progress). This GSH-independent oxygen consumption is possibly due to denaturation of the protein by the copper, with consequent exposure and oxidation of latent -SH groups. Gelatin, with its low sulfur content, is not similarly affected.

Summary. Native egg albumin and gelatin, in the presence of M/1000 $CuSO_4$ and M/200 glutathione, consume significant and approximately equal quantities of oxygen. When the $CuSO_4$ is replaced by M/1000 $FeCl_3$, the oxygen uptake is diminished by about 50%. If the M/1000 $CuSO_4$ is replaced by M/10 $CuSO_4$, the gelatin consumes less oxygen than before, and the egg albumin more.

11741

Calcification of Trichina Cysts *in vitro*.*

BERNARD D. ROSS. (Introduced by Franklin C. McLean.)

From the Department of Physiology, University of Chicago, Chicago.

Previous attempts to produce pathological calcification *in vitro*[1, 2] have failed to give consistent results, presumably owing to failure to employ a readily calcifiable tissue. Because trichina cysts become calcified in a uniform manner *in vivo,* we attempted to reproduce this phenomenon *in vitro,* employing the technics used for rachitic cartilage.[3] Preliminary experiments failed to produce calcification even when serum of high Ca^{++} x P product was used. In view of the findings of von Brand, Holtz and Vogel[4] and of Wantland[5] in which it was demonstrated that large doses of viosterol greatly accelerate the calcification of the cysts *in vivo,* calcification *in vitro* was attempted in infected muscle from rats which had received one or more toxic doses of viosterol. The experiments demonstrated that following this preliminary treatment, calcification of the cysts *in vitro* could be readily obtained.

Methods. Adult rats of varying strains, ages and weights, which

* This work was aided by a grant from the Josiah Macy, Jr., Foundation. We are indebted to Mead Johnson and Co. for supplies of viosterol, 1,000,000 units per gram.

1 Rosenheim, A. H., and Robison, R., *Biochem. J.,* 1934, **28**, 712.

2 Law, K. A. O., and Robison, R., *Biochem. J.,* 1936, **30**, 69.

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

4 v. Brand, T., Holtz, F., and Vogel, H., *Z. f. Parasitenk.,* 1933, **6**, 308.

5 Wantland, W. W., *J. Parasitol.,* 1938, **24**, 167.

had been infected with *Trichinella spiralis* 18 to 277 days before their tissues were incubated, were used. They were fed viosterol for from 1 to 5.5 days, the object being to predispose the cysts to calcification, but to avoid extensive calcification *in vivo*. The rats were then killed, and their diaphragms were removed aseptically and cut into pieces about 10 mm square. One piece from each diaphragm was fixed in 10% neutral formalin, as an unincubated control, and other pieces were placed in the incubating medium, which in each case was fresh sterile dog serum. In each experiment the $Ca^{++} \times P$ product was elevated in one-half the flasks by the addition of 0.1 cc of M/10 sodium phosphate mixture (20% NaH_2PO_4 + 80% Na_2HPO_4; pH 7.35) to 5 cc of serum. Incubation was carried out in tightly stoppered 50 cc Erlenmeyer flasks. No attempt was made to replace the lost carbon dioxide. Phenol red was added to each flask in order to observe any increased acidity due to bacterial contamination. The pH was not determined for individual flasks, but in representative samples was found to average about 7.5.

After incubating at 37°C for 24 hours, and for varying periods thereafter, the tissues were fixed in 10% neutral formalin, stained for calcium phosphate by the von Kóssa method, dehydrated with acetone, and cleared with isosafrol. In some cases they were also embedded in nitrocellulose, sectioned, stained by the von Kóssa method, and counterstained with hematoxylin and eosin or with eosin alone. The serum used was in each case analyzed for total calcium, phosphate, and protein, and the Ca^{++} concentration was estimated from total calcium and total protein.[6]

Results. No calcification of the cysts, either before or after incubation, was observed in the tissues of any of the animals which had not received viosterol, either with or without the addition of phosphate to the incubating medium. Tissues from 6 rats were so incubated, at from 48 to 229 days after infection. Calcification of the interstitial tissue occurred to a slight extent during incubation, as did calcification of the cut ends of the muscle. The cysts were free of any calcium deposits, and were, if anything, refractory.

Calcification of the cyst capsule *in vitro* was observed in tissues obtained from rats which had been infected for 35 days or more and which had received viosterol either as 150,000 units daily for at least 4 days or as 1,000,000 units in one dose at least 3.25 days previously. In the majority of instances this treatment initiated calcification *in vivo*, the amount of calcification being markedly increased by incubation with serum. The results in the tissues from

[6] McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, 1935, **108**, 285.

TABLE I.

Calcification of Trichina Cysts *in vitro* Following Administration of Viosterol to Infected Rats.

Rat No.	Age of infection (days)	Viosterol (USP XI units)	No. of doses	Days between first dose and incubation	$Ca^{++} \times P$ moles $\times 10^6$	Calcification of cysts 0	24	48	72 hr
36	251	150,000 daily	2	2	1.73	0	0	0	0
					4.59	0	0	0	0
35	269	150,000 daily	5½	5.5	1.95	++++	++++	++++	++++
					4.62	++++	++++	++++	++++
33	281	150,000 daily	4	4	1.85	++	++	++++	++++
					4.55	++	+++	++++	++++
					3.04	++	+++	++++	++++
40	110	150,000 daily	4	4	1.37	±	+++	++	
					4.18	±	++++	+++	
46	138	1,000,000	1	4	1.77	++++	++++		
					4.71	++++	++++		
47	138	1,000,000	1	3	1.77	±	++		
					4.71	±	+		
48	138	1,000,000	1	2	1.77	+	+		
					4.71	+	0		
49	138	1,000,000	1	1	1.77	±	0		
					4.71	±	±		
51	159	150,000 daily	4	4	1.45	+	++	++	
					3.99	+	+++	+++	
52	159	1,000,000	1	3.5	1.45	++	++++	++++	
					3.99	++	++++	++++	
53	159	1,000,000	1	3.25	1.45	±	+	++	
					3.99				
60	183 *18	1,000,000	1	4	1.76	0	0	0	
					4.64	0	0	0	
61	183 *18	1,000,000	1	3.5	1.76	+	+		
					4.64	+	+++	++++	
62	18	1,000,000	1	4	1.76	0	0	0	
					4.64	0	0	0	
63	18	1,000,000	1	3.5	1.76	0	0	0	
					4.64	0	0	0	
65	207 *35	1,000,000	1	4	1.47	+	++	+**	
					4.06	+	++	+++	
66	35	1,000,000	1	4	1.47	0	±	+**	
					4.06	0	+	+	
67	207 *35	1,000,000	1	3.5	1.47	+	+++	++**	
					4.06	+	++++	++++	
68	35	1,000,000	1	3.5	1.47	0	0	±**	
					4.06	0	±	0	

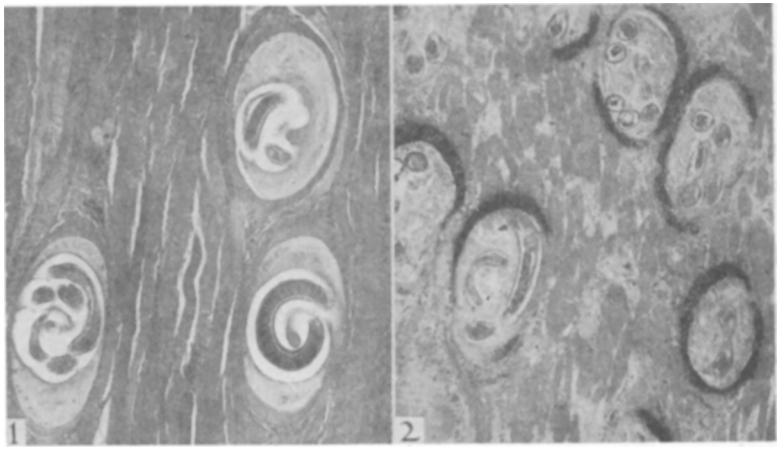
*Superinfection.

**Evidence of lowered pH owing to bacterial contamination.

animals treated with viosterol are summarized in Table I. Because the total calcium, total protein, and Ca^{++} concentrations varied but little, and because marked variation in the concentration of phosphate occurred only with the addition of phosphate to the medium, the extended data for these analyses are not given. Table I includes the figures for Ca^{++} x total P, when both are expressed in mols per kilogram of water.

The calcification process observed in the incubated tissues was similar to that observed *in vivo,* being localized in the capsules of the cysts, usually starting at the poles and involving the entire capsule as the process progressed (Fig. 2). This was usually accompanied by a small amount of interstitial calcification not exceeding that observed in tissues from animals not treated with viosterol. There was some tendency for calcifiability of the cysts to increase with their age.

Calcification of the cysts, when it occurred, was not found to be limited by the same Ca^{++} x P product found for the calcification of rachitic cartilage *in vitro.*[3, 7] In these experiments calcification occurred uniformly with sera having products from 1.37 to 1.95 x 10^{-6}, and when phosphate was added to raise the product above 3.0 x 10^{-6}, the critical product for the calcification of rachitic cartilage, the in-



FIGS. 1 AND 2.

Sections through diaphragm of rat No. 40, 110 days after infection with *Trichinella spiralis.* Silver nitrate-eosin. Photomicrographs, × 80. **Fig. 1.** Unincubated control. Whole mounts demonstrated minimal calcification of few cysts, not seen in sections. **Fig. 2.** After incubation for 48 hours in serum to which phosphate had been added. Heavy calcification of capsules of cysts.

[7] Robison, R., and Soames, K., *Biochem. J.,* 1930, **24**, 1922.

tensity of calcification was increased only slightly, if at all (Table I).

In an attempt to shed some light on the nature of the local changes associated with calcification, sections of infected muscles were stained for phosphatase by a modification of the method of Gomori.[8] Using this method, phosphatase was found to be present in all cysts which were old enough to be calcified following the use of viosterol. Its location was delimited to the region within the capsule, adjacent to the worm. In this respect it differed from the localization of calcification, for the latter characteristically occurred within the capsule substance, and not in the space confined by the capsule. The enzyme was present before any viosterol was administered, and such treatment did not visibly change its concentration or its distribution.

Discussion. The experiments reported were successful in producing a form of pathological calcification *in vitro,* the localization of the calcification and the conditions for its occurrence being predictable. Material is thus available for the further study of controlled pathological calcification. The fact that this calcification occurs at Ca^{++} x P products below those necessary for calcification of rachitic cartilage matrix indicates that the mechanisms of the two forms of calcification are not identical. This is consistent with the occurrence of various forms of pathological calcification in adults whose serum does not contain calcium and phosphorus in concentrations sufficient to produce calcification of rachitic cartilage.

In so far as the mode of action of viosterol is concerned, the experiments indicate that the action in this case is something more than to increase the absorption of calcium and of phosphate, and thus to raise the Ca^{++} x P product in the plasma. But it must be emphasized that the dosage employed was in the toxic range, and that the results do not necessarily bear any relation to the prophylactic and therapeutic actions of vitamin D in small doses. Instead it seems probable that the action is related to the known toxic actions of viosterol, and that the predisposition of the trichina cysts to calcification *in vitro* is the result of a toxic action upon the worm or its cysts, just as the soft tissues of the organism may be predisposed to calcification *in vivo* by the same action.

Demonstration of phosphatase in the cysts adds another instance to those in which this enzyme is found in tissues subject to calcification. In this instance, however, it is present in the cysts for some months before calcification usually occurs spontaneously in rats, and it has not been possible to demonstrate any change in its concentration or distribution under a procedure which predisposes the cysts to calcification.

[8] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

Summary. 1. Trichina cysts did not calcify spontaneously *in vitro,* even when exposed to media with high $Ca^{++} \times P$ products. 2. When calcification was initiated *in vivo,* by administration of toxic doses of viosterol over a period of several days, incubation with serum *in vitro* led to a marked increase in the amount of calcification of the cysts. 3. Calcification of the cysts, when it occurred at all, occurred at $Ca^{++} \times P$ products well below the critical point for the calcification of rachitic cartilage. 4. The action of viosterol in predisposing to calcification, both *in vivo* and *in vitro,* is attributed to a toxic effect upon the cyst or the worm. 5. An enzyme which hydrolyzes glycerophosphate is present in trichina cysts. It is present in the absence of administration of viosterol, which did not affect either its concentration or its distribution. It is present in the cysts for several months before calcification usually occurs spontaneously *in vivo* in rats.

11742 P

Tissue Response to p-Amino Benzene Sulfonamide (Sulfanilamide) in Mice.

ALF T. HAEREM.* (Introduced by G. F. Dick.)

From the Department of Medicine, University of Chicago.

With the greatly increasing use of the sulfonamide compounds in medicine in the past several years, a lively interest has arisen in regard to the effects of these drugs on the organism outside of their now well known activity in combating various types of infections. Numerous toxicological studies have therefore appeared on this subject. The somewhat equivocal relationship of p-amino benzene sulfonamide (sulfanilamide) to some of the aniline dyes suggested the possibility that the former might have mild carcinogenic properties similar to those reported for the aniline dyes,[1] and prompted the investigation described below. Subsequently, it came to the author's attention that a similar investigation had already been carried out by Lewis,[2] at the suggestion of Dr. E. K. Marshall. The results were subsequently confirmed by Zamecnik and Koletsky.[3] These

* Assistant, Department of Medicine, University of Chicago.

1 Hueper, W. C., Wiley, F. H., and Wolfe, E. J., *J. Ind. Hygiene,* 1938, **20**, 46.

2 Lewis, M. R., *Am. J. Cancer,* 1938, **34**, 431.

3 Zamecnik, P. C., and Koletsky, S., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 391.