CXCIX. CRITICISM OF SHUTE'S SERUM TEST FOR VITAMIN E DEFICIENCY

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IN 1935 Shute described a simple test which, he claimed, showed that the blood of aborting women contains a factor which inhibits proteolytic digestion. He was inclined to relate this finding to the actual abortion by postulating that the factor in question was responsible for increasing the resistance of the uterine walls to penetration by the placental villi [Shute, 1935, 1]. Investigating the matter further [Shute, 1935, 2] he found that the substance responsible for this effect could be removed from sera by chloroform extraction. From his observation that a normal rate of "proteolysis" was shown by sera withdrawn a few days after abortion, he was led to think that the placenta might be the source of the factor in question. Suspecting a fat-soluble substance in placenta he made experiments with certain sex hormones and found that the addition of oestrogens to normal sera produced an increased antiproteolytic action similar to that shown by the sera from cases of abortion.

Another important link in the chain of evidence which led him to put forward a theory regarding the physiological role of vitamin E was his observation that the sera of rats which were known to be in a condition of marked vitamin E deprivation showed similar behaviour to those of aborting women or normal sera to which oestrogens had been added [Shute, 1936]. Moreover, the response to the test became similar to that of normal sera after the vitamin E-deficiency condition had been successfully treated with wheat germ oil. On the basis of this experimental work he has put forward the view that "vitamin E and oestrin, or a substance much like it, exist in a sort of equilibrium during pregnancy. If there is too much of the oestrin-like substance the pregnancy is interrupted."

We are not here concerned with physiological criticisms of his theory; these are the subject of another communication [Drummond *et al.* 1939]. We are now reporting our inability to obtain results similar to his when comparative experiments were made with sera of normal rats, of others in a condition of vitamin E deficiency and of a group that had been fed on a diet containing a large excess of vitamin E.

In the first place, there are a number of points which appear unsatisfactory in his description of the test [Shute, 1935, 1; 1938]. Briefly, the test consists in placing 0.5 ml. of serum diluted with 3.5 ml. of a borax-sodium dihydrogen phosphate buffer of pH 9 in each of two tubes, one of which is heated to $55 \pm 5^\circ$ for 30 min. At the end of this time the tubes are cooled, a known amount of trypsin solution is added in equal amounts to each and the contents are incubated at a temperature between 37 and 42°. At intervals of 10 min. 0.5 ml. is withdrawn from each tube and, after dilution with 5 ml. of distilled water, is titrated with *N*/70 or *N*/100 NaOH to pH 9, using phenolphthalein as indicator. The titrations are regarded as a measure of increase in acidity due to "proteolysis", but Shute admits that

under the conditions of his test there is no increase in the formaldehyde titration. It is difficult to reconcile the view that a change in the reaction of the contents of the tube is due to "proteolysis", presumably due to the trypsin, with his statement that the "digestion" takes place over a range of temperature from 20 to 90°. His object in heating one tube to 55° for $\frac{1}{2}$ hr. is not, as one would expect, to have a control in which the antitryptic factor is inactivated, but, rather surprisingly, because he sometimes finds that the preliminary heat treatment gives a more definite result in the final test. He does not offer any explanation of this curious observation. Equally disturbing is his view that the digestion of the serum-buffer mixtures by trypsin under these conditions takes place according to an "all-or-none" principle, whilst the fact that he sometimes finds a "reversal" of his titration values in the later stages of the test is another observation which is difficult to explain. Of this "reversal" he himself remarks that it "seems to have eluded satisfactory explanation".

EXPERIMENTAL

Our first attempts to employ Shute's test as a means of differentiating between the sera of vitamin E-deficient and normal rats failed completely, although we followed as carefully as possible the details of the method as set out in his papers.

Fortunately, at this stage of the investigation we had the opportunity of availing ourselves of Dr Shute's personal advice and of seeing him carry out a demonstration of his method. For this willing co-operation we are greatly

Fig. 1.

indebted. In the course of discussion he agreed that the end-point of the titrations, which in his publications are stated to be "easily read", would be improved by the choice of a more appropriate indicator than phenolphthalein, it being borne in mind that a strong buffer mixture is being titrated from pH 8 to 9.

A much more suitable indicator was found in one of the methyl-red-methylene blue-phenolphthalein achromatic indicators described by Smith [1930], the one chosen showing a neutral grey tint at pH 9 and being sensitive to changes of

Fig. 2.

0.05 pH. With this indicator we have carried out a considerable number of experiments, following in all other respects the procedure laid down by Shute. The sera examined have been taken from rats (*a*) fed on an adequate, normal ration (N); (*b*) fed for from 9 to 12 months on a vitamin E-deficient diet (-E); and (*c*) fed for 6-9 months on a normal ration supplemented with 12% of wheat germ oil (+E).

The results fail to show any consistent differences. Typical titration curves are shown in Fig. 1.

These experiments were all carried out with a trypsin solution prepared according to Shute's method, namely 1 ml. of a 1% "Difco" trypsin in 75% glycerol diluted to 500 ml. with water. It was thought desirable to repeat the experiment with a stronger solution of the enzyme. Accordingly, a similar series of tests were made employing the stock 1% trypsin and dilutions of 1:10 and 1:100. The titration curves were exactly similar to those obtained when the greater dilution of 1:500 had been used. Much the same types of curves were obtained when the trypsin was omitted (Fig. 2).

This made us suspect that the test had little significance from the standpoint of vitamin E, but it seemed desirable, nevertheless, to repeat the experiment in which Shute found that oestrogens added to normal sera produced an inhibition of "proteolysis". Following closely his procedure we added 8-80 R.U. of "Theelin" (Parke Davis) per ml. to normal rat sera. The titration curves were not materially different from those of the controls (Fig. 3).

Fig. 3.

The general impression we formed from these experiments was that any small differences encountered were more probably due to absorption of CO_2 than to interaction of the trypsin and the serum. In order to clear up this point we carried out a test on a larger scale using four times the quantities employed in previous tests and taking special precautions against contact with air. We found no significant change in the reaction of the mixture during a period of 2 hr. 20 min., either when normal serum or that from a vitamin E-deficient animal was employed.

Parallel with this series of tests we carried out a number of experiments to ascertain whether there was a real increase in antitryptic action of the serum in vitamin E deficiency. For this purpose we employed a viscosimetric method. Mixtures were prepared with 2.4 ml. of 3.5% solution of gelatin in Clark and Lub's buffer pH 8, 0.1 ml. of serum and 0.5 ml. of *liquor trypticus* (B.D.H.). These were placed in an Ostwald viscosimeter in a thermostat at 40° and records were taken of the viscosity of the fluid over a period of 2 hr. In each experiment a comparison was made between a sample of serum which had been heated to 55° for $\frac{1}{2}$ hr. and an unheated specimen. The curves seen in Fig. 4 show that there is no significant difference between the rates of digestion of gelatin by trypsin in

the presence of normal rat serum and of serum taken from rats either deprived of vitamin E or fed liberally on wheat germ oil. The curves for the heated sera show exactly the behaviour that would be expected from the inactivation of the anti-tryptic factor. Another series of tests with serum to which had been added

Fig. 4.

Fig. 5. • Normal serum + water.
+ Normal serum + 80 R.U. per ml. "Theelin".

8–80 R.U. of "Theelin" (Parke Davis) per ml. failed to reveal any suppression of tryptic activity as a result of the addition. The curves are superimposable (Fig. 5).

In all these experiments the course of the digestion was quite normal and there was not the slightest indication of any "all-or-none" effect such as Shute claims to have observed.

Although we are inclined to regard the different responses to the test which Shute describes as artefacts we prefer to express no opinion on the possible value of the test as an aid to diagnosis in gynaecological practice [cf. Cooper, 1939]. We are satisfied that vitamin E has no such influence as Shute describes on the "antiproteolytic power" of the serum of rats.

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