subcellular Distribution of Serotonin in Rabbit Platelets.* (29877)

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Although serotonin (5-hydroxytryptamine) is known to be present in platelets(1), its subcellular localization has not been clearly established. Hughes and Brodie(2) reported that serotonin was not attached to subcellular particles. On the other hand, Baker *et al*(3) stated that most of the serotonin was concentrated in "cytoplasmic granules." Buckingham and Maynert(4) studied platelet fragments obtained by differential centrifugation after ultrasonic disruption and concluded that serotonin was stored either within "granules" or "some kind of subcellular particle."

The recent development of a method for separating subcellular platelet particles on a continuous sucrose gradient(5) has made it possible to examine the subcellular distribution of this biologic substance in a precise manner. The efficacy of the separation of the platelet granules and membranes by this technique has been confirmed by electron microscopy. Pilot experiments indicated that the procedure, originally designed for human platelets, could be utilized equally well for the study of rabbit platelets.

Methods. 1. Fractionation of rabbit platelets. Fifteen rabbits were bled from the carotid artery through a polyethylene cannula. To achieve rapid cooling, the blood was introduced into a large volume of mineral oil maintained at 0°C. Heparin-saline was provided to give a final concentration of 5 units/ml. The heparinized blood was processed at 5°C throughout. It was initially spun in a refrigerated centrifuge (International PR-2) at 100 $\times$ *g* for one hour. After decanting the supernatant, the sediment was resuspended in heparin-saline, 5 units/ml, and again centrifuged at 100 $\times$ *g* for one

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hour. The combined supernatants, which contained platelets, were spun at 10,000 × *g* in a Servall centrifuge. The sediment was resuspended twice in heparin-saline and centrifuged. The final platelet pellet was suspended in 0.44 M sucrose (0.001 M EDTA).

The platelets were disrupted in a glass homogenizer with a tightly fitting teflon plunger (A. H. Thomas B-7432) which was rotated by motor for 15 minutes. During the procedure the homogenizer was immersed in melting ice. When the homogenate was examined in the phase microscope, approximately 50% of the platelets appeared to be morphologically intact. In initial experiments, the "intact" platelets migrated in the granule band and undoubtedly affected the serotonin assays. In all subsequent experiments, therefore, the homogenate was centrifuged at 3000 rpm (2000 × *g*) for 30 minutes to remove the "intact" platelets; and the supernatant was then fractionated on the sucrose gradient.

All operations were carried out at 5°C. A continuous sucrose gradient (30-60%)† was prepared immediately prior to ultracentrifugation. The material to be fractionated was layered on the gradient and ultracentrifuged for 2 hours at 40,000 rpm (130,576 × *g*) in the SW-39 rotor of a Spinco model L ultracentrifuge. The 3 lusteroid tubes were then pierced at the bottom with a 26 gauge needle and the material was recovered in drop-wise fashion as fractions of approximately 0.25-0.5 ml. In other experiments, individual particulate bands were harvested in a single tube. The granule and membrane layers were washed in the following manner: each fraction was suspended in 5 ml 0.25 M sucrose (0.001 M EDTA) and spun at 40,000 rpm for one hour in the SW-39 rotor. This resulted in a pellet which was resuspended in 1 ml aliquots of 0.25 M sucrose (0.001 M EDTA). The membrane pellets were clear, whereas the granule pellets showed an opaque grayish-white color. Aliquots of the various fractions were frozen and thawed twice prior to addition to the organ bath for serotonin assays.

Rabbit aortic strip for assay of serotonin in platelets. The isolated rabbit aortic strip was used as described by Furchgott and Bhadrakom(6), and Wurzel *et al*(7). Spirally cut strips were suspended in Krebs-bicarbonate-Ringer solution at 37°C and aerated with a mixture of 95% oxygen and 5% CO_2. Isotonic contractions were recorded on a smoked drum kymograph. For bioassay, doses of serotonin creatinine sulfate (Sandoz Pharmaceuticals) and test samples from platelets were applied for a 3-minute period and then washed out. Doses were repeated at 7-minute intervals. A complete dose-response curve ranging from threshold to maximal response was obtained with sequentially doubled doses of norepinephrine and/or serotonin from 0.1 μg to 6.4 μg per 15 ml organ bath. The aortic strip was equally sensitive to both agents. The activity of the unknown samples was expressed as μg serotonin on the basis of this curve.

Results. 1. Subcellular fractions of platelets and their ability to contract aortic strip. A schematic drawing of the lusteroid tube after ultracentrifugation is shown in Fig. 1. The uppermost (surface) layer of the gradient (a), which showed a faint cloudiness in human experiments, consisted of solubilized protein and some membrane material(5). In the present studies, this layer contained dissolved serotonin. The area immediately beneath this (b) was transparent and showed a reddish tint, probably the result of hemoglobin contamination. A distinct and opaque particulate layer (c) followed, which in human experiments had been found to consist exclusively of membranes. A clear layer of sucrose (d) separated the membranes from the substantial granule layer below. In experiments with human platelets this clear layer was protein-free. The prominent granule layer (e) consisted, in parallel human experiments, of alpha granules and mitochondria. The sucrose layer beneath the granules (f) was clear and did not contain protein. In initial studies, the entire homogenate containing "intact" platelets was placed on the gradient, and a bottom layer beneath the granules was noted which contained a red sediment, probably red cell "ghosts." This was not seen when the initial homogenate was

† Details to be published later.

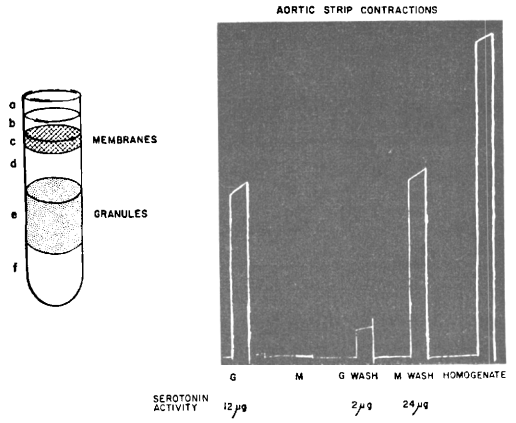

FIG. 1. Serotonin distribution in fractions of rabbit platelets obtained on a continuous sucrose gradient (30-60%). *Left:* Schematic drawing of separation. a) Top of gradient, cloudy; b) clear reddish layer; c) opaque layer, corresponding to membranes in human experiments; d) clear layer, found to be protein-free in human studies; e) "granules" (alpha granules and mitochondria); f) clear sucrose layer. Serotonin activity was found in the granules (e) and the upper layers of the gradient (a) and (b). No activity was found in (d) and (f). *Right:* Record of serotonin activity of membrane and granule layers assayed on a rabbit aortic strip. Washed granules recovered by centrifugation (G) retained their activity, whereas the supernatant (G wash) contained only about 15% of the serotonin activity of the granule layer. The washed membranes (M) had no contractile activity; however, the supernatant of the membrane wash (M wash) was very active. The contraction on the extreme right was given by the initial homogenate before fractionation. In these experiments a dose-response curve to norepinephrine was used for calibration of the aortic strip. N.B.: For the assay of sample G an aliquot of 0.1 ml was added to a 1 ml organ bath, whereas for M wash an aliquot of 0.5 ml was added to a 10 ml bath. Hence the total activity recovered in this experiment—12 μg for G, and 24 μg for M wash—is represented by the same contraction height.

centrifuged before being subjected to separation on the gradient.

As indicated on the kymograph record in Fig. 1, the granule layer (e) had substantial contractile activity. The bottom sucrose layer (f) and the protein-free area between the membranes and granules (d) showed no activity. The granule layer (e) retained its contractile activity even when washed; the supernatant was only moderately active (G wash in Fig. 1). On the other hand, there was no activity in the washed membrane fraction (c). The considerable contractile activity found in the membrane wash (M wash in Fig. 1) probably represented dissolved serotonin, although it is possible that serotonin had been loosely bound to the membranes. The surface layer (a) also exhibited contractile activity (not shown in Fig. 1) which probably represented dissolved serotonin.

2. Evidence that the aortic strip contracting agent was serotonin. The isolated rabbit aorta contracts upon exposure to norepinephrine, histamine, and angiotensin. However, purification with paper chromatography and high voltage paper electrophoresis indicated that most of the contracting material of rabbit platelets was probably serotonin. For these procedures, platelets from blood of 12 rabbits were suspended in a small volume of distilled water and disrupted by freezing and thawing. The platelet extract was layered on Whatman 3 MM filter paper with control spots containing serotonin alone and platelet extracts supplemented with serotonin. The paper was soaked in a solvent mixture consisting of pyridine:acetic acid:water (10:1:69, pH 6) and submitted to electrophoresis at 4,000 volts for one hour at room temperature in a thermo-regulated cooling tank (Savant Instruments Inc.). Strips of the paper were assayed after drying. Contraction of the aortic strip was obtained only with strips containing the spot which corresponded to the migration of exogenous serotonin. *A. Exclusion of histamine.* A descending chromatogram was developed for 5 hours on Whatman No. 4 paper with a solvent mixture of butanol:acetic acid:water (12:3:5). Control points which contained histamine alone and histamine mixed with platelet extract were established on the same sheet. Paper strips were cut out and immersed in the organ bath for assay. The contractile activity of the platelet extract was demonstrated only around an Rf of 0.5, whereas the site which corresponded to histamine (Rf around 0.25) showed no contractile activity. *B. Exclusion of norepinephrine.* Aortic strip contracting substance of the platelet extract was eluted from the paper and tested on the isolated guinea pig intestine. The result was a contraction rather than a relaxation, indicating that the contractile substance was not nor-

ephinephrine. *C. Exclusion of angiotensin.* High-voltage paper electrophoresis was carried out under conditions similar to those employed for the identification of serotonin (see *A.* above). Synthetic angiotensin migrated from the origin as two spots positioned at about half the distance from a spot of synthetic serotonin. Following electrophoresis of platelet extracts, the paper strips cut out from corresponding positions revealed no contractile activity. It was therefore concluded that the contractile substance in the platelet extract was serotonin.

Discussion. The qualitative subcellular distribution of serotonin in rabbit platelets as indicated by the results of these experiments may be summarized as follows: 1. The granule fraction contained tightly bound serotonin which could not be washed out by sucrose solution at 5°C. 2. The membrane fraction did not appear to contain bound serotonin, or, if it did, the amine was loosely attached, since the serotonin was easily washed out with 0.25 M sucrose. 3. Although the presence of serotonin in the uppermost (surface) layer of the gradient indicates the presence of a soluble form of this compound, its precise origin in the intact platelet cannot be determined. It may exist in the cytoplasm of the platelet in a free form. Therefore, these findings should not be interpreted as ruling out the existence of freely dissolved serotonin, as suggested by Hughes and Brodie (2). However, it is equally plausible that at least part of it appeared as a consequence of mechanical disruption of granules during the homogenization procedure. In human platelets, the granule fraction consists of so-called alpha granules and mitochondria, as seen in the electron microscope(5). We have tentatively presumed that the subcellular fractions of the rabbit platelet are similar to those of the human fractions studied in a similar manner. Should further studies demonstrate that this is actually the case, then the particle-bound serotonin of the rabbit platelet is definitely confined to the alpha granules or mitochondria. It remains to be determined which of these two types of granules actually contains the bound serotonin. Data on the quantitative distribution of the serotonin were not obtained. Thus it is not known how much of the total platelet serotonin the granule fraction represents.

Recently, Schulz and associates(8) reported that 95% of the serotonin was associated with the "hyalomeres," and only 5% with the granules. Our results are at variance with these findings. Their studies were carried out by harvesting subcellular particles from platelets with the technique of freezing and thawing, followed by differential centrifugation. In our experiments, freezing and thawing was used only to release serotonin from previously separated fractions. It is unlikely that platelet disruption by freezing and thawing can adequately reflect the subcellular distribution of serotonin. Actually, we interpret their findings to be a consequence of the liberation of serotonin from the granules during the freezing and thawing process.

Our results, although mainly qualitative, would appear to establish the presence of tightly-bound serotonin in the granules. There are also indications that a higher concentration is present in the granule than in the membrane. This suggests that the granules may possess a mechanism for the storage of serotonin, or they might be a site of synthesis of this compound.

Humphrey and Jaques reported that another substance in rabbit platelets which causes contraction of the aortic strip was histamine(9). Histamine was not demonstrated in our experiments, possibly because of inadequate amounts for purification.

Heparin was used as anticoagulant because earlier work showed that cellular elements of heparinized blood (4-8 units/ml, *i.e.*, 40-80 μg/ml) incubated at 37°C did not release contractile substances into plasma(10) and it was felt that release would also fail to occur at 4°C.

Summary. In rabbit platelets, serotonin has been qualitatively localized in the granule fraction in a bound form. The platelet membranes do not appear to contain bound serotonin. The existence of free (soluble) serotonin is likely, although at least part of the dissolved serotonin may have resulted from mechanical disruption of platelet granules.

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Renal Histopathology Associated with Different Degrees of Amphotericin B Toxicity in the Dog. (29878)

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It has been reported recently that administration of amphotericin B to patients with systemic fungal diseases is followed by the development of renal tubular lesions, characterized by focal necrosis and calcification(1-4). Such lesions have been observed in this laboratory in 7 consecutively treated patients and in 3 dogs which had received large doses of amphotericin B and the description of these findings has been reported(4).

The present report contains descriptions of the gross and microscopic renal abnormalities in dogs given various doses of amphotericin B for periods of a few days to several months. Abnormalities of renal function in some of the dogs have been described elsewhere(5-7).

Materials and methods. Sixteen adult male mongrel dogs in good general health were given amphotericin B (Fungizone for infusion), prepared and administered intravenously as described previously(8). Blood urea nitrogen (BUN) determinations were made using an autoanalyzer (Technicon Co., Chauncey, N. Y.).

Renal biopsies were performed under anesthesia with sodium pentobarbital, using the Vim-Silverman-Franklin needle (V. Mueller Co., Chicago, Ill.) or by excisional wedge biopsy. The needle biopsies were performed after exposure of the left kidney by a muscle splitting subcostal incision, or after previous subcutaneous explantation of the left kidney by the technique of Page(9). Entire kidneys were obtained for histologic observation by nephrectomy or at post-mortem examination.

Specimens for histologic observation were obtained from 15 dogs before and after administration of amphotericin B and from an additional dog after drug administration. The specimens obtained prior to amphotericin B were all needle or wedge biopsies except for a single nephrectomy specimen. The specimens obtained following amphotericin B were whole kidneys except 2 which were biopsy specimens. Renal specimens were also obtained from 9 other dogs which had not received amphotericin B.

Biopsy specimens were fixed in 10% buffered formalin or formol sublimate, and entire kidneys were fixed in 10% buffered formalin after being bisected in a frontal plane. The sections were embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin, alizarin and von Kossa stains.

Observations were made by one of us