cleared to the same degree. The absolute efficiency of these organs cannot be determined, as the blood flow per unit of tissue is not known in either instance. The percent removal of the bacteria remained high even when the arterial levels were maintained at 50,000 and 100,000 per ml. When one considers that the splanchnic circulation receives one-fifth of the cardiac output and that approximately 75% of the organisms are removed in a single circulation, it becomes clear why such a large number of bacteria is required to maintain a blood level of 100 organisms per ml.

The tissues of the hind limb showed no consistent ability to remove organisms from the circulating blood. The percent removed in a single circulation through the hind extremity was 12 ± 14% (S.D.). No organisms were lost in circulation through the lungs of the dog.

In the present series of rabbits the total splanchnic removal of Klebsiella pneumoniae type B averaged 20 ± 10% (S.D.). The effect of variation in the parasite on the rate of removal by the host will be discussed in a later communication.

Other studies[4,5] have shown that in the course of some experimental bacterial infections, the organisms are cleared partially or completely from the blood stream after a variable period of 5 to 48 hours, the variation depending apparently on the virulence of the infecting organism and the susceptibility of the host. After a period of several hours, the blood culture may become positive again and may be associated with a progressively unfavorable clinical course leading to the death of the animal. The reason for the ineffective clearing of the blood stream in the later stage of the infection has not been determined. It may be that the removal mechanism is blocked by accumulation of organisms in the cells of the reticuloendothelial system, but this seems unlikely because the mechanism still functions efficiently at a level of 100,000 organisms per ml. The organisms may proliferate in the cells and so alter their physiologic functions. Observations on the sites from which the bacteria return to the blood stream and the state of the reticuloendothelial system at that time are needed.

Summary. 1. A method of constant intravenous infusion of bacteria combined with the determination of bacterial counts in the circulation at various sites by venous catheterization is described, which provides a means of determining the site and of quantitating the rate of removal of bacteria from the blood stream of the intact animal.

2. The total canine splanchnic removal of hemolytic *Micrococcus aureus* averaged 74 ± 16% (S.D.); splenic removal, 78 ± 17% (S.D.). No organisms were lost in circulation through the lungs.

3. The total rabbit splanchnic removal of encapsulated *Klebsiella pneumoniae* type B averaged 20 ± 10% (S.D.).

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The Reversible Inactivation of Calcification *in vitro*.* (17334)

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Some aspects of normal calcification can be explained by physico-chemical concepts. For example, new calcification depends on the concentration of calcium and phosphate ions in excess of a critical product.[1-10] Furth-

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1 Howland, J., and Kramer, B., *Tr. Am. Pediat. Soc.*, 1922, **34**, 204.

2 Shipley, P. G., *Bull. Johns Hopkins Hosp.*, 1924, **35**, 304.

3 Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, **20**, 379.

ermore the composition of the mineral matter found in bone, is related to that of the body fluids as would be expected from phase rule considerations for a solid solution.[11-14] However such physico-chemical principles do not explain why calcification takes place at specific sites and at a definite time in the life of the preosseus cells.

Calcification *in vitro* of the hypertrophic epiphyseal cartilage has served as a useful indicator of the cellular factors.[2-4,7,8,15-21] The appearance of new calcification depends on the functioning of the complete system essential for mineralization. Most of our knowledge of the "local factors" comes from such studies. Robison[15,16,18] by such *in vitro* studies indicated the importance of phosphatase. Gutman[19] has presented evidence that phosphoralative glycogenolysis is an integral part of the calcification process. However, one can demonstrate the presence of phosphatase and phosphoralative glycogenolysis in tissues other than bone. Moreover Waldman[21] has shown that calcification *in vitro* can take place when phosphatase is inactivated. Furthermore, in rickets due to strontium, calcification *in vitro* is inactivated without affecting phosphatase activity.[17,22] Thus it can not be unequivocally stated that enzymes acting on organic phosphates are responsible for mineralization.

It occurred to us that by extending the studies of the injury to calcification in strontium rickets which is reversible *in vivo* and *in vitro*,[17] the role of a system handling calcium may be explored. (In addition we were stimulated by the statement of McLean[23] "Our search for a method of producing a reasonably complete but reversible inhibition of the local mechanism has so far failed.") This observation with strontium rickets and that of Robison[24,25] who caused mineralization of the hypertrophic epiphyseal cartilage *in vitro* by replacing Ca^{++} ions with Sr^{++} ions suggested that strontium competes with calcium for some constituent of the bone cell necessary for calcification. The more recent experiments using radioactive strontium as an indicator of calcium metabolism are in harmony with such a concept.[26]

It was reasoned that if rachitic bone sections (produced by the usual high calcium, low phosphorus diet) were shaken with Sr^{++} ions of high concentration, subsequent calcification would be inhibited by the competitive combination of strontium with a factor in the cell which is necessary for calcification. Moreover since inhibition did in fact take place, as

4 Shelling, D. H., Kramer, B., and Orent, E. R., *J. Biol. Chem.*, 1928, **77**, 157.

5 Kramer, B., Shear, M. J., and Siegel, J., *J. Biol. Chem.*, 1931, **91**, 271.

6 Kramer, B., Shear, M. J., and Siegel, J., *J. Biol. Chem.*, 1931, **91**, 723.

7 Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 708.

8 Niven, J. S. F., and Robison, R., *Biochem. J.*, 1934, **28**, 2237.

9 Logan, M., *Physiol. Rev.*, 1940, **20**, 522.

10 Huggins, C., *Physiol. Rev.*, 1937, **17**, 119.

11 Eisenberger, S., Lehrman, A., and Turner, W. D., *Chem. Rev.*, 1940, **26**, 257.

12 Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, 1945, **159**, 159.

13 Sobel, A. E., and Hanok, A., *J. Biol. Chem.*, 1948, **176**, 1103.

14 Sobel, A. E., Hanok, A., Kirshner, H. A., and Fankuchen, I., *J. Biol. Chem.*, 1949, **179**, 205.

15 Robison, R., *Biochem. J.*, 1923, **17**, 286.

16 Robison, R., and Soames, K. M., *Biochem. J.*, 1924, **18**, 740.

17 Sobel, A. E., Cohen, J., and Kramer, B., *Biochem. J.*, 1935, **29**, 2640.

18 Robison, R., Significance of phosphoric esters in metabolism, 1932, New York University Press.

19 Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, 1942, **95**, 461.

20 Robison, R., *Ann. Rev. Biochem.*, Stanford University Press, 1936, **5**, 181.

21 Waldman, J., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 262.

22 Sobel, A. E., Cohen, J., and Kramer, B., *Biochem. J.*, 1935, **29**, 2646.

23 McLean, F. C., Lipton, M. A., Bloom, W., and Barron, E. S. G., 14th Conference on metabolic aspects of convalescence, Symposium on bone metabolism, 1946, pp. 18, Josiah Macy, Jr. Foundation, New York.

24 Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 684.

25 Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1936, **30**, 66.

26 Norris, W. D., and Kisieleski, W., *Symposia on Quantitative Biology*, Cold Spring Harbor, L. I., N. Y., 1948, **13**, 164.

TABLE I.

Reversible Inactivation of Calcification *in vitro* by $SrCl_2$ and NaCl Solutions.

Control* No treatment	Inactivation				Reactivation			
Degree of calcification†	Solution mE/L	Hr of shaking	Ca×P‡ product	Degree of calcification†	Solution mE/L	Hr of shaking	Ca×P product	Degree of calcification†
	$SrCl_2$-150	2	50	0(0), 0(0)	$CaCl_2$-100	1	50	2(++++), 2(++++)
	$SrCl_2$-150	2	50	0(0), 0(0)	$CaCl_2$-660	1	50	4(++++), 4(++++)
2(++++)	$CaCl_2$-666	2	45	4(++++)	$CaCl_2$-666	1	45	4(++++), 4(++++)
2(++++)	$SrCl_2$-666	2	45	0(0), 0(0)	$CaCl_2$-666	1	45	4(++++), 4(++++)
	$NaCl_2$-666	2	45	0(0), 0(0)	$CaCl_2$-666	1	45	4(++++), 4(++++)
1½(++++)	$CaCl_2$-666	3	40	3(++++), 4(++++)	$CaCl_2$-666	1	40	4(++++), 4(++++)
							20	2½(++++), 1(++++)
1½(++++)	$SrCl_2$-666	3	40	0(0), 0(0)	$CaCl_2$-666	1	40	3(++++), 1½(++++)
							20	1½(++++), 1½(++++)
2(++++)	NaCl-666	3	40	0(0), 0(0)	$CaCl_2$-666	1	40	2½(++++), 4(++++)
							20	2(++++), 0(0)
0(0)[5]			20					
2(++++)	$CaCl_2$-666	3	45	2(++++), 2(++++)	$CaCl_2$-666	1	45	3(++++), 4(++++)
					$CaCl_2$-666	1	20	0(0), 2(++++)
					$CaCl_2$-666	1	0	0(0), 1(+++)
	NaCl-167	3	45	1(++), 1(+)	$CaCl_2$-666	1	45	4(++++), 3(++++)
					$CaCl_2$-666	1	20	0(0), 1(++), 1(++)
					$CaCl_2$-666	1	0	0(0), 0(0)
	$SrCl_2$-167	3	45	0(0), 1(+++)	$CaCl_2$-666	1	45	4(++++), 4(++++)
					$CaCl_2$-666	1	20	0(0), 0(0)
					$CaCl_2$-666	1	0	0(0), 0(0)
2(++++), 2(++++)	Saline adjusted to pH3 with HCl§	½	45	0(0), 0(0)	$CaCl_2$-666	1	45	3(++++), 1½(++++)
					$CaCl_2$-666	1	20	1(+)
					$CaCl_2$-666	1	0	0(0)
	Saline adjusted to pH9.6 with NaOH§	½	45	1(+), 1(+++)	$CaCl_2$-666	1	45	4(++++), 4(++++)
					$CaCl_2$-666	1	20	3(++++)
					$CaCl_2$-666	1	0	0(0)

* Specimens incubated in calcifying solution for 18-24 hr.
Calcifying solution contains: 0.7 eq/L NaCl
0.05 eq/L NaCl
0.22 eq/L $NaHCO_3$
Ca×P product = 10 mg % Ca×5 mg % P unless otherwise noted.

† The degree of calcification is indicated as follows: 0(0) no calcification; 1(+) trace; 1(++) broken thin line; 1(+++) almost complete thin line across the provisional zone; 1(++++) complete thin line across the provisional zone; 2(++++) heavy line across the provisional zone including the primary tongues of cartilage; 3(++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4(++++) practically complete calcification of the metaphysis.

‡ Ca×P products are obtained by keeping Ca concentration constant (Ca = 10 mg %) and varying P concentration thus: Ca×P = 10×0 = 0; Ca×P = 10×2 = 20; Ca×P = 10×4.5 = 45; and Ca×P = 10×5.0 = 50.

§ Physiological saline was used; NaCl = 0.145 eq/L.

‖ Ca×P product for this control = 20.

TABLE II.
Reversible Inactivation of Calcification *in vitro* with $CuCl_2$ in the Presence of $CaCl_2$.

Control* No treatment	Inactivation—2 hr shaking			Reactivation—1 hr shaking	
Degree of calcification†	Solution, mE/L $CaCl_2$	$CuCl_2$	Degree of calcification†‡	Solution, mE/L $CaCl_2$	Degree of calcification†‡
2(++++)	150	0.0	4(++++), 4(++++)		
2(++++)	150	0.1	4(++++), 4(++++)	150	4(++++), 4(++++)
2(++++)	150	0.3	4(++++), 4(++++)	150	4(++++), 4(++++)
3(++++)	150	0.5	1(++++), 1(++++)	150	4(++++), 4(++++)
3(++++)	150	0.5	1(+), 0(0)	150	4(++++), 4(++++)
2(++++)	150	0.5	1(+), 1(+)	150	4(++++), 4(++++)
2(++++)	150	0.75	2(+++), 2(++)	150	4(++++), 4(++++)
2(++++)	150	1.0	1(+), 1(+)	*660*	4(++++), 3(++++)
	150	1.0	1(+), 2(++)	150	4(++++), 3(++++)
	150	1.0	3(++++), 4(++++)	150	4(++++), 4(++++)
	150	1.0	0(0), 0(0)	150	4(++++), 4(++++)
	150	2.0	0(0), 0(0)	150	4(++++), 4(++++)
	150	3.0	0(0), 0(0)	*660*	3(+++), 1(+)
	150	3.0	0(0), 0(0)	150	4(+++), 1(++++)
	150	4.0	0(0), 0(0)	150	1(+), 1(+)
	150	5.0	0(0), 0(0)	150	0(0), 0(0)
	150	6.0	0(0), 0(0)	150	1(+), 1(+)
	150	10.0	0(0), 0(0)	150	0(0), 0(0)

* Specimens incubated in calcifying solution for 18-24 hr.
Calcifying solution contains: 0.7 eq/L NaCl
0.05 eq/L KCl
0.22 eq/L $NaHCO_3$
Ca×P product =[10 mg % Ca] [5 mg % P] = 50, unless otherwise noted.

† The degree of calcification is indicated as follows: 0(0) no calcification; 1(+) trace; 1(++) broken thin line; 1(+++) almost complete thin line across the provisional zone; 1(++++) complete thin line across the provisional zone; 2(++++) heavy line across the provisional zone including the primary tongues of cartilage; 3(++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4(++++) practically complete calcification of the metaphysis.

‡ Calcifying solution same as that used previously (Table I).
Ca×P product kept constant at 10×5.0 = 50.

shown in Table I, it was further reasoned that the reverse reaction should take place, by shaking with Ca^{++} ions of high strength. This actually turned out to be the case as shown in Table I. The calcification was usually more extensive than in the untreated control sections. Further experiments showed that similar reversible inactivation is possible by preliminary shaking with a high concentration of NaCl, NaCl of physiological strength adjusted to a pH of 3, and to a smaller degree with physiological saline adjusted to a pH of 9.6.

These experiments demonstrate that it is possible to reversibly inactivate calcification in vitro by electrolytes but do not prove that it takes place by competitive retardation. One can also explain these results as due to the simple removal of Ca^{++} ions from the cell when shaking with calcium free solutions. In the reactivation step, these Ca^{++} ions are replaced so that subsequent calcification can proceed. That reversible inactivation does not depend on the mere removal of Ca^{++} ions was shown in the following experiments. When rachitic bone sections were shaken with 150 mE/L of $CaCl_2$ in the presence of as little as 0.5 mE/L of $CuCl_2$, calcification was inactivated as shown in Table II. When the section was subsequently shaken with the same concentration of $CaCl_2$ in the absence of the $CuCl_2$, reactivatioin took place which again was more extensive than in the untreated control sections.

These experiments indicate that Cu^{++} ions combined reversibly with a necessary portion of the calcifying system. They further suggest that an important step in calcification is the combination of calcium with some constituent of the bone cell, probably part of an

enzyme system. In a calcium free medium, calcium is removed and is probably replaced by one of the competing ions. Cu^{++} ions may compete so avidly for this local constituent that they preferentially combine with this factor even in the presence of large amounts of Ca^{++} ions. On shaking with $CaCl_2$ solution free of the competing ions, the formation of the calcium complex is favored as an initial step to calcification.

Summary. It was possible to demonstrate the reversible inactivation of calcification *in vitro* of the hypertrophic epiphyseal cartilage. When rachitic bone sections are shaken with strontium chloride, sodium chloride, calcification *in vitro* is inhibited. On subsequent shaking with calcium chloride calcification *in vitro* takes place. In addition, inactivation takes place with ½ mE of cupric chloride in the presence of 150 mE of calcium chloride. On subsequent shaking with 150 mE of calcium chloride (in the absence of cupric chloride) reactivation takes place.

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The Influence of Alloxan Diabetes on Cholesterol Atheromatosis in the Rabbit.*† (17335)

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It has long been suspected, largely on the basis of statistical studies, that humans suffering from diabetes mellitus develop arteriosclerosis not only in greater numbers than do non-diabetics, but also develop it earlier. However, conclusive experimental evidence that diabetes enhances arteriosclerosis has been lacking. It seemed to us that it might be instructive to test the influence of alloxan diabetes in the rabbit, despite the fact that the relationship of both experimental entities to their human counterparts are poorly understood.

Methods. The general plan of the experiment was as follows: 3 groups of rabbits, one group of which had been made diabetic with alloxan, were fed a cholesterol enriched diet; and animals from each group were examined at intervals after commencement of the diet to see if there was any difference in the time of onset or character of the vascular lesions in the different groups. Young Dutch rabbits weighing from 900 to 1400 g were given intravenous injections of alloxan monohydrate (Eastman) amounting to 80 to 100 mg per kilo of body weight daily for 2 consecutive days. Animals that were not diabetic on the third day were given similar injections on the third and fourth successive days. Although this method had been found in previous experiences with alloxan to be very effective, nevertheless in this instance a large number of animals died either during the course of the injections or within a week thereafter. Only approximately one half of the animals developed diabetes and survived long enough to be of value in the experiment.

Blood sugar determinations were performed by the method of Somogyi,[1] using a Klett-Summerson photoelectric colorimeter, both before and after alloxan injection. During the period of feeding the diet, the urines of the diabetic group were tested periodically with Benedict's qualitative reagent, and just before each animal was sacrificed, another blood

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† Since preparation of this manuscript the results of G. L. Duff and G. C. McMillan (abstracted in *Am. Heart J.*, 1948, **36**, 469) have come to our attention. These results confirm and extend those presented in this paper in showing that alloxan diabetes inhibits experimental cholesterol atherosclerosis in the rabbit.

[1] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 61.