XXV. A NOTE ON THE HOPKINS AND COLE MODIFICATION OF THE ADAMKIEWICZ TEST FOR PROTEIN.

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INTRODUCTION.

The following brief piece of research is the outcome of an unexpected failure in class-work to obtain the well-known reaction with "reduced oxalic" acid [Hopkins and Cole, 1901, 2] protein and concentrated sulphuric acid. In the previous experience of the writer at Cambridge and elsewhere, the test had never failed, and a high estimate of its reliability had been formed, so that this case, when nothing but a yellow to a yellow-brown ring was obtained, needed investigation.

It was soon found that the failure was due to an accidental admixture of the bench sulphuric acid with nitric acid, but during, and arising out of, the search for the cause, the phenomena recorded below were observed.

It became obvious that three disturbing factors were involved: (a) the presence of small quantities of impurities in the sulphuric acid, (b) the absence of traces of impurities, and (c) physical factors influencing the rise and fall of temperature of the reacting fluids. These have been investigated in turn.

(1) *The presence of impurities in supraminimal amounts.*

The cause of the trouble recorded above was the presence in the sulphuric acid of nitric acid.

The test, as has been pointed out by Salkowski [1888] and Hopkins and Cole [1901, 1], and confirmed by Rosenheim [1906], is spoilt by oxidising agents. This occurs at small concentrations, especially if, as a criterion, rather than the formation of a ring, the colour of the resultant mixture be

taken¹. Thus a final concentration of 1 part by volume of hydrogen peroxide (10 vols. comm.) in 50, of 1 part by weight of ferric chloride in 1000, of 1 part by weight of potassium chlorate in 25,000, of 1 part by volume of concentrated nitric acid in 25,000 or of 1 part by weight of sodium nitrite in 50,000 parts by volume prevents the formation of a violet or purple coloration.

In all cases except one (nitric acid) a yellow ring is formed, which, on shaking the tube gently from side to side, spreads throughout the mixture. With nitric acid there appears a purple ring above the yellow ring at the junction of the fluids. When the test tube is shaken the purple ring moves up the test tube, is overtaken by the yellow coloration and is destroyed. In no case is there charring.

For laboratory practice, therefore, if, instead of a lilac, mauve or violet coloration, a yellow, a red, a red-purple or a brown ring is formed, it is probably due to contamination of the sulphuric acid with oxidising agents. Commercial sulphuric acid, to judge by the samples investigated, never has enough oxidising agents present to prevent the reaction.

(2) *The influence of oxidising agents in minimal amounts*

In the course of a large number of experiments it has become clear that Rosenheim's statement that an oxidising agent is necessary to the reaction is justified. The observations on which this is based are as follows (for experimental details consult Appendix, § 2):

(a) Some very pure samples of sulphuric acid (supplied by Messrs Evans, Lescher and Webb, Messrs Baird and Tatlock, Liverpool, and Messrs Towers and Sons, Widnes and Liverpool) give with freshly made or with old "reduced oxalic," whether strong or weak, or with Benedict's magnesium-reduced oxalic [1909] a lilac or lilac-mauve colour instead of the typical violet (see Appendix, § 2, Exp. LVIII etc.). But if traces of oxidising agents are added to the "reduced oxalic" the blue-violet or violet develops at once (Appendix, § 2). The optimal concentration is, for hydrogen peroxide (10 vols. comm.) *circa* 1 part by volume in 1000 of the resulting mixture, for ferric chloride 1 part by weight in 20,000 and for sodium nitrite 1 part in 375,000.

(b) Subsequent addition of oxidising agents after the lilac or lilac-mauve coloration with pure sulphuric has developed converts this colour to a blue-violet of great density and beauty (Appendix, § 1, Exps. LII—LIV)². This

¹ For experimental details see Appendix, § 1, Exps. I, II, XXX, LII—LV.

² The paradox of the greater stability of the colour when once formed towards oxidising reagents is simply explained as the decrease in velocity of a chemical reaction concurrent with fall of temperature.

does not always take place if the reacting fluids have cooled. But in this case, warming, with previous or subsequent addition of an oxidising agent, yields the typical blue-violet (Appendix, § 3 and § 6). Continued heating alters the test through a series of colours typically the result of the addition of increasingly greater quantities of oxidising agents to the "reduced oxalic" (Appendix. Compare § 3, with § 2 Exp. LIX and § 4 Exp. LXIV). Obviously change in colour is a sign of advancing oxidation.

(c) Commercial sulphuric acid will withstand the addition of less oxidising agent than will pure sulphuric acid if the test is to be typical. The reaction is not improved but spoilt by the addition of even small traces (Appendix, § 4, Exp. XXXVI, etc.).

(d) Commercial sulphuric acid will withstand the addition of more formaldehyde than will pure sulphuric acid (Appendix, § 4, Exp. XLI and XLII).

(e) If the violet fluid resultant from the use of commercial sulphuric acid be boiled, the colour changes seen in the parallel example quoted in section (b) above are imitated with exactitude (Appendix, § 4, Exp. LXIV). Oxidation by means of the oxidising agents present in the commercial sulphuric acid is proceeding.

These five observations accord well with Rosenheim's theory that oxidising agents are necessary to the reaction. They suggest that a coloured substance arises from the oxidation of a tryptophane-formaldehyde product formed from the protein and that further oxidation of this blue-violet substance modifies this colour and ultimately destroys it. We may assume that this reaction proceeds at a velocity very much greater at high temperatures than at lower temperatures and that, in the optimal conditions of the test, the cooling is rapid enough to prevent the oxidation going too far.

That there is a change proceeding at a definite rate may be seen by carrying out the test under conditions in which a high temperature is not attained, or if attained, is rapidly reduced. For instance the initial colour may be profoundly modified in a very wide tube, or by cooling the test tube under the tap. A rose colour at first develops, but in the first case after the test tube has stood some time the colour has deepened to mauve and after a day to blue-violet. It is doubtful if this be due to the oxygen of the air, for no observable difference was found when the test was carried out under an atmosphere of carbon dioxide.

To the question whether a balance of formaldehyde and oxidising agents is necessary [see Rosenheim, 1906] (another case of the two classical antagonistic principles) my experiments give no certain answer. If these be

both in the "reduced oxalic" and in optimal equipoise, then reduction of the strength of the "reduced oxalic" should render the equipoise more susceptible to tilting agencies. Oxidisers should have more effect in the presence of weak "reduced oxalic" than of strong. There is no evidence to offer that this is the case (App. § 5, Summary of Exps. XVIII to XXXV).

(3) *The influence of physical conditions.*

It was thought that physical conditions, such as the size and shape of the test tube, area of reacting fluids compared with their volume, and rapidity with which the fluids reached their maximal temperature and subsequently cooled would affect the results. This is indeed so but, except in the extreme cases given above, is not of much importance. Thick test tubes give a lighter result than thin; very narrow than normal size; and very large tubes with inadequate amounts of fluid than normal tubes or large tubes with adequate amounts. Cooling under the tap produces a rose colour shot with green which subsequently, unless oxidising agents are added, does not darken much. In three days it is lilac in colour (see App. § 6).

One reason for the different colours observed when different volumes of the reagents are used is to be found in the dichromatism of the coloured substance. It is lilac in thin layers or great dilutions, and bluer in thicker layers and greater concentrations. This, of course, has its parallel in its absorption band in the spectrum. A mixture that was lilac showed an absorption band from λ 592 to λ 547, on adding a drop of ferric chloride the band stretched from λ 646 to λ 511, diluting the mixture with pure sulphuric acid brought the band to its original dimensions and the mixture to its original colour. On warming the diluted mixture, with consequent alteration in colour to purple, the band stretched from λ 610 to λ 531, and the blue and violet were reduced in intensity. This band is identical with one described by Hopkins and Cole [1901, 1].

Finally, the different colours observed with weaker solutions of protein are due to the oxidation of the chromogen beyond the optimum. Weaker solutions give with either pure or commercial sulphuric acid results that are redder in tint than the controls. This is not dichromatism, for dilution with sulphuric acid leaves the control many degrees bluer than the others. The relation of this phenomenon to the others given above is clear if we assume progressive oxidation.

SUMMARY AND CONCLUSIONS.

(1) The modification of the Adamkiewicz reaction introduced by Hopkins and Cole fails in the presence of small amounts of oxidising agents.

(2) But traces of these improve the reaction when carried out with "pure" sulphuric acid.

(3) Probably, therefore, the result with "commercial" sulphuric acid is due to the presence of contained oxidising agents.

(4) Whether the trace of oxidising agent which gives the lilac to mauve tint with "pure" sulphuric acid is in the reduced oxalic or in the sulphuric acid cannot be settled. In view of Miss Homer's work [1911] it is probably in the sulphuric acid.

(5) The resultant coloration of the mixture depends on the extent of a combination of a chromogen with oxygen. This combination has a definite rate of reaction which is naturally more rapid at high temperature and at greater concentrations of the oxidising agent.

(6) It is therefore best to keep the volumes and percentage compositions of the reacting fluids as constant in proportion as possible and their absolute measure should depend on the size of the test-tube¹.

(7) The following method of carrying out the test yields excellent results: 1 c.c. of strong "reduced oxalic" acid (Benedict's "reduced oxalic" will do just as well) is mixed with 1 c.c. of 1 % Witte's peptone, and then 2 c.c. of sulphuric acid are run down the side of the test-tube which is held as near horizontal as convenient. The fluids are then rapidly mixed by shaking from side to side with the test-tube vertical. The quantities given are for a test-tube 1.5 cm. in diameter (the usual laboratory size). Subsequent addition of one drop of 1 % FeCl_3 solution usually increases the density and the blueness of the coloration.

The quantities given may be varied greatly and yet a good coloration be obtained. 1 c.c. or 5 c.c. sulphuric acid to 2 c.c. of the mixture yield an unmistakable result and the quantities may be guessed and not measured. More sulphuric acid gives a bluer result than less.

¹ I can see no reason for abandoning the excellent reagent known as "reduced oxalic" acid in favour of formaldehyde and doctored sulphuric acid. As long as the test is performed under conditions that lead to a maximal development of heat, all samples of sulphuric acid give a reasonable coloration. It is only when the reacting fluids are cooled or contaminated that an imperfect result is obtained; whereas the balance of oxidising agent and formaldehyde is easily tilted in an adverse direction, especially in the hands of students.

"Reduced oxalic" acid is a reagent easily made, particularly now that magnesium powder is a requisite in the biochemical laboratory. It does not diminish in strength even when more than a year old.

The details of the experiments on which the statements above made are based are relegated to an appendix.

I have to thank Messrs Hopkins and Cole for reading through the MS. of this paper and for suggestions they have made. Their explanation of the phenomena recorded must be left to a future communication from them.

APPENDIX.

1. *Experiments on the destructive effect of oxidising agents.*

In most of the experiments performed the production of a coloration was taken as a criterion and not the formation of a ring. In the experiments based on the plan of Exp. XXX (*v. inf.*)—the majority—all the test tubes were prepared before the shaking of the contents took place. This naturally does not give a maximal rise of temperature. The first test tube has to wait a minute or so before the fluids it contains are mixed and the heat evolved by the natural admixture at the junction of the fluids is to some extent lost. This exaggerates the influence of oxidising agents as adjuvants of the reaction, and decreases the influence as deterrents.

Exp. I. Equal parts stock solution of "reduced oxalic" undiluted and (*circa*) 1 % acid albumin mixed.

Mixture	Pure sulphuric	Oxidiser	Remarks
25 c.c.	25 c.c.	1 drop conc. HNO_3	Yellow colour, faint effervescence.
25 c.c.	25 c.c.	Small amt. FeCl_3	Normal violet, disappeared on adding excess FeCl_3 .
25 c.c.	25 c.c.	1 c.c. 10 vols. H_2O_2	Light yellow coloration.

Exp. II. Same mixture as above.

Mixture.	Comm. sulph.	Oxidiser	Remarks
2 c.c.	2 c.c.	Nil	Violet ring.
2 c.c.	2 c.c.	2 drops 0.01 % HNO_3	Yellow ring.
2 c.c.	2 c.c.	1 drop " "	Faint violet above, yellow ring below.
2 c.c.	2 c.c.	(1 drop water)	Violet ring.

Exp. XXX. Mixture 1 % peptone and strong "reduced oxalic." Equal parts.

Mixture	H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks	Coloration
2 c.c.	0 drops	5 drops	2 c.c.	Ring not clear.	Lilac-mauve.
2 c.c.	1 "	4 "	2 c.c.	Good ring.	Clear violet.
2 c.c.	2 "	3 "	2 c.c.	Good ring.	Purple, then rose.
2 c.c.	3 "	2 "	2 c.c.	Vanishing ring.	Light red.
2 c.c.	4 "	1 "	2 c.c.	Vanishing ring.	Yellow.
2 c.c.	5 "	0 "	2 c.c.	Vanishing ring.	Straw colour.

Throughout the experiments based on this plan the same pipettes were kept for each reagent. The drops were frequently estimated and their

average volume was 0.04 c.c. Of course in the measurement of the above volumes, whether large or small, no great accuracy was obtained. A 1 c.c. pipette has a high percentage error (*circa* 5 %).

Exp. LII. NaNO_2 . *Same mixture as in Exp. XXX.* In the experiments following, not the formation of a ring, but the final coloration is used as a criterion.

Mixture	0.1 % NaNO_2	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac coloration (deep blue-violet on adding drop of NaNO_2).
2 c.c.	1 "	4 "	2 c.c.	Dense purple coloration.
2 c.c.	2 "	3 "	2 c.c.	Red coloration.
2 c.c.	3 "	2 "	2 c.c.	Orange coloration.
2 c.c.	4 "	1 "	2 c.c.	Yellow coloration.
2 c.c.	5 "	0 "	2 c.c.	Yellow coloration.

Exp. LIII. FeCl_3 .

Mixture	4 % FeCl_3	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (dense blue on adding 1 drop FeCl_3).
2 c.c.	1 "	4 "	2 c.c.	Purple coloration.
2 c.c.	2 "	3 "	2 c.c.	Claret "
2 c.c.	3 "	2 "	2 c.c.	Red-brown "
2 c.c.	4 "	1 "	2 c.c.	Brown "
2 c.c.	5 "	0 "	2 c.c.	" "

Exp. LIV. KClO_3 .

Mixture	0.2 % KClO_3	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac coloration (purple on addition of 1 drop KClO_3).
2 c.c.	1 "	4 "	2 c.c.	Purple coloration.
2 c.c.	2 "	3 "	2 c.c.	Yellow "
2 c.c.	3 "	2 "	2 c.c.	" "

Exp. LV. HNO_3 .

Mixture	HNO_3 2 vols. per 1000	H_2O	Pure sulphuric	Remarks
2 c.c.	0 drops	5 drops	2 c.c.	Lilac (no deepening on adding HNO_3).
2 c.c.	1 "	4 "	2 c.c.	Purple.
2 c.c.	2 "	3 "	2 c.c.	Red-brown.
2 c.c.	3 "	2 "	2 c.c.	Yellow.
2 c.c.	4 "	1 "	2 c.c.	Yellow.
2 c.c.	5 "	0 "	2 c.c.	Yellow.

In this experiment a purple ring was formed above the junction of fluids with a yellow ring beneath. On gently shaking, the above colours were produced.

A number of other experiments on similar lines were performed with similar results.

2. *Experiments on the beneficial effect of traces of oxidising agents.*

(See also Experiments immediately preceding.)

Exp. VIII. Peptone mixture.

	Mixture	10 % H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks
(a)	{ 2 c.c.	0.0 c.c.	1.0 c.c.	2 c.c.	Ring obtained in all. On shaking, (a) (i) and (ii) were the most violet, (b) the deepest and (c) the weakest. After two hours (b) was nearly bleached and (c) yellow.
	{ 2 c.c.	0.0 c.c.	1.0 c.c.	2 c.c.	
(b)	{ 2 c.c.	0.5 c.c.	0.5 c.c.	2 c.c.	
	{ 2 c.c.	0.5 c.c.	0.5 c.c.	2 c.c.	
(c)	{ 2 c.c.	1.0 c.c.	0.0 c.c.	2 c.c.	
	{ 2 c.c.	1.0 c.c.	0.0 c.c.	2 c.c.	

Exp. IX.

2 c.c.	0 drops	5 drops	2 c.c.	Blue-violet coloration.
2 c.c.	1 „	4 „	2 c.c.	Dense violet, showed up most rapidly.
2 c.c.	2 „	3 „	2 c.c.	Purple.
2 c.c.	3 „	2 „	2 c.c.	Purple.
2 c.c.	4 „	1 „	2 c.c.	Red-purple.
2 c.c.	5 „	0 „	2 c.c.	Red-purple.

A number of other experiments with different concentrations of H_2O_2 gave similar results. Exp. XXI is quoted. Strong reduced oxalic diluted five times and then mixed with an equal volume of 1 % Witte's peptone.

Exp. XXI.

	Mixture	4 % H_2O_2 (10 vols.)	H_2O	Pure sulphuric	Remarks
	2 c.c.	0 drops	5 drops	2 c.c.	Pale mauve.
	2 c.c.	1 „	4 „	2 c.c.	Violet-purple. Three times as dense.
	2 c.c.	2 „	3 „	2 c.c.	Purple.
	2 c.c.	3 „	2 „	2 c.c.	Rose-purple.
	2 c.c.	4 „	1 „	2 c.c.	Purple-rose.
	2 c.c.	5 „	0 „	2 c.c.	„ „

Exp. LVIII. Mixture: 1 part freshly made reduced saturated oxalic and 1 part 1 % Witte's peptone.

	Mixture	0.5 % FeCl_3	H_2O	Pure sulphuric	Remarks
	2 c.c.	0 drops	5 drops	2 c.c.	Lilac. (Deepened to royal blue on subsequent addition of FeCl_3).
	2 c.c.	1 „	4 „	2 c.c.	Deep violet.
	2 c.c.	2 „	3 „	2 c.c.	Clear violet.
	2 c.c.	3 „	2 „	2 c.c.	Purple-violet.
	2 c.c.	4 „	1 „	2 c.c.	Purple.
	2 c.c.	5 „	0 „	2 c.c.	Red-purple.

Exp. LIX. Mixture as above.

	Mixture	0.04 % NaNO_2	H_2O	Pure sulphuric	Remarks
	2 c.c.	0 drops	5 drops	2 c.c.	Lilac (deepening remarkably on addition of NaNO_2 solution).
	2 c.c.	1 „	4 „	2 c.c.	Violet.
	2 c.c.	2 „	3 „	2 c.c.	Purple-violet (strongest at first, bleaching later).
	2 c.c.	3 „	2 „	2 c.c.	Purple.
	2 c.c.	4 „	1 „	2 c.c.	Brown-purple.
	2 c.c.	5 „	0 „	2 c.c.	Purple-brown.

Very many other experiments were performed, some of which are quoted in other sections, with the same results.

3. *Experiments on the subsequent addition of oxidising agents.*

As will be seen in a number of the above experiments, addition of oxidising agents after the lilac coloration was formed produced a deep blue coloration. In some earlier experiments on cooling the fluids while the mixture was being made, it was noticed that the cold mixture of sulphuric acid reduced oxalic and peptone did not turn blue on the addition of ferric chloride, but after the addition warming gave the intense blue coloration. This on standing turned violet. Other tests showed that rewarming the mixture and then adding ferric chloride produced the same result, and that further warming, or rather boiling, made the colour proceed through violet, purple, to red and ultimately bleached it almost completely. Further notes will be found on similar results obtained in the experiments on the effect of cooling the test tube while the sulphuric and the other reagents are mixing (App. § 6).

4. *Comparison between pure and commercial sulphuric acid.*

Exps. XLI and XLII.

Mixture	0.04 % form- aldehyde	H ₂ O	2 c.c. Commercial sulphuric	2 c.c. Pure sulphuric
2 c.c.	0 drops	5 drops	Violet	Lilac-mauve (gives blue ring on running in H ₂ O ₂).
2 c.c.	1 "	4 "	Dense violet	Olive green.
2 c.c.	2 "	3 "	Brown-violet	" "
2 c.c.	3 "	2 "	Brown-purple	" "
2 c.c.	4 "	1 "	Purple-brown	" "
2 c.c.	5 "	0 "	Light purple-brown	" "

Exp. LXIV. Experiments on same plan as above made with different samples of acid.

B. & T. commercial sulphuric	Purple-violet	} On heating passed through purple to purple-red and brown.
T. " "	Purple-violet	
E. L. W. " "	Purple-violet	
T. pure "	Lilac	} On heating slowly altered to purple- brown obtained in XLI, 6.
E. L. W. " "	Bluish-lilac	

Exp. LXI. When various samples of acids, commercial and pure, were used, but to 2 c.c. of mixture and 4 drops of H₂O in each case 1 drop of 0.04 % NaNO₂ was added, the following results were obtained :

Sulphuric	Remarks
B. & T. pure, 2 c.c.	Blue-violet.
B. & T. comm., "	Purple.
T. pure, "	Blue-violet.
T. comm., "	Purple.
E. L. W. pure, "	Blue-violet.
E. L. W. comm., "	Purple.

Exps. XXXVI to XL and LX with varying strengths oxidising agents added showed that the commercial sulphuric used had the optimal amount of oxidising agents present. Compare with tests in §§ 2 and 3. As an example XXXVIII is quoted.

Mixture	2% 10 vols. H ₂ O ₂	H ₂ O	Commercial sulphate	Coloration
2 c.c.	0 drops	5 drops	2 c.c.	Violet (densest).
2 c.c.	1 "	4 "	2 c.c.	Purple-violet.
2 c.c.	2 "	3 "	2 c.c.	Purple.
2 c.c.	3 "	2 "	2 c.c.	Red-purple.
2 c.c.	4 "	1 "	2 c.c.	Purple-red.
2 c.c.	5 "	0 "	2 c.c.	Red-brown.

5. *Experiments on the supposed antagonism between formaldehyde and oxidising agents and on the occurrence of the latter in reduced oxalic acid.*

"weak" Hopkins' reagent = reduced saturated oxalic diluted 5 times.

"normal" " " = " " " 2½ "

"strong" " " = " " " undiluted.

The experiments were carried out as usual, six tests in each, with increasing strengths and amounts of hydrogen peroxide; thus:

Exp. XXXIII. Mixture of equal parts of strong Hopkins' reagent and 1% peptone.

	Mixture	2% 10 vols. H ₂ O ₂	H ₂ O	H ₂ SO ₄	Coloration
(a)	2 c.c.	0 drops	5 drops	2 c.c.	Lilac-mauve.
(b)	2 c.c.	1 "	4 "	2 c.c.	Violet-mauve.
(c)	2 c.c.	2 "	3 "	2 c.c.	Violet
(d)	2 c.c.	3 "	2 "	2 c.c.	Violet } densest.
(e)	2 c.c.	4 "	1 "	2 c.c.	Purple-violet.
(f)	2 c.c.	5 "	0 "	2 c.c.	Purple.

Summarising the results it was found:

10 vols. H ₂ O ₂	Densest colour		
	Strong reagent	Normal reagent	Weak reagent
10 %	(b) (violet)	(b)	(b)
4	(b) & (c) (purple-violet)	(b)	(b)
2 %	(c) & (d) (violet)	(d) & (e)	(c)
1.3 %	(d) (violet)	(d)	(e)
1 %	(b) to (d) (much the same colours)	—	(f)

No evidence is seen of oxidising agents deflecting the optimum towards nil concentration when weak Hopkins' reagent is used. This should be the case if oxidising agents are in the reduced oxalic.

6. *Experiments on effect of preventing, or of accelerating, rise to maximal temperature.*

Exp. LXV.

Mixture	Pure sulphuric	Procedure	Coloration
2 c.c.	2 c.c.	Shaken under cold tap	Pale rose with green fluorescence.
2 c.c.	2 c.c.	„ „ warm tap (45° C. circ.)	Rose.
2 c.c.	2 c.c.	Shaken in air	Lilac-mauve.
2 c.c.	2 c.c.	„ „ Bunsen flame	Violet-mauve, changing to brown-violet.
Repeated first experiment left to stand...			Mauve after three days.
„	„	„ added 1 drop 4% FeCl ₂	Pale rose slowly alters to deep rose, lilac-mauve and on gentle warming to violet.
„	„	„ very thoroughly cooled, added 1 drop 4% FeCl ₃	Slowly darkening in about an hour to lilac-mauve.

7. *Experiment on dilution of peptone.*

	Peptone	Strong Hopkins' reagent	Sulphuric	Coloration.
(a)	1 c.c. (1%)	1 c.c.	2 c.c. (pure)	Lilac-mauve.
(b)	1 c.c. „	1 c.c.	2 c.c. (comm.)	Violet-purple.
(c)	1 c.c. (0.2%)	1 c.c.	2 c.c. (pure)	Pale lilac-violet.
(d)	1 c.c. „	1 c.c.	2 c.c. (comm.)	Pale purple.
(e)	1 c.c. (0.1%)	1 c.c.	2 c.c. (pure)	Very pale lilac-purple.
(f)	1 c.c. „	1 c.c.	2 c.c. (comm.)	Light rose-purple.

When (a) and (b) are diluted with pure sulphuric the result is bluer than any parallel experiment with weaker peptone. Addition of commercial sulphuric acid to (b) produced a redder colour than addition of pure sulphuric acid to (b). These observations accord with the theory of progressive oxidation of a chromogenic substance by oxidising agents in the sulphuric acid.

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