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THE FERMENT-ACTION OF BACTERIA.

BY

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"The Ferment-action of Bacteria." By T. LAUDER BRUNTON, M.D., F.R.S., and A. MACFADYEN, M.D., B.Sc. Received March 23,—Read April 4, 1889.

In the course of the research the following micro-organisms were used:—

1. Koch's comma spirillum (Flügge, 'Die Mikro-organismen,' Leipzig, 1886, p. 334).
2. Finkler's comma spirillum (Flügge, 'Die Mikro-organismen,' Leipzig, 1886, p. 382).
3. A putrefactive micrococcus.
4. Scurf bacillus (Klein).
5. A bacillus isolated from milk by Dr. Klein, which for convenience we may call the "Welford Bacillus."

All of these liquefy gelatine, the two last most energetically. Anthrax was not used, on account of the resistance of its spores and the consequent difficulty of completely sterilising the culture media. The experiments were made in each case with pure cultures.

The first question which we tried to solve was, What is the nature of the substance by which bacteria liquefy gelatine? Is it an enzyme? There are two ways in which they might do this. They might secrete some fluid which would dissolve the gelatine mechanically, without altering it chemically, as saliva dissolves sugar in the mouth; or they might do it by secreting a specific enzyme, which would dissolve the gelatine by altering it chemically, as the ptyalin of the saliva effects the solution of starch. If the solution were effected in the first way by the secretion of a mere solvent, we should expect that when the microbes were removed or destroyed, either by heat or chemical means, the portion of the medium already dissolved would not have any extensive action on fresh media. But if it had any such solvent action, it would probably continue after the solution had been heated to a temperature sufficient to destroy the action of an enzyme. If, on the other hand, the microbes liquefied the media by secreting an enzyme, we should expect that the liquefied portion would probably dissolve a considerable amount of new medium when added to it, but that its solvent action would be arrested by exposure to a temperature sufficient to inhibit enzyme action.

The culture medium was made by adding to meat broth: gelatine, 10 per cent.; peptone, 1 per cent.; and sodic chloride, 0·5 per cent. The reaction was rendered faintly alkaline with carbonate of soda. In all the experiments Koch's methods to ensure sterile media and pure cultures were followed out.

Tubes of 10 per cent. gelatine were inoculated with the five microbes, and placed in the incubator at 37° C., with the exception of the putrefactive micrococcus, which was kept at 22° C.

When liquefaction was complete the fluid was filtered into sterile tubes, the bacterial deposit being washed with a small quantity of sterile distilled water.

Of the filtrate, one, three, and five drops were added respectively to fresh gelatine, and the tubes placed in the incubators as before. The gelatine liquefied, and in all cases bacteria were present.

This liquefied gelatine was in its turn taken and subjected to a temperature of 50° C. for one hour. Then one, three, and five drops were added to fresh gelatine. After incubating, some of the cholera comma tubes did not liquefy, but in all cases where liquefaction took place it was due to the active bacteria, as proved by their growth on control plates. The control plates were made by adding a few drops of the liquefied gelatine to fresh gelatine, and pouring it out in a sterile glass dish. After incubating at 22° C., the gelatine was examined microscopically, and the presence or absence of bacterial colonies noted.

The liquefied gelatine was next subjected to a temperature of 100° C. for fifteen minutes. The same number of drops were added to gelatine. This fresh gelatine did not liquefy. Finally, 5 c.c. were added to fresh gelatine, but still it did not liquefy.

The control plates showed no colonies.

We therefore conclude that exposure to a temperature of (I) 100° C. destroys—

(*a.*) The bacteria.

(*b.*) The liquefying power of the fluid.

(II) 50° C. does neither. It was not deemed advisable to continue the sterilisation too long, having regard to the injurious action of heat on soluble ferments.

It was next necessary to determine the temperatures between 50° C. and 100° C., which would be sufficient to kill the bacteria without rendering any ferment which might exist inactive. A series of experiments led to the following results:—

60° C. for half an hour killed Koch's and Finkler's spirillum.

75° C. for fifteen minutes, on two successive days, killed the scurf and "Welford" bacilli.

70° C. for fifteen minutes, on two successive days, destroyed the putrefactive micrococcus.

Having established these facts, a series of cultures at 37° C. were made in small glass flasks, each containing about 100 c.c. of 10 per cent. gelatine. The liquefied gelatine was filtered, and the deposit washed with sterile distilled water.

These filtrates from the five series of cultures were sterilised as described above. Then 5—10 c.c. of each were added to 10 per cent. gelatine (20 c.c.) and kept at 37° C., as well as control tubes of sterile gelatine.

On the third day the tubes were removed from the incubator and placed in ice-cold water.

Results :—

Scurf bacillus, Welford bacillus: The gelatine does not stiffen, but remains liquid.

Koch's spirillum, Finkler's spirillum: The gelatine is semi-liquid, and does not completely re-gelatinise.

Putrefactive micrococcus, Control gelatine: The gelatine stiffens.

Control plates. No bacteria.

Kept at the ordinary room temperature, these phenomena persisted, the liquid gelatine remaining liquid, and the solid gelatine not liquefying.

Here, then, we have complete liquefaction of the gelatine produced in the first two cases, partial liquefaction in the next two, and no effect in the last.

That this liquefaction was brought about without the presence of active bacteria is proved by the fact that control plates inoculated from the liquefied gelatine remained sterile. The complete liquefaction was produced by the sterile fluid from the microbes which were more active liquefiers of gelatine than the others. In the case of the two comma spirilla the enzyme action in gelatine was evidently more feeble. The negative result with the putrefactive micrococcus, and also the fact that tubes inoculated from it, and kept at the optimum temperature of 22° C., also gave negative results, were probably due to the preliminary sterilisation having destroyed both the microbes and any enzyme which they might have formed.

These introductory experiments led to the following conclusions :—

1. 100° C. destroyed both the bacteria and the liquefying power.

2. 50° destroyed neither the bacteria nor the liquefying power.

3. Temperatures between 60° and 75° C. destroyed the bacteria, but not the liquefying power in four cases.

4. The liquefied gelatine treated as under 3, and added to fresh gelatine, liquefied it, although active bacteria were proved to be absent.

5. The liquefaction must, we think, be due to a soluble enzyme, inasmuch as liquefaction still took place after the elimination of the microbes, while it was prevented by exposure to such a temperature as would destroy the activity of an enzyme but would not be likely to affect the action of a simple solvent.

II.

Having regard to the fact that the peptonising action in gelatine was slow, and in two cases partial, it was next sought to determine whether more active liquefaction of the gelatine could be obtained by growing the microbes in some other albumenoid soil.

Two culture fluids were made with meat broth as follows:—

A. Meat broth—	B. Meat broth—
Peptone, 1 per cent.	NaCl 0·5 per cent.
NaCl 0·5 ,,	

Both were rendered faintly alkaline with the carbonate of soda.

The bacteria grew well in both of these media, and so rapidly and abundantly in B. that further experiments were made with it only, *i.e.*, without peptones. For each culture, 100 c.c. meat broth were used. After inoculation and four days' incubation at 37° C., the broth was filtered, and the bacterial deposit washed with sterile distilled water. It was then sterilised as already described, and 10 c.c. added to tubes of 10 per cent. gelatine. These tubes were placed in the incubator, as well as control tubes of sterile gelatine. When taken out, and placed in ice-cold water, the following results were obtained:—

(1.) After 24 hours:

Scurf bacillus Welford bacillus	Liquid.
Koch's spirillum Finkler's spirillum	Semi-liquid.
Putrefactive micrococcus Control gelatine	No liquefaction.
Control plates. No colonies.	

(2.) After 48 hours:

Koch's spirillum Finkler's spirillum	Liquid.
Putrefactive micrococcus Control gelatine	No liquefaction.
Control plates. No growth.	

From these experiments it will be seen that the enzyme developed in meat broth is more active than that formed in gelatine. In twenty-four hours the gelatine was liquefied by the scurf and Welford bacilli; in forty-eight hours by Koch's and Finkler's comma spirilla. Again the putrefactive micrococcus gave negative results.

Conclusions:—

1. An enzyme is formed in meat broth which liquefies gelatine,

and does so more surely and quickly than the enzyme formed in gelatine itself.

2. The liquefaction is produced by a soluble ferment, since its action can be demonstrated apart from the microbes which produce it.

III.

Instead of using heat sterilisation some experiments were made with menthol and thymol.

It was found that when these substances were added in amounts sufficient to prevent the growth of the bacteria—the ferment action was likewise inhibited.

IV.

The presence of a soluble ferment being demonstrated, can we isolate it?

(1.) From gelatine.

(2.) From meat broth.

(1.) *From Gelatine Cultures.*

Flasks containing 250 c.c. of 10 per cent. gelatine were inoculated with the five microbes. They were left in the incubator at 47° C., (putrefactive micrococcus, 32° C.), till liquefaction was complete. The liquefied gelatine was treated with absolute alcohol and filtered. The precipitate was extracted with glycerine, and finally reprecipitated with alcohol. The precipitate, after drying in a sterilised flask, was taken up in a small quantity of sterile distilled water, and allowed to stand over night. About 5 c.c. were then added to 10 per cent. gelatine, and incubated at 37° C.

Results.—Negative. No liquefaction was produced.

(2.) *Meat Broth Cultures.*

In each case 250 c.c. were treated in a similar manner—with alcohol and glycerine, and the precipitate and sterile distilled water added to 10 per cent. gelatine.

Results :—

Koch's spirillum Finkler's spirillum Putrefactive micrococcus	No liquefaction.
Scurf bacillus Welford bacillus	In a few tubes the gelatine was viscid. The rest resolidified.
Control plates.	No colonies.

Concluding that the prolonged method of extraction had weakened

the action of the enzyme, a modification of the process was next made in the following manner:—500 c.c. of meat broth were inoculated with the microbes, and left in the incubator for seven days. The precipitate, with an excess of alcohol, was allowed to stand overnight, and, after drying, was dissolved in sterile distilled water, and then reprecipitated by alcohol. This precipitate was dried and taken up in distilled water. The next day about 20 c.c. were added to 100 c.c. of a 5 per cent. gelatine, and placed in the incubator at 37° C.

Results after four days:—

The only positive results were obtained with the scurf bacillus and the Welford bacillus. In these cases the gelatine remained liquid, while the control gelatine resolidified. The control plates gave no colonies.

Conclusion.—The bacteria do form a soluble enzyme which can be isolated, and its action demonstrated on albumenoid gelatine.

V.

Are the microbes which liquefy gelatine capable of exerting a like action on other proteid bodies?

To test this, experiments were made with—

(*a.*) Egg-albumen.
(*b.*) Fibrin.

In the first place, it was necessary to find out what resulted from the direct action of the microbes.

Faintly alkaline meat broth, as developing the most active enzyme, was used.

(a.) *Egg Albumen.*

To flasks containing 100 c.c. of meat broth were added small pieces of coagulated egg albumen. The flasks were then sterilised and inoculated with Koch's spirillum, Finkler's spirillum, the scurf and Welford bacilli. They were then placed in the incubator at 37° C.

Results:—

(1.) Scurf bacillus.
Welford bacillus:—
1st day. No marked change.
2nd day. Albumen broken up into small fine flocculent fragments.
3rd day. Disintegration almost complete.
4th day. Disintegration complete.

(2.) Koch's spirillum.
Finkler's spirillum :—
1st day. No marked change.
2nd day. Translucent.
3rd day. Thinned and transparent.
5th day. Disintegration.

The bacteria are therefore able, by means of their peptonising action, to disintegrate egg albumen.

(b.) *Fibrin.*

To 100 c.c. of the meat broth small pieces of boiled fibrin were added, and after sterilisation the flasks were inoculated with the same microbes, then placed in the incubator at 37° C.
Results :—

(1.) Scurf bacillus.
Welford bacillus.
1st day. No marked change.
2nd day. Fibrin eroded.
3rd day. Breaking up.
4th day. Disintegration complete.
5th day. Fluid has become turbid.

(2.) Koch's spirillum.
Finkler's spirillum :—
1st day. No change.
2nd day. Slight erosion.
3rd day. Frayed appearance.
4th day. Commencing to break up.
5th day. Disintegrated.
6th day. Turbidity.

Here again we have a marked disintegrating action on fibrin.
Conclusion.—The bacteria exert a disintegrating action on egg albumen and fibrin, as well as on gelatine.

VI.

Can we demonstrate the action of the enzyme on proteid bodies such as egg albumen and fibrin, in the same way that its action was demonstrated on gelatine?

The alcoholic precipitate from 500 c.c. of the meat broth culture was dried at 35° C., and then dissolved in sterile distilled water. It was then reprecipitated by alcohol and filtered. This precipitate was dried in sterile plugged flasks, and to it were added 50 c.c. of sterile

distilled water, and 5 c.c. of a $\frac{1}{2}$ per cent. chloroform water. Carbonate of soda was finally added to render the fluid faintly alkaline.

In each flask was placed a small piece of boiled fibrin. After four days in the incubator they were taken out and examined :—

A. From each, gelatine plate cultures were made.

B. The appearance of the fibrin was noted.

C. After filtration the filtrate was tested for digestive products.

A. Some of the plates showed bacteria. The flasks from which these had been made were rejected; only those were used which had remained sterile.

B. In none did the fibrin break up and disappear. But it became thinned and frayed at the edges. This was most marked with the scurf and Welford bacilli.

C. The filtrate was examined for soluble products :—

On neutralising with dilute hydrochloric acid a precipitate appeared. This was filtered off and the filtrate tested for peptones. A solution of caustic soda was added, and then a highly dilute solution of cupric sulphate was filtered down the side of the test tube. At the line of demarcation the rose-coloured peptone reaction was strongly marked.

The simple boiled solution of the ferment only gave the faintest peptone reaction.

These results were obtained with the scurf and Welford bacilli, and Koch's and Finkler's spirillum. To sum up :—

1. The fibrin was visibly affected.
2. Neutralisation produced a precipitate.
3. The peptone reaction was very distinct.

The enzyme therefore, apart from the bacteria, *can* form soluble products from fibrin, and amongst these peptones.

VII.

Are the microbes capable of forming a diastatic, as well as a peptonising ferment ?

A. Scurf bacillus.

Welford bacillus :—

Starch was heated with water so as to form a thin paste. To this was added sodic chloride (0·5 per cent.). About 100 c.c. were placed in each flask, which was then plugged with cotton wool and sterilised.

After inoculation they were placed in the incubator (37° C.) along with flasks of sterile starch paste.

Flasks were opened on successive days and examined :—

2nd day. Starch has lost its opalescence. Iodine gives a blue colour.
3rd day. Iodine gives a red colour.
5th day. No reaction with iodine.
6th day. Was tested for a reducing sugar. The reactions were as follows :—

(1.) Iodine.—No reaction.
(2.) Caustic soda.—On gently boiling fluid becomes yellow.
(3.) Cupric sulphate and caustic soda.—A yellow precipitate on boiling.
(4.) Fehling's reagent.—A red precipitate.
(5.) Barfoed's reagent.—No reaction on gently heating.

(*Barfoed's Solution* :—One part of neutral acetate of copper dissolved in 15 parts of water, and then to 200 c.c., 5 c.c. of acetic acid (38 per cent.) added.)

The control starch gave blue colour with iodine, but none of the above reactions.

B. Putrefactive micrococcus—
Results were negative.
C. Koch's spirillum.
Finkler's spirillum :—

The same starch solution was used, but a few drops of meat broth were added in each case. The usual control experiments were made :—

3rd day. Starch has lost its opalescence. Iodine strikes a blue colour.
4th day. Iodine gives a violet colour.
5th day. Iodine gives red reaction.
7th day. Iodine.—Red.
Caustic soda.—Yellow on boiling.
Cupric sulphate and caustic soda.—No reduction.
Fehling's solution.—No reduction. On previous addition of H_2SO_4 a slight reduction.
Barfoed's reagent.—No reduction.
Control starch.—Iodine strikes blue.

From these experiments the following conclusions may be drawn :—

1. The putrefactive micrococcus did not grow on the carbohydrate soil, and so we are left in doubt as to its diastatic action.

2. The scurf bacillus and Welford bacillus were both capable of cultivation, and evinced a marked diastatic action, in addition to their peptonising power. The failure of the iodine test, and the

precipitates obtained with Fehling, &c., indicate the presence of a reducing sugar. The failure with Barfoed's reagent suggests that the sugar is in great part, at any rate, maltose.

3. With regard to Koch's spirillum and Finkler's, though they evinced a diastatic action, it was feebler than in the former case, only traces of a reducing sugar being detected after the addition of sulphuric acid. The red and violet coloration with iodine points to the formation of dextrin (erythro- and achroo-dextrin).

At any rate, in the scurf and Welford bacilli we have two microbes which evince a marked diastatic action; and a demonstration of the fact that the same germ can produce both a diastatic and a peptonising ferment.

VIII.

Can we demonstrate the action of the diastatic enzyme apart from the bacteria?

Starch cultures of the scurf bacillus and the Welford bacillus (two days' growth) were treated with chloroform water (1 per cent.) till they became sterile.

The fluid was then added to fresh starch, and incubated at 37° C.

In eight to ten days the iodine reaction had disappeared. On boiling with caustic soda the fluid became yellow. Fehling's solution was reduced. The fluid lost its opalescence. Control plates—no growth.

These experiments point strongly to the existence of a diastatic enzyme capable of isolation, and of acting apart from the bacteria.

IX.

That the peptonising enzyme bears the closest analogy to the pancreatic ferment will be seen from the following experiments. Sterile meat broth, in which Finkler's spirillum and the Welford bacillus had been cultivated, was added to 10 per cent. gelatine tubes of differing reaction:—

	Gelatine.	*Results.*
A.	Acidified with dilute hydrochloric acid..	No liquefaction.
B.	Alkaline by adding sodic carbonate	Liquefied.
C.	Neutral	Liquefied.
D.	Boiled after adding the ferment.....	No liquefaction.

X.

The digestive action of the microbes was tested on several other bodies.

1. *Fats.*—Alkaline meat broth and olive oil, 2 per cent.

The results were negative.

Experiments which were made by Manfredi* tend to show that fat-containing media impair the vegetative energy of bacteria.

2. *Dextrose.*—The culture fluid was prepared as follows:—

Dextrose	2 per cent.
Peptone.....	1 „
Sodic chloride	0·5 „
Reaction	Neutral.

After sterilisation, the flasks were inoculated with the scurf bacillus and Welford bacillus. Incubated at 37° C. They were examined on the fourth day.

Fehling's solution was no longer reduced. The fluid gave a marked acid reaction.

The control solution reduced Fehling's solution. Reaction was unchanged.

3. *Cane-sugar.*—Cane-sugar, 2 per cent.
Peptone, 1 per cent.
NaCl, 0·5 per cent.
Reaction, neutral.

Inoculated with scurf bacillus and Welford bacillus, and incubated at 37° C.

The results were negative. No reducing sugar detected.

Muscle.—Alkaline meat broth cultures were used. Inoculated with Finkler's spirillum and Welford bacillus.

With the Welford bacillus a marked effect—the muscular tissue becomes disintegrated, and the striæ indistinct.

These experiments, though incomplete in themselves, are sufficient to show that the bacteria which liquefy gelatine and diastase starch, can also exert a digestive influence on dextrose and muscle. The exact determination of the products of this action in the case of these and some other organic bodies must be reserved for further investigation.

To sum up briefly the results of this inquiry:—

1. The bacteria which liquefy gelatine do so by means of a soluble enzyme.

2. This enzyme can be isolated, and its peptonising action demonstrated apart from the microbes which produce it.

3. The most active enzyme is that formed in meat broth.

4. Acidity hinders, alkalinity favours its action.

5. The bacteria which form a peptonising enzyme on proteid soil can also produce a diastatic enzyme on carbohydrate soil.

* 'Accademia dei Lincei, Rendiconti,' vol. 3, sem. 1, 1887, p. 535.

6. The diastatic enzyme is not so readily separated from the microbes which produce it, but where that has been accomplished its action on starch can still be demonstrated.

7. The diastatic enzyme has no effect on gelatine, and *vice versâ*.

8. The bacteria are capable of evincing an adaptiveness to the soil in which they grow.

9. The microbes are capable of digesting other similar bodies such as dextrose and muscle.

10. Fatty matter was not affected.

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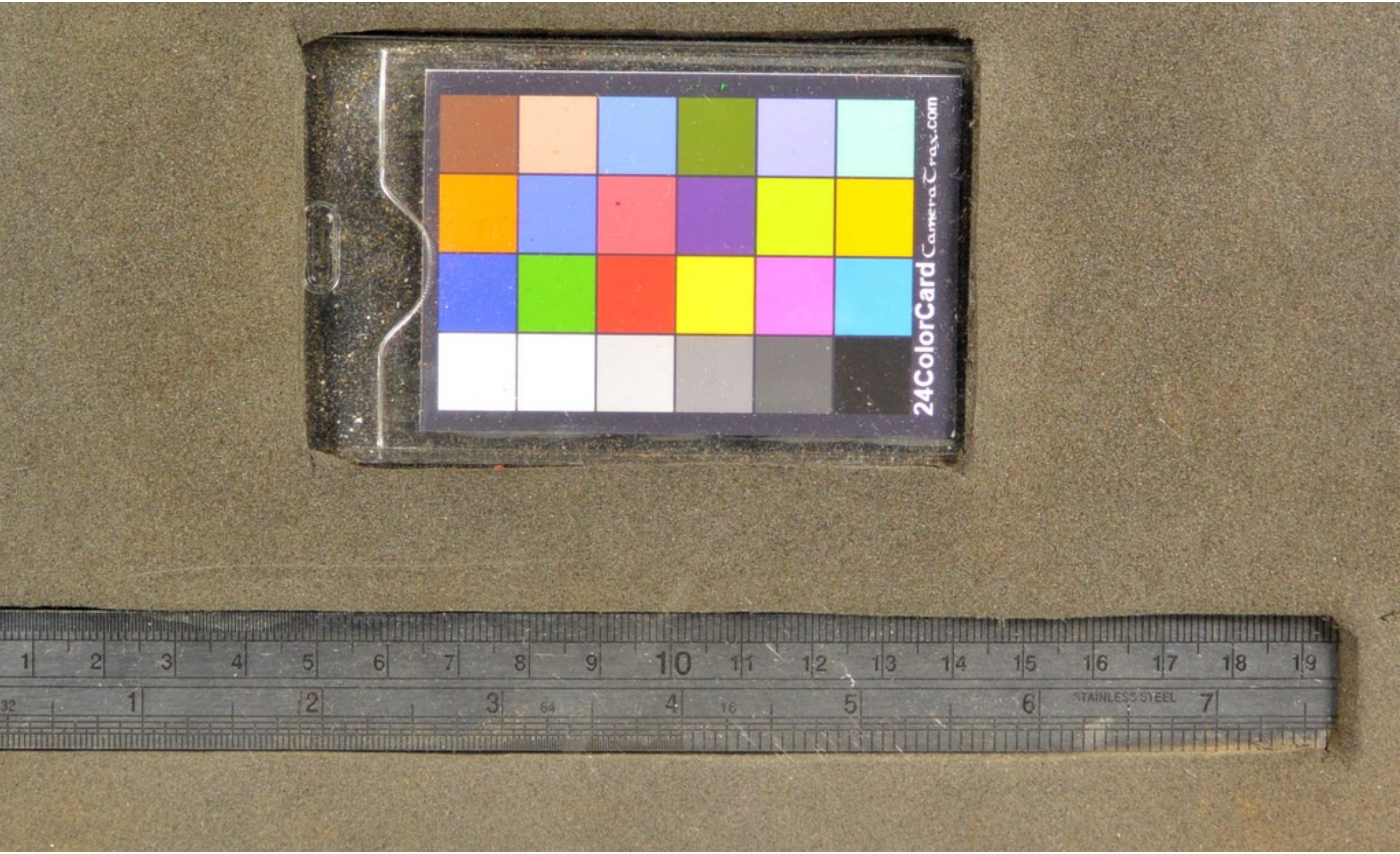
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