THE DETECTION OF ACETO-ACETIC ACID BY SODIUM NITROPRUSSIDE AND AMMONIA

By VICTOR JOHN HARDING AND ROBERT FULFORD RUTTAN.

(Received September 17th, 1912)

The use of sodium nitroprusside and ammonia followed by addition of an acid insufficient in amount to completely neutralize the ammonia, was first suggested by Le Nobel¹, as a method of detecting small quantities of acetone. The test, of course, is based on the original test of Legal, but as the two tests differ in result, it is proposed to call the former the Le Nobel test and to reserve the term Legal's test exclusively to the action of sodium nitroprusside and potassium (or sodium) hydroxide followed by acidification. The two tests differ in the following points: (a) The Le Nobel test gives with acetone a much bluer shade of purple than the Legal test; (b) The Le Nobel test will detect aceto-acetic acid, as will be shown in this paper.

The two most important variations of the Le Nobel test are those of Jackson-Taylor² and Rothera³. Jackson-Taylor in detecting acetonuria dispenses with any previous acidification, and obtains in presence of acetone a petunia coloured ring. This modification is highly recommended by Saxe⁴ for clinical practice, and is in general use in many hospitals. Rothera recommends the addition of ammonium sulphate to the acetone solution, either in water or in urine, followed by the addition of sodium nitroprusside solution and concentrated aqueous ammonia. Other ammonium salts may be used, and the authors can confirm Rothera's experiments that such a procedure greatly enhances the value and delicacy of the test. Experiments on the Legal test also confirm Rothera's conclusions on the mechanism of the reaction; the condensation of the sodium nitroprusside and acetone by means of alkali giving rise to the production of a compound which acts as an indicator, but which, in the Le Nobel test, is unaffected by a low concentration of OH ions. The authors examined the action of a great number of organic acids in this respect. The method of applying the Le Nobel test was to acidify the acetone solution with the organic acid, add 0.5 c.c. M/10 sodium nitroprusside

(1 c.c. = 0.0298 grs. $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO} \cdot 2 \text{H}_2\text{O}$) and then overlies the solution with concentrated aqueous ammonia. When examining a suspected case of acetonuria, the organic acid invariably used was acetic acid.

When one comes to apply the Le Nobel test to the detection of acetonuria, one meets several anomalies which, so far, do not seem to have been put on record. The difficulties met with may be summarized as follows:—

(1) Solutions of acetone in water and in urine of concentrations similar to those occurring in cases of acetonuria do not give the test as distinctly as the natural cases.

(2) If some samples of urine which give a marked response to the Le Nobel test be distilled with acids, the test given by the distillate, where the acetone is presumably ten to twenty times more concentrated than in the original urine, is usually much less marked. It was a knowledge of these anomalies which attracted the attention of the authors to this subject.

In a paper on the output of acetone under physiological and pathological conditions, Engel⁵ determined the percentages excreted in various diseases and under varied conditions of diet. The acetone was determined by the Messenger-Huppert method, thus the figures given include acetone from aceto-acetic acid as well as free acetone, and are consequently too high. The highest amount of acetone found was 0.076 per cent. The authors, however, have found a much higher percentage of acetone in one case of severe 'diabetes mellitus,' accompanied by 'acidosis.' The percentage of acetone in the twenty-four hour sample, the day preceding death, was 0.134. This was determined by the Messenger-Huppert method. The percentage of free acetone as determined by the Folin method was 0.057. If a solution of 0.134 per cent. acetone be made in water or in urine and the Le Nobel test applied to it, a light purple coloration results at the end of about three minutes, while a solution of 0.057 per cent. acetone will only give a very faint reddish-violet ring at the end of about twenty minutes, a coloration which might easily be overlooked. The 'acidosis' case in question gave a strongly positive Le Nobel test at the end of about a minute. Thus, in this case, the test as given by solutions of acetone in water or in urine of a concentration the same as that occurring in a natural case of acidosis is not as distinct as in the natural urine.

In cases where the Le Nobel test is doubtful, it is always recom-

mended to acidify the urine with either sulphuric or acetic acid and distil, usually taking about 100 c.c. of urine and collecting from 5 to 10 c.c. of distillate, and then to apply the test to the distillate. Such a concentration of acetone would be expected to lead to an intensification of the colour reaction with sodium nitroprusside. In the acidosis case previously mentioned, and also in a second case examined by the authors, such an intensification did not occur. In the first case, the Le Nobel reaction was much weaker in the distillate than in the original urine, while in the second it had disappeared entirely.

It is evident from a consideration of these facts that there must be present in certain cases of acetonuria some constituent or constituents which either of themselves respond to the Le Nobel test or act as intensifiers to the reaction with acetone. Such substances might be ammonium salts, hydroxy butyric acid and aceto-acetic acid. From Rothera's experiments³, it might be expected that the increased excretion of ammonium salts which occurs in acidosis would account for the increased delicacy of the Le Nobel test when applied to a natural sample of acetonuria. That this is not the explanation is easily proved in the following manner. Fifty cubic centimetres of urine, which gave a strong Le Nobel reaction, were acidified with oxalic acid, and gently distilled, the first five cubic centimetres of distillate being collected. The distillate gave a weaker reaction than the original urine, and the residual urine gave a very faint reaction. A second specimen of the urine was then distilled under exactly similar conditions and the distillate added to the residual urine. This mixture gave a very faint Le Nobel reaction, much less intense than the original, thus showing that ammonium salts in the urine were not present in sufficient amount to greatly increase the delicacy of the Le Nobel reaction with acetone, and also that the substance to which the intensifying action was due was destroyed by heat. This was confirmed by boiling a sample of the urine, acidified with oxalic, acetic or sulphuric acid, under a reflux condenser for fifteen minutes, when it was found that an application of the Le Nobel reaction gave a negative result. As the specimen in question contained aceto-acetic acid, the urine at the end of the experiment would contain more acetone than at the beginning, and yet the Le Nobel test failed to show its presence. This pointed to the fact that it was the presence of aceto-acetic acid in the urine which caused the marked Le Nobel reaction. Aceto-acetic acid, however, is stated in the literature to give the following colorations:—

$\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}$ Alkali = Reddish-brown, unchanged by organic acids or metaphosphoric acid.⁶

$\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}$ Ammonia = Orange-red.

„ „ Solid ammonium salt.

Orange-red.

„ „ KOH = Orange-red, unchanged by acidification by phosphoric acid.⁷

No mention has been found of a violet or purple coloration such as it produced in the application of the Le Nobel reaction to aceto-aceturia. That the violet coloration given by the Le Nobel reaction with aceto-aceturia is due to the aceto-acetic acting independently of the acetone is evident from the following experiment. A natural sample of urine containing aceto-acetic acid was acidified with oxalic acid, saturated with sodium chloride and all the free acetone removed by aspiration by means of a current of air, as in the Folin method of estimating acetone. This was continued for an hour. At the end of that time the free acetone left in the urine was determined by the Folin method and found to be nil. The acetone-free urine, however, still gave a strong Le Nobel reaction, almost undiminished in intensity, which reaction became negative if the acetone-free urine was boiled under a reflux condenser. A solution of aceto-acetic acid was next prepared in the following manner: Thirteen (13) grams of ethyl aceto-acetate were added to a solution of six (6) grams of potassium hydroxide in 240 c.c. of water, the mixture shaken and allowed to remain at room temperature for twenty-four hours. It was then diluted with water to 1,000 c.c., and this solution was used as a solution of aceto-acetic acid. It contains also some unhydrolysed ethyl aceto-acetate and some acetone. If the Le Nobel reaction be applied to this solution, it will be found to give a violet coloration, indistinguishable from that given by urine containing aceto-acetic acid. If the acidification with acetic acid be omitted as in the Jackson-Taylor modification, the coloration appears at the end of a few seconds and is a distinctly redder shade of violet. If the solution is acidified and boiled under a reflux condenser, the test becomes negative; an acetone-free solution, obtained by removing the acetone by a current of air as in the Folin method of estimation of acetone, gives the test with undiminished intensity. Thus there is no doubt in the minds of the authors that the intense violet colorations obtained by the application of the Le Nobel test to urine are due to aceto-acetic acid, and not to acetone. This diminishes still further the value of this test as a

test for acetone alone. The authors, however, are at a loss to explain the results obtained by Rothera⁷, who, by the addition of sodium nitroprusside and ammonia to aceto-acetic acid, obtained an orange-red coloration, a colour similar to that given by ethyl aceto-acetate. If a few drops of our solution of aceto-acetic acid be added to normal urine, such a urine behaves exactly as the urine of a case of aceto-aceturia.

The point next investigated was the influence of other substances upon the test, which were likely to occur in urine, and the influence of acids, other than acetic. The following table shows quite clearly that the test is unaffected by the ordinary constituents of acidosis or diabetic urine.

Substance	Result	Remarks
Creatinin	—	No appreciable effect on coloration
Glucose	—	No appreciable effect on coloration
NaH ₂ PO ₄	—	Coloration as with acetic acid
β Oxybutyric acid	—	Coloration as with acetic acid
Formic acid	—	Coloration as with acetic acid
Oxalic acid	—	Coloration as with acetic acid
Sulphuric acid	—	Coloration not so intense as with acetic acid
Hydrochloric acid	—	Coloration not so intense as with acetic acid

In the first two cases the substances were added to the solution of aceto-acetic acid and the Le Nobel test applied as usual. In the other cases the acids named were used to replace acetic acid in the Le Nobel test. It next became important to compare the delicacy of this test for aceto-acetic acid with that of ferric chloride. The tests were carried out as follows: The urine containing aceto-acetic acid was rendered acetone-free by the Folin method, and the aceto-acetic acid then determined by the Messenger-Huppert method. A sample of the acetone-free urine was then tested by the ferric chloride test and by the Le Nobel at varying dilutions, and the dilutions noted at which the urines just failed to respond to the tests. The dilutions were performed with normal urine. Two cases were examined.

A. Percentage aceto-acetic acid = 0·139.

„ free acetone = nil.

Dilution	1 part aceto-acetic acid in	Le Nobel test	Fe ₂ Cl ₆ test
1	720	+	+
1/5	3,600	+	+
1/10	7,200	+	—
1/20	14,400	+	—
1/40	28,800	+ (very faint)	—
1/60	43,200	—	—

B. Percentage aceto-acetic acid = 0.0273.

„ free acetone = nil.

Dilution	1 part aceto-acetic acid in	Le Nobel test	Fe ₂ Cl ₆ test
1	3,663	+	+
1/2	7,326	+	—
1/4	14,652	+	—
1/8	29,304	+ (very faint)	—
1/12	43,956	—	—

It will thus be seen that in both the cases examined the limit of detection of aceto-acetic acid in urine by the Le Nobel test is about 1 part in 30,000, whereas the ferric chloride test fails to show aceto-acetic acid when present 1 part in 7,000. The Le Nobel test is thus at least four times as delicate as the ferric chloride test, and the authors feel quite confident in recommending its use as a test for aceto-acetic acid in urine in place of the ferric chloride test.

The delicacy of the test in aqueous solution cannot be easily determined, and the figures obtained would not, in our opinion, be of sufficient interest to justify the extremely complicated processes which would have to be undertaken to obtain pure aceto-acetic acid. As a rough indication, however, a sample of our solution of aceto-acetic acid was rendered acetone-free and the aceto-acetic acid determined by the Messenger-Huppert method, and the test applied at varying dilutions. The experiment indicated that the limit of detection of aceto-acetic acid in aqueous solution by the Le Nobel test is about 1 part in 80,000. The error consists in the presence in the solution of ethyl aceto-acetate, which would make the percentage of aceto-acetic acid appear too high. The limit of detection is thus greater than the actual experimental figures.

REFERENCES

1. Le Nobel, *Archiv. für exper. Path.*, XVIII, p. 10, 1884.
2. Jackson-Taylor, *Lancet*, I, p. 805, 1907.
3. Rothera, *Journ. Physiol.*, XXXVII, p. 491, 1908.
4. Saxe, *Examination of Urine*, Sec. Ed., p. 168, 1909.
5. Engel, *Zeit. für klin. Med.*, XX, p. 514, 1892.
6. Bitto, *Ann.*, CCLXVII, p. 867, 1892.
7. Rothera, *Journ. Physiol.*, XXXVII, p. 492, 1908.