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A laboratory determination of the destruction of α-amylase and salmonellae in whole egg by heat pasteurization

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INTRODUCTION

When liquid whole egg is heat-pasteurized in a continuous flow plant (Editorial 1960) at 64·4° C. (148° F.) for 2½ min., the baking properties are not impaired and no salmonellae have been recovered from samples which have received large inocula of *Salmonella typhimurium* before the heat treatment (Heller, Roberts, Amos, Smith & Hobbs, 1962). Heat pasteurization is thus a practicable process, but before it can be widely used it is desirable to know whether the more heat-resistant salmonellae can also be eliminated in this way further, a test is required which will indicate whether the pasteurization treatment has been adequate. Such a test, based on the reduction of the activity of α-amylase by heat and analogous with the phosphatase test for milk, was proposed by Brooks (1962) after he had shown that the extent of the reduction was directly related to the heat applied and was not influenced by any factor other than heat.

The object of the work reported in this paper was to test this proposal by comparing the conditions of heating necessary to destroy the activity of α-amylase and the most heat-resistant salmonellae and, if appropriate, to develop an amylase-test suitable for routine use.

MATERIALS AND METHODS

Apparatus

The complexity of the temperature-flow relation in continuous flow pasteurizers, described by Burton (1958), is such that a batch method of heating was the only possible one to use for estimating the destructive effect of heat on the activity of α-amylase and of salmonellae. Following the principles of Stern & Proctor (1954), stainless steel tubing with an internal diameter of 3 mm., wall-thickness of 0·5 mm. and length of 130 cm., was wound into a single-turn spiral. This gave a holding tube with a volume of approximately 7 ml. Each end was closed with a steel screw-cap and washer and a sensitive thermocouple was sealed into the centre of one of the caps and machined so that it did not touch the walls of the tube. This thermocouple was connected to an electronic temperature recorder which responded to a change in e.m.f. in less than 5 sec.

Following the methods of Franklin, Williams & Clegg (1958), the tube was

heated and cooled by total immersion in water-baths. The procedure was to fill the sterilized tube with egg inoculated with salmonellae, then to pre-heat to 45° C. (113° F.) and, as soon as the recorder indicated that the egg had reached this temperature, to transfer the tube first to the water-bath at the selected pasteurizing temperature and then to a cold water-bath.

Cleaning and sterilization of apparatus

All glassware was immersed in Lysol immediately after use and subsequently cleaned with N/10 sodium hydroxide followed by rinses with N/10 hydrochloric acid, hot water and distilled water. The pasteurizing tube was immersed for 10 min. in boiling water and then rinsed in cold water; coagulated egg was removed with a wire pull-through, and alcohol and ether were passed through and evaporated with compressed air.

Bacteriological methods

Strains of salmonellae

A streptomycin-resistant (S^R) strain of *Salm. typhimurium*, MM 2871, belonging to phage type 14 (Callow, 1959) was used because *Salm. typhimurium* of this phage type is frequently found in egg products (*Salm. typhimurium* was responsible for 45 % of the reported incidents of food poisoning from all sources in the United Kingdom in 1960, see Report, 1961).

Salm. senftenberg, N.C.T.C. 9959 (775 W), was also used because this particular strain is the most heat resistant of all salmonellae tested hitherto (Osborne, Straka & Lineweaver, 1954; Anellis, Lubas & Rayman, 1954; Angelotti, Foter & Lewis, 1961), and it was originally isolated from egg products.

Preparation of inoculum

The cultures were held on slopes of Dorset's egg medium which were used to inoculate a nutrient agar slope 48 hr. before the experiment. After incubation of the slope for 16 hr. at 37° C. a loop was used to inoculate 5 ml. of nutrient broth, which was then incubated for 24 hr. at 37° C.

To obtain an inoculum which would give a concentration of 10^5 to 10^6 per ml. in 200 ml. of whole egg, the overnight broth culture was diluted 1 in 3 for *Salm. typhimurium* and 1 in 15 for *Salm. senftenberg* with nutrient broth, 0·5 ml. of the diluted culture being used for inoculum. A uniform distribution of the inoculum through the whole egg was obtained by inoculating 10 ml. of the egg, and adding successive amounts of 10 ml. The contents of the flask were stirred continuously with a magnetic stirrer and, after each addition of egg, a portion of the contents was twice drawn into and ejected from a wide-mouthed pipette into the flask.

Isolation and counting of salmonellae

After each experiment the contents of the pasteurizing tubes were poured into screw-capped bottles which were held at 5° C. for not more than 2 days. These samples were taken from Cambridge to Colindale (1½ hr.) in an insulated container, and salmonella counts were carried out on heated and control samples as soon

as possible, usually within 1 hr. after the egg had been received. The samples, consisting of 2 to 5 ml. of egg, were thoroughly shaken and 1 ml. was removed from each. Suitable dilutions of each 1 ml. quantity were made in quarter-strength Ringer's solution, and duplicate quantities of 0·02 ml. from each dilution were dropped on to MacConkey and deoxycholate citrate agar plates according to the technique of Miles & Misra (1938). In addition 0·2 ml. quantities of a 1/2 dilution of each heat-treated sample were spread over the surface of MacConkey and deoxycholate citrate agar plates. The colonies were counted after 48 hr. incubation at 37° C. and the average count for each sample was estimated. The identification of the salmonellae was checked by serology. The results were expressed as the number of salmonellae per ml. of egg. Counts of less than 10 per ml. were not recorded and liquid enrichment cultures were not made.

Chemical methods

Determination of the activity of α-amylase

(*a*) For the laboratory experiments, the method of Brooks (1962) was used with appropriately reduced quantities of reagents. (*b*) For routine use, a method is described in the appendix of this paper.

Origin of the liquid whole egg

This was prepared in the laboratory from freshly laid eggs. It was used for all experiments except those which were designed to test the proposed routine method of detecting the activity of α-amylase, when commercially prepared egg pulp was used.

RESULTS

Because an objective of the experiments was the destruction of salmonellae, care was taken to check that the initial numbers of bacteria in the pulp were reasonably constant, that the manipulative methods themselves did not kill the organisms, and that the egg pulp was actually heated at the temperature and for the time stated.

The results shown in Table 1 indicate that the method of inoculation resulted in a uniform distribution of bacteria through the pulp. Magnetic stirring alone

Table 1. *Numbers of viable* Salmonella typhimurium *in samples taken from different sites within a flask* of inoculated pulp*

Site from which sample was taken	*Salm. typhimurium* $\times 10^6$/ml.
(1) Top centre	3·25
(2) Bottom centre	3·40
(3) Middle-side (*a*)†	4·20
(4) Middle-side (*b*)†	3·50
(5) Middle-side (*c*)†	4·15

* A conical flask of 250 ml. containing 200 ml. of pulp.
† These sites were evenly placed round the flask.

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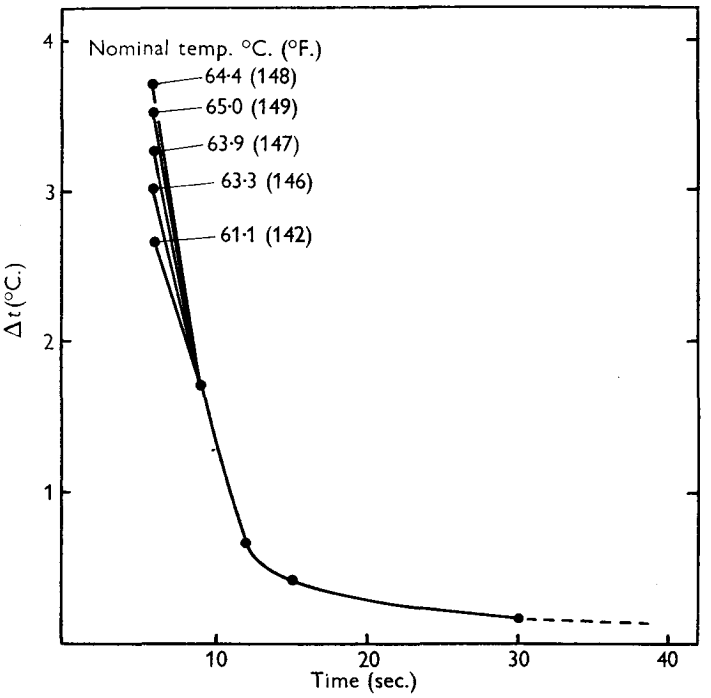

Fig. 1. Rate at which the temperature of the egg approaches that of the water-bath (Δt = temperature of water-bath − temperature of egg).

Table 2. *Effect of different conditions of pasteurizing on the destruction of* Salmonella senftenberg *N.C.T.C.* 9959 (775 W)

Holding time (min.)	Experiment no.	Survival of salmonellae as a percentage of the initial count*						
		65·5° C. (150° F.)	65·0° C. (149° F.)	64·4° C. (148° F.)	63·9° C. (147° F.)	63·3° C. (146° F.)	62·8° C. (145° F.)	61·1° C. (142° F.)
1	1	0·736	—	0·058	—	—	1·020	0·177
	2	0	—	0·003	—	—	—	—
	3	—	0·095	0	0	0	—	—
1½	1	0·078	—	0·003	—	—	0·113	0·082
	2	0	—	0·001	—	—	—	—
	3	—	0	0	0	0	—	—
2	1	0·002	—	0·001	—	—	0·067	0·026
	2	—	—	0	—	—	—	—
	3	—	0	0	0	0	—	—
2½	1	0	—	0	—	—	—	—
	2	—	—	0	—	—	—	—
	3	—	0	0	0	0	0·001	0·007

—, Not done; 0, salmonellae not found by the techniques used.

* the initial count was approximately 10^6 organisms per ml. and survival was determined in samples of 1 ml. from the holding tube (capacity 7 ml.)

No salmonellae were recovered from samples heated for 3, 3½, 4 and 5 min. at these temperatures except for the isolation of one colony (0·001 % survival) from egg heated at 62·8° C (145° F.) for 3½ min.

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did not distribute the salmonellae uniformly. The results of control experiments indicated that none of the manipulative procedures nor the holding period at +5° C. caused a reduction in the count of salmonellae.

A temperature/time curve was obtained for every experiment and Fig. 1 has been plotted from these graphs. The greatest difference in temperature between the pulp and the bath occurred initially, the difference being greater for higher

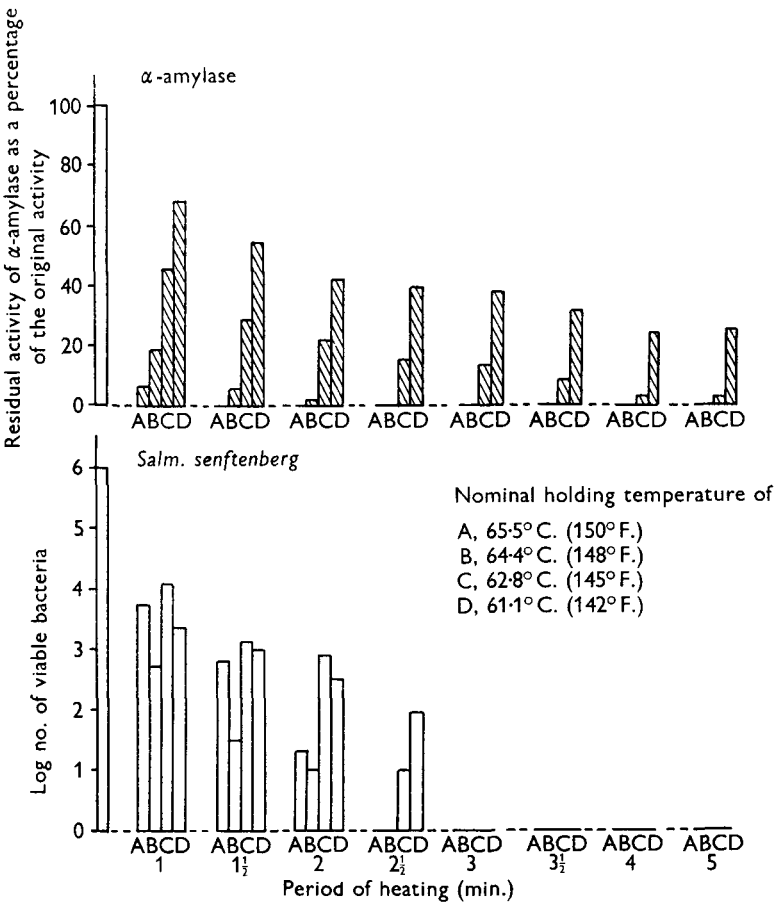

Fig. 2. Susceptibility to heat of α-amylase and *Salmonella senftenberg* N.C.T.C. 9959 (775 W).

water-bath temperatures; but these differences disappeared after immersion for 10 sec., and after approximately 20 sec. the temperature of the pulp was within 0·16° C. (0·35° F.) of that of the water-bath; any subsequent change was small.

All conditions of pasteurization, from the mildest at 61·1° C. (142° F.), for 1 min. to the most severe at 65·5° C. (150° F.) for 5 min., resulted in the complete destruction of *Salm. typhimurium* according to the method of assessment used. The more resistant salmonella strain, *Salm. senftenberg*, N.C.T.C. 9959 (775 W), was killed consistently at the lower temperature only when the holding time was 3 min. or more, but in three separate experiments in which infected egg was pasteurized at 64·4° C. (148° F.) for 2½ min. viable organisms could not be recovered.

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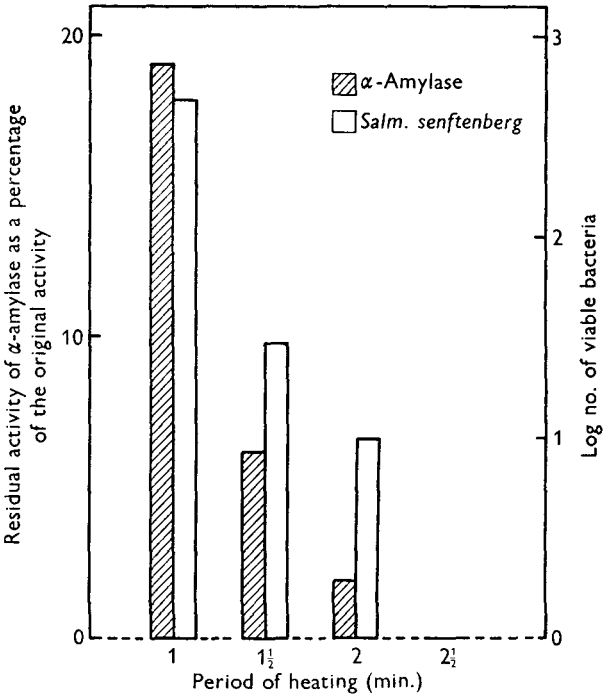

Fig. 3. Comparison of the susceptibility to a period of heating at 64·4° C (148° F.) of α-amylase and *Salmonella senftenberg* N.C.T.C. 9959 (775 W).

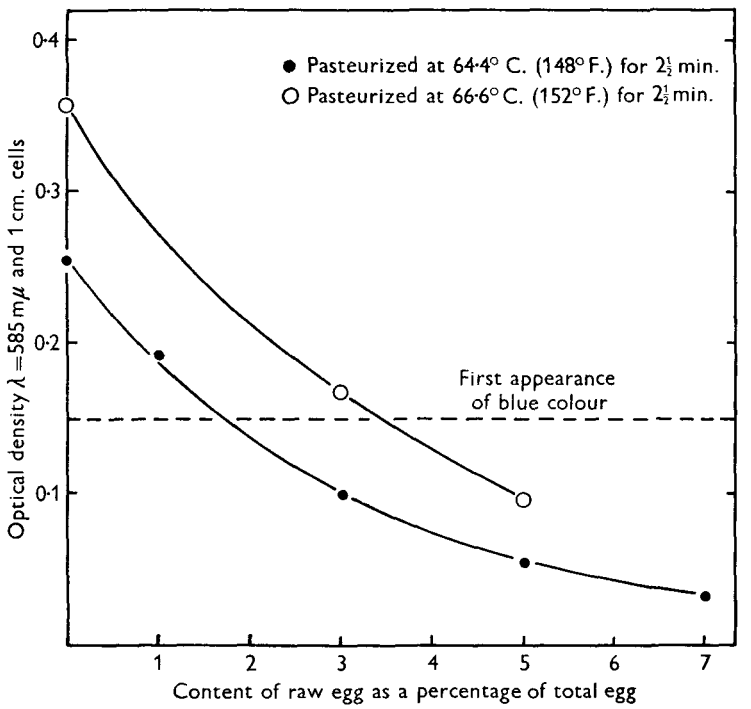

Fig. 4. Sensitivity of routine test to presence of raw egg in pasteurized egg.

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The results of other conditions of pasteurizing, in addition to these, are shown in Table 2. The inconsistent results in percentage survival at 1, 1½ and 2 min. may be due to a variation in the heat-resistance of the few remaining cells, and also to the chances of isolating very small numbers of organisms by direct methods of counting. The effects of conditions listed in Table 2 upon the survival of *Salm. senftenberg* and upon the activity of α-amylase are compared in Fig. 2.

Differences in temperature are reflected more in the residual activity of α-amylase than in the survival of *Salm. senftenberg* when short periods of heating are used.

Complete inactivation of α-amylase in less than 5 min. was accomplished only when the temperature of exposure was 64·4° C. (148° F.), or above. At this temperature a period of 2½ min. or more was sufficient, these conditions also being suitable for the destruction of *Salm. senftenberg*. Fig. 3 shows the relation between the destruction of α-amylase and *Salm. senftenberg* at 64·4° C. (148° F.).

The method for detecting the activity of α-amylase which is proposed for routine use (see Appendix) has been tested on commercially produced egg pulp which had been pasteurized at 64·4° C. (148° F.) for 2½ min. and also on the same pulp after the addition of increasing amounts of raw pulp. The results are shown in Fig. 4. The broken line indicates the optical density at 585μ which corresponds to a tube where the blue-violet colour can first be discerned. Any sample which gave an absorption of less that 0·15 under these conditions would be considered to have active α-amylase. By this means it is possible to detect contamination of the pasteurized egg with approximately 2% of raw egg. However, when this raw pulp was batch-pasteurized in the laboratory at 66·6° C. (152° F.) for 2½ min. the sensitivity of the test for detecting pollution was decreased, so that 3·5% of raw egg had to be added before the activity of α-amylase could be detected.

DISCUSSION

Murdock, Crossley, Robb, Smith & Hobbs (1960) recommended that when liquid egg was pasteurized by the high-temperature short-time method the following precautions were desirable: (1) automatic control of the temperature differential between the water used for heating the egg and the egg itself, (2) provision of an automatic flow-diversion valve, (3) provision of a positive pump to feed the egg into the plant, (4) provision of equipment to enable holding times to be determined by injection methods, and (5) the use of accurate recording thermometers at the entrance and exit of the holding section. Clearly, a sixth precaution is desirable, namely, a simple test which will rapidly demonstrate that the liquid egg has been adequately pasteurized and that the equipment is operating as intended; but no such test was available when these precautions were recommended.

The results of the experiments reported here indicate that a test based on the destruction of the activity of the α-amylase naturally present in liquid egg would be suitable. The critical heat treatment for the destruction of the activity of the α-amylase, 64·4° C. (148° F.) for 2½ min., is identical with that required for the killing of the most heat-resistant strains of *Salm. senftenberg*. Moreover, this treatment is one which does not impair the baking properties of the egg (Heller

et al. 1962). Although the design of these experiments precludes the calculation of F_t and D_t values, the conditions found for the destruction of *Salm. typhimurium* and *Salm. senftenberg* N.C.T.C. 9959 (775 W) are in general agreement with those previously reported (Winter, Stewart, McFarlane & Solowey, 1946; Goresline, Hayes, Moser, Howe & Drewniak, 1951; Anellis *et al.* 1954; Osborne *et al.* 1954; Murdock *et al.* 1960).

Such a test would be less satisfactory if different conditions of heat treatment were specified because periods of heating at lower temperatures could destroy salmonellae but not the activity of the α-amylase, whilst shorter periods of heating at higher temperatures would destroy the enzyme but not the heat-resistant strains of *Salm. senftenberg*. Indeed, the condition of 64·4° C. (148° F.) for 2½ min. is a unique one in that both the enzyme and the salmonellae are destroyed together.

Thus the destruction of the activity of α-amylase could be considered as a test for the adequacy of pasteurization in conjunction with the precautions 1–5 listed above, provided that the minimum treatment required was one of 2½ min. at 64·4° C. (148° F.).

Recent experience using the amylase test in the manner described for routine use has indicated that under factory conditions the test is simple to apply and effective for determining the adequacy of pasteurization at 64·4° C. (148° F.) (Monsey & Shrimpton, 1962). In a factory where the precautions of Murdock *et al.* (1960) were followed, a fall in temperature of 1° C (0·5° F.) was detected enabling an appropriate correction to be made to the plant within one hour of the occurrence of the fault. Any increased sensitivity is difficult to obtain because of the nature of the iodine-starch complex formed in the test and possibly because of a residual α-amylase-like activity which cannot be detected under standard conditions.

SUMMARY

The conditions of heating necessary to destroy salmonellae in liquid whole egg have been compared with those necessary to destroy the activity of the α-amylase of whole egg. All conditions of pasteurizing from the mildest at 61·1° C. (142° F.) for 1 min. to the most severe at 65·5° C. (150° F.) for 5 min. eliminated *Salm. typhimurium*. The heat-resistant strain of *Salm. senftenberg* N.C.T.C. 9959 (775 W) was not recovered after heating at 64·4° C. (148° F.) for 2½ min. and at the lower temperatures when the heating period was 3 min. or more. The activity of α-amylase was also destroyed by heating at 64·4° C. (148° F.) for 2½ min. but not at lower temperatures.

Because the baking properties of egg are not impaired by heating at 64·4° C. (148° F.) for 2½ min. it is proposed that the inactivation of the α-amylase of whole egg can be used as a test for controlling the pasteurization process, and a routine test has been developed which can be completed within 1 hr.

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APPENDIX

A method for the routine determination of the activity of α-amylase in liquid whole egg

(1) *Principle of the method*

The typical starch-iodine complex has a characteristic blue colour, but degradation of the starch causes the disappearance of this blue colour. The enzyme α-amylase, a natural constituent of egg-yolk, can degrade starch so long as the enzyme is active, so that the addition of iodine to mixtures of whole egg and starch will no longer give the characteristic blue colour. But destruction of the enzyme leaves the starch intact and the normal colour reaction will develop. Thus the test can be applied to the assessment of α-amylase activity in liquid whole egg.

(2) *Reagents*

(*a*) Distilled water used for all dilutions. (*b*) Soluble starch, A.R. (nominal 0·75 % w/v). A fresh solution containing 0·70 g. dry weight of starch (dried at 100° C for 16 hr. or at 160° C. for 1 hr.) in 100 ml. of water is made up monthly and held at room temperature with the addition of three drops of toluene. The solution is prepared by first making a paste of the weighed starch with cold water. This paste is poured quantitatively into 50 ml. of boiling water, boiled for 1 min.,

cooled by immersion in cold water and then made up to volume with cold water. (*c*) Trichloroacetic acid (for deproteinization)—15% (w/v) of A.R. quality trichloroacetic acid in water. (*d*) Potassium iodide, A.R. quality. (*e*) Iodine solution—12·70 g. of iodine are dissolved in a solution of potassium iodide (25 g. in 30 ml. of water) and made up to 1 l. with water to give an approximately N/10 solution. This stock solution may be kept for 6 months; before use it is diluted 1 in 100 with water and 0·25 g. of potassium iodide is added.

(3) *Apparatus*

Pipettes, Grade B bulb 2 ml., 5 ml. and 10 ml., or Grade B bulb 2 ml. and Grade A 10 ml. graduated straight-sided pipettes. *Volumetric flasks*, Grade B, 100 ml. and 1000 ml. *Measuring cylinder*, 50 ml. *Filter funnels*, 3 in. diameter. *Filter paper*, 12·5 cm. diameter, Whatmans No. 12 (fluted). *Conical flasks* (wide neck), 25 ml. and 100 ml. capacity. *Test tubes*, approximately 6 by 5/8 in. and 7 by 1 in. *Water-bath* at 44° ± 0·5° C.

(4) *Cleaning*

All glassware should be soaked in Chloros or Lysol after use. Adhering egg should be washed off with water, and if necessary, with N/10 sodium hydroxide, and the glassware washed with chromic acid followed by thorough rinsing with water and distilled water. Particular care is necessary to avoid contamination with saliva. Because it has been shown that salmonellae are killed within a few seconds after the addition of trichloroacetic acid to the egg, no precautions are necessary to sterilize apparatus after this stage.

(5) *Method*

15 gm. of whole egg are incubated for 30 min. with 2 ml. of starch solution in a 25 ml. conical flask or a 7 by 1 in. boiling tube at 44° C. After incubation 5 ml. are pipetted into 5 ml. of 15% (w/v) trichloroacetic acid solution contained in a 100 ml. conical flask and shaken thoroughly. 15 ml. of water are then added by pipette, and after thorough mixing the suspension is filtered. 10 ml. of the filtrate are pipetted into 2 ml. of iodine solution contained in a test tube. A blue-violet colour indicates that the egg has been adequately pasteurized.

It is preferable to compare the colour obtained against distilled water with a standard series of colours in a Lovibond comparator using a cell of 25 mm. depth and a disc for pasteurized egg. A blue colour above 3 indicates satisfactory pasteurization. The reagents and procedure can be checked by preparing two control tubes at the same time that the experimental material is tested. In one of these (*a*) the egg is replaced with an equivalent amount of water and in the other, (*b*) the starch is replaced with an equivalent amount of water. The colour in tube *a* will be a deeper blue and that in tube *b* will be paler than any shade on the disc.