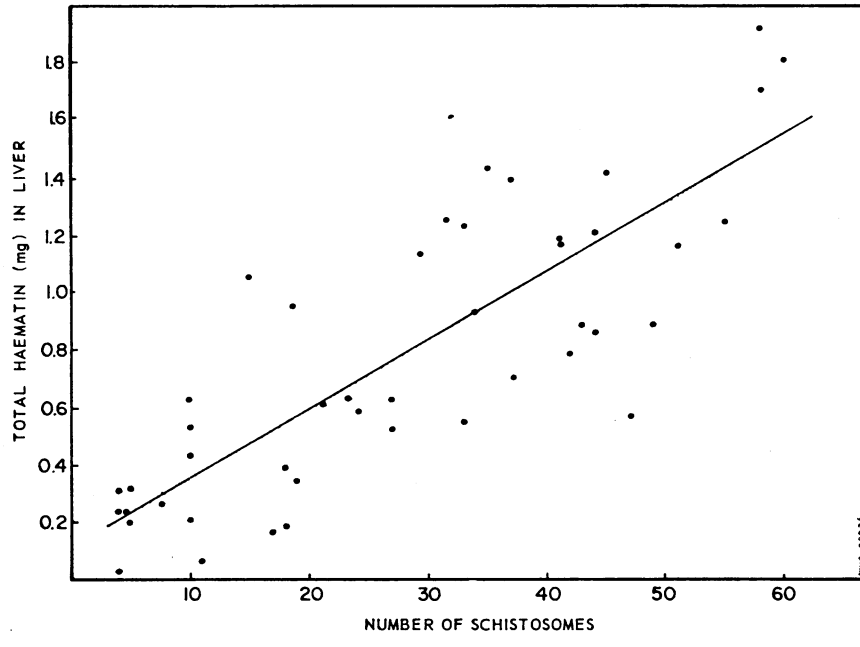
CORRELATION BETWEEN LIVER HAEMATIN AND SCHISTOSOME INFECTION



Conditions Affecting the Accuracy of Potassium Hydroxide Digestion Techniques for Counting *Schistosoma mansoni* Eggs in Tissues

by ALLEN W. CHEEVER, *Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, US Department of Health, Education, and Welfare, Bethesda, Maryland 20014, USA*

In schistosome infections of man or experimental animals, it is often important to know the number of eggs present in the tissues. In persons infected with *Schistosoma mansoni*, egg numbers were related to the number of female worms present and to the age of the subject.[a]

Caustic (KOH or NaOH) digestion is a convenient and rapid means of preparing tissues for the counting of schistosome eggs, but since the eggs are themselves digested in time, the accuracy of techniques employing caustic reagents is critically dependent upon the conditions of the digestion.[b] These conditions have varied widely. Several workers have used fresh or frozen tissues digested at 37°C in 4%–5% KOH for periods of 18 hours or less.[b, c, d] Schwink[e] used 5% NaOH at 55°C for 6–8 hours. Gelfand et al.[f] digested partially fixed tissues at 57°C for 16 hours in 10% KOH. Wright

[a] Cheever, A. W. (1968) *Amer. J. trop. Med. Hyg.*, **17**, 38-64.

[b] Cheever, A. W. & Warren, K. S. (1964) *Trans. roy. Soc. trop. Med. Hyg.*, **58**, 406-412.

[c] Szumlewicz, A. P. & Olivier, L. J. (1963) *Science*, **140**, 411-412.

[d] Kloetzel, K. (1967) *Amer. J. trop. Med. Hyg.*, **16**, 293-299.

[e] Schwink, T. M. (1955) *J. Parasit.*, **41**, No. 6, Sect. 2 (Supplement), p. 26.

[f] Gelfand, M., Hunt, R. H. & Clarke, V. de V. (1965) *J. trop. Med. Hyg.*, **68**, 245-247.

& Bennett [g] used 3% KOH and did not specify the time or temperature for digestion.

In the present study, fixed and unfixed tissues were digested under a variety of conditions. The counts from KOH digests were also compared with those from peptic digests, and with counts from compression preparations or homogenized tissue.

Materials and methods

Female white Swiss mice, infected with a Puerto Rican strain of *S. mansoni*, were sacrificed 7–22 weeks after infection. Worms were recovered using the technique of Duvall & DeWitt.[h] The livers of 8–10 mice were divided into approximately equal portions weighing 300 mg – 400 mg. These were assigned randomly to the test groups, weighed to the nearest 2 mg and treated as indicated in the table. All unfixed tissues were stored at −20°C prior to digestion. Fixed tissues were left 4–120 days in 10% formalin (1 part USP formaldehyde solution to 9 parts water) buffered with 20 g sodium acetate per litre.

For KOH digestion, 100 ml of 4% KOH solution were added to the tissue. Tissues were not minced prior to KOH digestion, with one exception (see table, column E). Peptic digestion was done following homogenization (trituration in a 300-ml Monel-metal cup using a Waring Blendor) for 1 minute in 100 ml of saline containing 0.3% Difco (1 : 10 000) pepsin and 1.5% concentrated HCl. These preparations were incubated at 37°C and thoroughly shaken at least 4 times each day. The eggs in duplicate 1-ml samples of peptic or caustic digests were counted in Sedgwick-Rafter chambers.[i]

Eggs in undigested liver samples were counted after crushing 5 mg–15 mg portions of liver between a glass slide and coverslip, and also in unfixed liver homogenized for 1 minute in 50 volumes of saline. After mixing, two 0.15-ml samples from the homogenized specimens were placed on a glass slide from a half-filled 1-ml serological pipette or a Stoll pipette (0.15 ml capacity). The aliquots were covered with a 22 mm × 50 mm coverslip and examined immediately.

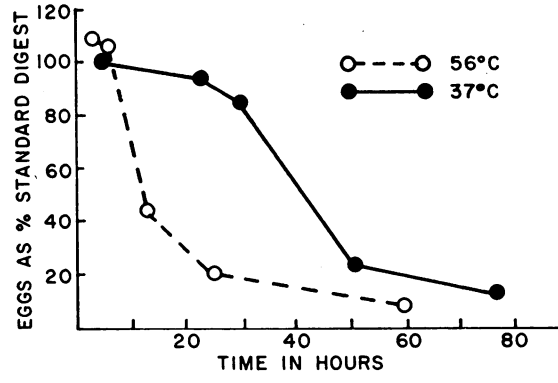
In undigested samples, eggs were classified according to their stage of development, as described by Pellegrino et al.[j]

The number of eggs per g of liver following digestion of unfixed tissue at 37°C for 3–6 hours in 4% KOH (here termed the " standard digest ") and the number found under other conditions were compared by Student's *t*-test for paired samples.[k]

Results

Unfixed tissues incubated in 4% KOH at 37°C were digested in 3–6 hours (see table, column A). Few eggs were digested under these conditions in the next 20–24 hours of digestion (Fig. 1). Overnight

FIG. 1
PATTERN OF DESTRUCTION OF EGGS IN UNFIXED TISSUE IN 4 % KOH AT 56°C AND 37°C



digestion gave similar results (see table, column B). Incubation of the tissues overnight at room temperature (22°C) shortened the time needed for digestion at 37°C and did not significantly affect the counts (see table, column C). Homogenization of tissues before digestion also had no significant effect on the number of eggs found after KOH digestion (see table, column D). Digestion in 4% KOH at 56°C gave satisfactory results when the eggs were counted immediately after digestion (see table, column E), but with further incubation, eggs were rapidly destroyed (Fig. 1).

The number of eggs found in peptic digests increased gradually with more prolonged digestion (Fig. 2). A slight (Fig. 2) to moderate increase in the average number of eggs counted was obtained

[g] Wright, C. A. & Bennett, M. S. (1967) *Trans. roy. Soc. trop. Med. Hyg.*, **61**, 221-227.

[h] Duvall, R. H. & DeWitt, W. B. (1967) *Amer. J. trop. Med. Hyg.*, **16**, 483-486.

[i] Obtained from A. H. Thomas Co., Philadelphia, Pa., USA. Sedgwick-Rafter chambers are slides measuring 3.5 cm × 7.5 cm with ground-glass strips fused to the surface to form a chamber 5 cm × 2 cm × 0.1 cm which holds 1.0 ml when the coverslip is in place.

[j] Pellegrino, J., Oliveira, C. A., Faria, J. & Cunha, A.S. (1963) *Amer. J. trop. Med. Hyg.*, **11**, 201-215.

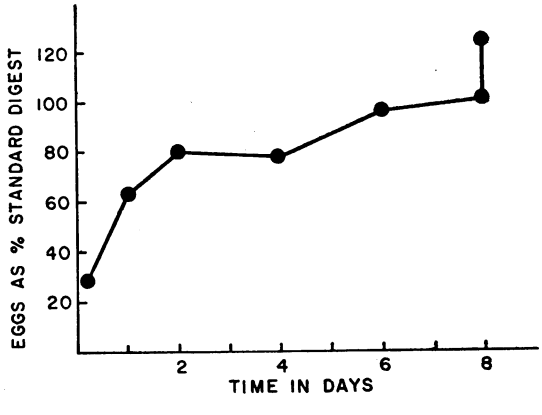
[k] Worcester, J. (1966) *New Engl. J. Med.*, **274**, 27-36.

EFFECT OF VARIOUS CONDITIONS OF DIGESTION ON THE NUMBER OF *S.MANSONI* EGGS FOUND IN MOUSE LIVERS [a]

Experiment no.	Duration of infection (weeks)	Average no. of worm pairs	Unfixed tissues								Formalin-fixed tissues			
			KOH concentration (%)											
			4	4	4	4	4	4	0	0	4	4	4	10
			Treatment before digestion											
			none	none	13–20 h at 23°C	homogenized	none	pepsin 48–192 h	compression	homogenized	none	61 h at 23°C	homogenized	none
			Digestion temperature (°C)											
			37	37	37	37	56	37	not digested	not digested	56	56	56	56
			Time for digestion (hours) [b]											
			3–6	18	1–2	2–3	3	0.2–1	not digested	not digested	17–24	17	15–16	7
			A	B	C	D	E	F	G	H	I	J	K	L
1	18–22	5	100	ND	ND	ND	109	123	ND	ND	64 [c]	36 [c]	85	65 [e]
2	16–20	5	100	112	114	94	ND	99	ND	ND	64 [c]	ND	40 [c]	57 [e]
3	11	2	100	100	91	ND	ND	109	ND	ND	81	ND	ND	ND
4	9	ND	100	ND	ND	77	ND	ND	64 [d]	147 [d, e]	62 [c]	ND	ND	ND
5	7	5	100	ND	ND	ND	ND	ND	ND	92 [e] and 67 [d]	ND	ND	ND	ND

[a] Values are given as the percentage of the number of eggs per gram of liver found in the "standard digest" (see col. A). ND = not done.
[b] Minimum time required for complete digestion of tissues, with the exception of column B.
[c] $p < 0.01$.
[d] $p < 0.05$ as compared to the "standard digest" in column A.
[e] Results from samples removed from the homogenized specimen with a serological pipette. For experiment 5, the second figure is for samples removed with a Stoll pipette (see text).

FIG. 2
EGG COUNTS AFTER VARIOUS PERIODS OF DIGESTION IN PEPSIN SOLUTION [a]



[a] 4 g per 100 ml of KOH were added on day 8, after which repeat egg counts were made immediately.

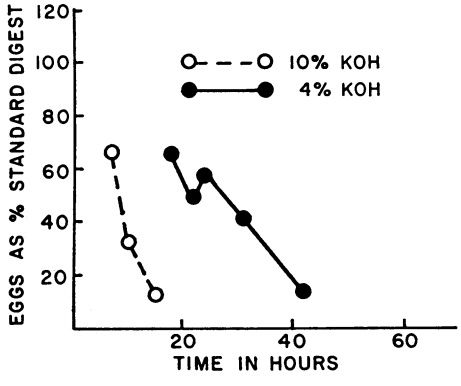
in each of 3 experiments after adding 4 g KOH to 100 ml of the pepsin digest. All granulomata and other tissue remnants disappeared within seconds after the addition of KOH to peptic digests, and the number of eggs found did not differ significantly from the "standard digest" (see table, column F).

In spite of the use of only 5 mg–15 mg of tissue in compression preparations, significant numbers of eggs were apparently overlooked on these slides (see table, column G). The number of eggs found in undigested liver specimens homogenized in saline was significantly greater than the number in the "standard digest" in one experiment in which aliquots were removed with a serological pipette. In a second experiment, the number of eggs found using this technique was equivalent to the number found in the "standard digest", but significantly fewer eggs were counted in samples removed with a Stoll pipette (see table, column H). Only insignificant numbers of eggs were found adherent to the walls of either type of pipette.

After KOH digestion, egg counts were done without difficulty using a scanning lens (3×– 3.5×). In pepsin digests and in crushed or homogenized tissue it was frequently necessary to use a 10 × objective lens to count eggs within granulomata.

When fixed tissues were examined, significant numbers of eggs were destroyed before digestion was complete under all conditions tested (see table, columns I, J, K, L). The rate of egg destruction was more rapid with higher concentrations of KOH (Fig. 3). Low counts were also obtained after digestion of fixed tissue at 56°C in 2% KOH and at 37°C in 4% KOH.

FIG. 3
PATTERN OF DESTRUCTION OF EGGS IN FIXED TISSUES IN 10% AND 4% KOH RESPECTIVELY AT 56°C



In undigested samples, eggs were classified according to their stage of development, using the criteria described by Pellegrino et al.[j] Eggs of comparable appearance were not seen following KOH digestion. Shells surrounding shadowy remnants of what appeared to have been miracidia were present. The number of these eggs was variable and bore no consistent relation to the number of viable or mature eggs found in undigested tissue from the same livers.

Discussion

All tested variants of the KOH digestion technique yielded satisfactory preparations when used with unfixed tissues for the minimum time required for complete digestion. When digests were held beyond this minimal time, rapid destruction of eggs occurred when the incubation temperature or KOH concentration was high (Fig. 1 and 3). Digestion in 4% KOH at 37°C is recommended for routine use, as the tissues are completely digested several hours before detectable numbers of eggs are destroyed. If counts cannot be done soon after digestion, the digest can be stored for days at room (22°C) or refrigerator temperature without loss of eggs.[b]

Significant numbers of eggs were always destroyed prior to complete digestion of formalin-fixed tissues; but if conditions were carefully controlled, digests from fixed tissues could presumably give useful information as to the approximate number of eggs.

Caustic digestion can be recommended for its convenience and sensitivity. Preparations with sufficient transparency for examination in Sedgwick-Rafter chambers are usually obtained with tissue concentrations up to 100 mg/ml, and 10-fold concentration by centrifugation gives digests which are usually clear enough for accurate counting. Large masses of tissue can be digested easily, thus minimizing the sampling problems which arise when egg distribution is uneven, as it is in the human intestine and in the liver with Symmers' fibrosis.[a] In addition, KOH is inexpensive, and infectious organisms, including tubercle bacilli[i] are rapidly destroyed. The principal disadvantage of caustic digestion is the difficulty in classifying developmental stages of the eggs after digestion, although Kloetzel seems to have obtained meaningful results following KOH digestion.[d]

Since schistosome eggs sediment rapidly, vigorous stirring and rapid handling of samples are important factors in dilution counting. Sedgwick-Rafter chambers allow rapid removal and preparation of 1-ml samples. The high counts obtained after dispensing 0.15-ml samples of triturated specimens from a 1-ml serological pipette were considered to have been caused by rapid sedimentation of eggs and granulomata in the pipettes.

Several disadvantages were associated with the use of triturated fresh tissues for egg counting. It was frequently necessary to use 100 × magnification to check for the presence of eggs within granulomata; and the variation in duplicate samples was greater than in digests. Adequate dispersal of eggs would be even more difficult in human bladders or livers having marked fibrosis. Adequate preparation of fibrous tissue would also be difficult with peptic digestion.

Unpublished preliminary results in this laboratory indicate that *S. haematobium* eggs, particularly those which are calcified, may be more resistant to KOH digestion than are *S. mansoni* eggs.

* * *

I wish to thank Mr T. R. Megginson and Mr R. T. Rusten for their assistance with these experiments. I am grateful to Dr Louis Olivier for his critical review of the manuscript.

[i] Krasnow, I. & Wayne, L. G. (1966) *Amer. J. clin. Path.*, **45**, 352-355.