A METHOD OF TESTING THE STABILITY OF FINELY DIVIDED EMULSIONS OF OILS AND FATS, WITH REFERENCE TO THE VALUE OF YOLK OF EGG, AND DECOCTUM CHONDRI AS EMULSIFYING AGENTS

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I. Introduction

In 1932 and 1934, Myers showed that superlethal doses of diphtheria toxin when mixed with emulsions of olive oil, in a fine state of division, and injected subcutaneously into guinea-pigs and rabbits, failed to produce lethal effects. He also noted that such olive-oil emulsions protected against the effects of superlethal doses of the toxins of *B. tetani*, *C. Welchii* and *Clostridium oedematis-maligni* (Koch).

The emulsion used consisted of 50 % olive oil in water, with gum acacia as the emulsifying agent, and the whole emulsified by means of a mechanical emulsifying apparatus, or homogenizer, until the oil globules were in a fine state of subdivision (0·5–1 μ). Walsh & Frazer (1934), using emulsions of cod-liver oil and of olive oil mixed with diphtheria toxin, obtained similar results. They used 3·5 % of oil in water, with sodium carbonate as the emulsifying agent and adjusted to *p*H 8. Although the emulsions used by these investigators varied in both the quantity of oil and the emulsifying agents used, they all protected animals against the lethal effects of bacterial toxins.

The present investigation is devoted to a study of other emulsifying agents which may be used to stabilize olive-oil emulsions. It is important to note

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that three conditions must be complied with in order to render the bacterial toxins harmless. First, the emulsions must be in a very fine state of subdivision; the finer the particles the more efficient they are. The size of the particles is limited by the design of the machines in general use to-day, but it is relatively easy to produce an average size of particle of the order of 0·5–1 μ. Secondly, the finished emulsion must remain stable for a week or more, and this can be achieved by the use of a suitable emulsifying agent. Thirdly, there must be a suitable interval of time allowed (at least 20–30 min.) to elapse between the moment the toxin is mixed with the finished emulsion at 37·5° C. and the injection of the mixture into the animal. One other important point is that no substance should be introduced into the toxin-mixture which will upset the stability of the emulsion and coarsen the particles. For this reason normal saline or Ringer's solution cannot be used for the dilution of the concentrated bacterial toxins when they are to be mixed with the emulsion; distilled water is used. Many workers keep syringes sterile by immersion in rectified spirit and wash them out immediately before use. This was the practice of the author until it was found that even minute traces of alcohol can upset the stability of carefully made emulsions, and so interfere with the experimental results. Since then, this method has been abandoned, and all syringes are sterilized in boiling water immediately before use.

As emulsifying agents play such an important part in the stabilization of these finely divided emulsions, it was thought that a study of the various agents in common use might be made, using the protective effects against toxins as the test of their ability to stabilize emulsions.

II. Methods

From the previous paragraph it is apparent that when a lethal dose of a bacterial toxin is mixed with a stable emulsion in a fine state of division and injected into an animal death does not follow; should the emulsion be unstable, however, through the fault of the emulsifying agent, then death of the animal will take place after an interval of time, depending on the toxin employed.

In all these experiments the emulsion contained 50 % of olive oil. Yolk of egg and Irish moss were used as emulsifying agents in an endeavour to stabilize the emulsion, and retain it in a fine state of subdivision.

The toxins used in this investigation were kindly supplied by Dr O'Brien of the Wellcome Laboratories, and the emulsions made by Mr E. S. Peck of Cambridge.

A preliminary determination of the minimum lethal doses of the toxins for guinea-pigs was carried out.

Egg yolk. This is used extensively in commerce as an emulsifying agent. It is claimed that such emulsions do not separate on the addition of acids, salts, glycerine, syrup or sugar solutions. The stability of egg-yolk emulsions is said to increase with keeping, but, unfortunately, putrefaction occurs. This can be overcome by the addition of a small quantity of benzoic acid should it

be necessary to keep the emulsions over long periods. In these experiments it was unnecessary to add benzoic acid as the emulsion was not required for more than 1 or 2 days. To emulsify 4 oz. of a fixed oil, 3 or 4 drachms of egg-yolk are sufficient and can be obtained from an average-sized hen's egg.

Preparation of olive oil emulsion with egg yolk

A fresh egg was broken and the yolk separated from the albumen. The yolk was placed in a large mortar and triturated with a pestle. Four ounces of olive oil were slowly added and the trituration continued until the mixture was homogeneous. Two ounces of water were slowly introduced as the trituration proceeded. The whole was now filtered through finely woven muslin and the filtrate made up to 8 oz. with water and well mixed by shaking. The coarse emulsion was then passed through a mechanical emulsifier until the average size of the particles was of the order of $\frac{1}{2}$–1 μ.

Irish moss (Chondrus)

Chondrus crispus is a seaweed found on the northern coasts of the Atlantic Ocean. A mucilage can be prepared from the dried thallus of the seaweed and is used as an emulgent. Such emulsions will stand for many months without creaming, but putrefactive decomposition is liable to occur. In these experiments it was unnecessary to add preservatives as the emulsion was always used within a few days of preparation.

Preparation of olive-oil emulsion with Irish moss

Four ounces of olive oil were triturated in a mortar with 2 oz. of decoction chondri (1–40). Eight ounces of water were then slowly added and the trituration continued. When the mixing was completed the whole was passed through an emulsifying machine until the average size of particle was 0·5–1 μ.

In all cases the injections were given subcutaneously and the lethal doses of the toxins were calculated on the basis of the animal's weight. After the toxin was mixed with the emulsion the mixture was allowed to stand 30 min. at 37·5° C. before it was injected into the animal.

III. EXPERIMENTS

The efficiency of yolk of egg as an emulsifying agent

(*a*) *Diphtheria toxin.*

Nine guinea-pigs were divided into two groups, one of six animals, the other of three. Four guinea-pigs of the first group were injected with a mixture of 4 M.L.D. of the toxin in emulsion, while the remaining two received 8 M.L.D. in emulsion. The second group of animals served as controls, two were injected with 4 M.L.D. of the toxin alone, and the third 8 M.L.D. Table I shows that all the animals died. This result suggested that the yolk of egg failed to maintain the emulsion in a fine state of subdivision, or that it was perhaps responsible for the death of the first group.

Table I. *The influence of olive oil emulsion, 50 %, upon the resistance of guinea-pigs to diphtheria toxin*

Emulsifying agent, yolk of egg

No.	Wt. g.	Amount of toxin injected M.L.D.	Duration of life hr.	Mortality rate %
1	430	4	36	
2	350	4	70	
3	335	4	42	100
4	330	4	48	
5	290	8	40	
6	400	8	36	
A	350	4	48	
B	380	4	48	100
C	410	8	38	

1–6, emulsion animals; A–C, controls.

In order to decide this question a second experiment was conducted in which four guinea-pigs were injected with yolk of egg alone in quantities equivalent to those injected into the animals which died in the previous experiment.

Preparation of egg yolk for injection. The yolk (3–4 drachms) of one fresh egg was placed in a mortar and broken up by triturating with a pestle. Four ounces of water were slowly added and the trituration continued until a

Table II. *The influence of yolk of egg when injected subcutaneously into guinea-pigs*

No.	Wt. g.	Duration of life	Mortality rate
1	280	Survived	
2	260	Survived	Nil
3	275	Survived	
4	290	Survived	

homogeneous mass was obtained. The fluid was filtered through fine muslin and the filtrate made up to 8 oz. with water. The whole was well shaken and passed several times through a mechanical emulsifier. Of this mixture 1 c.c. was injected into each of four guinea-pigs and the results are given in Table II. Since all the animals survived, it is evident that yolk of egg when injected subcutaneously in these quantities is not harmful to guinea-pigs. It seems, therefore, that the deaths in the first group were due to instability of the emulsion, and that yolk of egg is not a satisfactory substance for the stabilization of olive-oil emulsions.

(*b*) *Tetanus toxin.*

Exp. 1 was repeated, using tetanus toxin instead of diphtheria toxin. Table III shows that all the animals died. These results are in complete agreement with those of the first experiment.

Table III. *The influence of olive oil emulsion, 50 %, upon the resistance of guinea-pigs to tetanus toxin*

Emulsifying agent, yolk of egg

No.	Wt. g.	Amount of toxin injected M.L.D.	Duration of life days	Mortality rate %
1	320	4	2·5	
2	300	4	2·5	
3	340	4	3·0	
4	280	4	2·0	100
5	310	8	2·0	
6	300	8	2·5	
A	310	4	2·0	
B	340	4	2·5	100
C	300	8	2·0	

1–6, emulsion animals; A–C, controls.

(*c*) *Toxin of C.* Welchii.

This experiment was conducted on the same lines as Exp. 1 with the substitution of the toxin of *C. Welchii* for diphtheria toxin. Six animals were injected with 4 M.L.D. of the toxin which had been previously mixed with the emulsion, and three animals, injected with 4 M.L.D. of the toxin alone, were used as controls. Table IV shows that all the animals died. All these three sets

Table IV. *The influence of olive oil emulsion, 50 %, upon the resistance of guinea-pigs to the toxin of* C. Welchii

Emulsifying agent, yolk of egg

No.	Wt. g.	Amount of toxin injected M.L.D.	Duration of life days	Mortality rate %
1	330	4	3·0	
2	310	4	2·5	
3	300	4	3·0	
4	310	4	3·5	100
5	320	8	3·0	
6	325	8	2·5	
A	300	4	3·0	
B	325	4	2·5	100
C	310	8	2·5	

1–6, emulsion animals; A–C, controls.

of experiments show conclusively that yolk of egg is not a satisfactory emulsifying agent for the purposes of such experiments, presumably because it does not render the emulsion stable over a sufficiently long period of time.

The efficiency of Irish moss as an emulsifying agent

(*a*) *Diphtheria toxin.*

For the purposes of this experiment four guinea-pigs were injected subcutaneously with 4 M.L.D. of diphtheria toxin in the emulsion, two with

8 M.L.D. of the toxin in emulsion, and three animals were used as controls, two being given 4 M.L.D. and the other 8 M.L.D. of the toxin alone. Table V shows that all the animals died. In order to make sure the Irish moss had no toxic properties when injected subcutaneously, four guinea-pigs were injected with a decoction of Irish moss. Two ounces of decoctum chondri were diluted to 8 oz. with water and well mixed. The mixture, after it had been homogenized by means of a mechanical emulsifier, was ready for use. Each animal

Table V. *The influence of olive oil emulsion*, 50 %, *upon the resistance of guinea-pigs to diphtheria toxin*

Emulsifying agent, Irish moss

No.	Wt. g.	Amount of toxin injected M.L.D.	Duration of life hr.	Mortality rate %
1	350	4	36	100
2	320	4	40	
3	300	4	70	
4	390	4	48	
5	360	8	36	
6	420	8	36	
A	350	4	48	100
B	380	4	48	
C	410	8	38	

1–6, emulsion animals; A–C, controls.

Table VI. *The influence of mucilage of Irish moss when injected subcutaneously into guinea-pigs*

No.	Wt. g.	Duration of life	Mortality rate
1	305	Survived	Nil
2	290	Survived	
3	310	Survived	
4	330	Survived	

was given subcutaneously an amount equivalent to that injected in the previous experiment (1 c.c.). Table VI shows that none of the animals died, and proves the absence of any toxic properties in Irish moss when used in this way. From these experiments it is evident that the olive-oil emulsion made with Irish moss fails to protect the animals against the lethal effects of the diphtheria toxin, presumably through instability. It is therefore not a satisfactory emulsifying agent as measured by the standards used in this investigation.

(*b*) *Tetanus toxin.*

This experiment was conducted in exactly the same way as the others except that tetanus toxin was substituted in the place of diphtheria toxin. Table VII shows that all the animals died, suggesting that Irish moss does not produce a stable emulsion. Further evidence in support of this conclusion is offered by the results of the following experiment.

(*c*) *Toxin of* C. Welchii.

A mixture of the emulsion and 4 M.L.D. of this toxin was injected subcutaneously into each of four, and 8 M.L.D. into each of two guinea-pigs. Three other animals served as controls, two receiving 4 M.L.D. subcutaneously of the

Table VII. *The influence of olive oil emulsion*, 50 %, *upon the resistance of guinea-pigs to tetanus toxin*

Emulsifying agent, Irish moss

No.	Wt. g.	Amount of toxin injected M.L.D.	Duration of life days	Mortality rate %
1	330	4	2·0	
2	350	4	3·0	
3	320	4	2·5	100
4	350	4	2·5	
5	340	8	2·0	
6	345	8	2·0	
A	355	4	2·5	
B	360	4	3·0	100
C	340	8	2·5	

1–6, emulsion animals; A–C, controls.

Table VIII. *The influence of olive oil emulsion*, 50 %, *upon the resistance of guinea-pigs to the toxin of* C. Welchii

No.	Wt. g.	Amount of toxin injected M.L.D.	Duration of life days	Mortality rate %
1	330	4	3·0	
2	355	4	3·5	
3	320	4	2·5	100
4	330	4	3·0	
5	320	8	3·0	
6	350	8	2·5	
A	320	4	3·5	
B	350	4	2·5	100
C	340	8	2·0	

1–6, emulsion animals; A–C, controls.

toxin alone and one 8 M.L.D. All the animals died within $3\frac{1}{2}$ days (Table VIII), proving that this particular emulsion offered no protection against the lethal effects of the toxin.

Pathology

A post-mortem examination was conducted on all the animals which died after the injection of the diphtheria toxin emulsion mixture in order that the pathological changes in them might be compared with those in the control animals. No differences between the two groups were observed, and all showed changes indicating that death was due to acute toxaemia caused by diphtheria toxin.

The following is a characteristic protocol of a post-mortem examination held on a guinea-pig which had died 3 days after the injection of the yolk of egg emulsion mixed with 4 M.L.D. diphtheria toxin.

Macroscopical appearances. There was general enlargement, congestion and oedema of all the organs and amber-coloured exudate in the peritoneal, pleural, and pericardial sacs. Petechial haemorrhages were seen scattered throughout the submucous regions of the small and large intestines. Free blood was sometimes present in the lumen of the caecum, colon and rectum. The heart was pale and all the chambers dilated. Small petechial haemorrhages were seen beneath the pericardial surface. On opening the heart anti-mortem thrombi were found in the cavities of both ventricles, but the valves and coronary arteries appeared normal. The myocardium was soft and easily torn.

Microscopical appearances. Heart: The muscle fibres were swollen and a few looked atrophic and fragmented. Many of the fibres had undergone hyaline degeneration and lost their striated appearance. Separation of the muscle fibres was seen in many places, and red cells lay freely between the fibres, indicating small haemorrhages into the myocardium. The nuclei of the myocardial fibres were pyknotic and had lost their staining reaction. Focal necrosis was never seen. The blood vessels, especially the veins, were dilated, and the blood in the lumen showed an increase in lymphocytes, while a lymphocytic infiltration was seen in the tissues around the vessels. All sections stained with osmic acid showed globules of fat inside the muscle fibres. This fatty degeneration was most marked adjacent to the pericardial and endocardial surfaces. The liver and kidneys showed cloudy swelling and some fatty degeneration. Similar changes were seen in guinea-pigs which had succumbed to the injection of 4 M.L.D. of diphtheria toxin mixed with an emulsion containing Irish moss.

IV. Discussion

In this investigation an attempt has been made to measure the stability of olive-oil emulsions by means of a toxin standard. The test is based on the observation that stable oil emulsions in a fine state of division when mixed with bacterial toxins, incubated at 37·5° C. for 30 min. and injected subcutaneously into guinea-pigs, render the toxins harmless. A positive result is yielded only if the emulsion remains stable over a considerable interval of time; unstable emulsions give a negative result, that is, the animal dies from the effect of the bacterial toxin. Since the stability of an emulsion is largely dependent upon the emulsifying agent used, this method can be used as a means of determining the powers of various agents to stabilize emulsions.

Myers (1932, 1934 *a*, *b*) showed that gum acacia gave positive results and is therefore an excellent emulsifying agent as judged by this standard, which is a very critical one. Walsh & Frazer (1934) used sodium carbonate and reported a positive result. The experiments of the present investigation were directed towards other agents in common use, namely, yolk of egg and decoctum chondri (B.P.C.). The results have shown that these two agents, although they are considered excellent stabilizers in commercial practice, yield negative results when this toxin test is applied to them. They do not seem to stabilize emulsions sufficiently well to render bacterial toxins harmless, and so apparently for

this reason all the animals injected with the emulsion-toxin mixture died. Three different bacterial toxins were used in three separate tests; diphtheria toxin, tetanus toxin and the toxin of *C. Welchii*. That the emulsifying agents did not cause death is clearly shown in the experiments in which yolk of egg or decoctum chondri (B.P.C.) were alone injected subcutaneously into guinea-pigs. Moreover, all these animals which died after the injection of emulsion diphtheria toxin mixtures showed the characteristic lesions due to diphtheria toxin.

V. SUMMARY

1. A new test for the stability of emulsions of oils or fats is described. It is based on the observation that, when stable emulsions of oils and fats in a fine state of division are mixed with lethal quantities of bacterial toxins and incubated for 30 min. at body temperature, and the mixtures when injected into guinea-pigs do not cause death.

2. Emulsions of olive oil (50 %) made with either egg yolk or Irish moss do not protect animals against the effects of lethal doses of bacterial toxins, and are therefore regarded as unstable as judged by this standard.

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