Studies on the Metabolism of Semen

9. EFFECT OF SURFACE ACTIVE AGENTS WITH SPECIAL REFERENCE TO THE OXIDATION OF SUCCINATE BY SPERMATOZOA

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In the course of investigations on the respiration and the cytochrome-succinic oxidase system of ram spermatozoa (Mann, 1945, 1951*a, b*; Humphrey & Mann, 1949) two phenomena have been observed: (i) it was found that as the result of freezing and subsequent thawing, or after prolonged storage of semen *in vitro*, there was a leakage of cytochrome *c* from the spermatozoa into the surrounding fluid; (ii) it was noticed that, whereas the oxygen consumption of suspensions of fresh, washed spermatozoa was but slightly increased by the addition of succinate, sperm cells which have become senescent or have undergone some damage with resulting lowered motility and respiration, responded to the addition of succinate by a markedly increased uptake of oxygen.

We have now found that it is possible to produce the stimulating effect of succinate on the rate of sperm respiration, characteristic of senescent or damaged spermatozoa, by exposing the sperm cells to the action of certain ionic and non-ionic detergents. Under the influence of the surface-active agents, motility, fructolysis and respiration were suppressed, but it was possible to raise the rate of oxygen consumption of the detergent-treated sperm by the addition of sodium succinate.

EXPERIMENTAL

Spermatozoa. The material used in the present investigation consisted of ram semen collected by means of an artificial vagina (Walton, 1946).

Oxygen uptake and fructolysis. In whole semen, respiration and fructolysis were determined at 37°, after a tenfold dilution of the semen with 'Ringer-phosphate-fructose' which had the following composition: 100 ml. 0.9% (w/v) NaCl + 4 ml. 1.15% (w/v) KCl + 1 ml. 2.11% (w/v) KH_2PO_4 + 1 ml. 3.82% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ + 21 ml. 0.05% fructose solution in 0.25M phosphate buffer, pH 7.4 (20 ml. 3.55% Na_2HPO_4 + 1 ml. N-HCl). From the semen thus diluted, 2 ml. were used for the manometric determination of oxygen uptake in a Barcroft differential manometer, and 6 ml. were placed in an incubation tube (0.8 cm. in diameter), from which 1 ml. samples were withdrawn at 30 min.

intervals, deproteinized, and used for fructose determination (Mann, 1946, 1948*a, b*). Deproteinization was carried out in centrifuge tubes by diluting each 1 ml. sample with 3 ml. water and adding 0.5 ml. 5% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 ml. 0.3N-Ba(OH)₂; in the centrifuged extract the colour reaction was developed according to Roe (1934): to 2 ml. of the extract were added 2 ml. 0.1% ethanolic solution of resorcinol and 6 ml. 10N-HCl, the mixture was heated 10 min. at 85° and, after cooling, the colorimetric determination was carried out in the Hilger Spekker Absorptiometer (1 cm. cell; Spectrum Blue-green Ilford filter no. 603, transmission 470–520 mμ.).

In some experiments, washed sperm suspensions were used instead of whole semen. For this purpose, semen diluted ten times with Ringer-phosphate-fructose, was centrifuged for 10 min. at 1200 *g* and supernatant fluid was removed; the sperm were resuspended by gentle mixing with a small amount of Ringer-phosphate or Ringer-phosphate-fructose, and brought up to the volume of ten-times-diluted semen.

Effect of succinic acid. The effect of succinic acid on the respiration of spermatozoa was followed manometrically, in the presence of 6 mg. sodium succinate. When cytochrome *c* has been used, the amount added was 0.2 ml. of a 1% solution; cytochrome *c* was prepared from heart muscle by the method of Keilin & Hartree (1945). For manometric experiments, spermatozoa were used either as semen suitably diluted with Ringer-phosphate-fructose or Ringer-phosphate, or as a suspension of washed cells.

Detection of cytochrome c. The leakage of cytochrome *c* from the spermatozoa into the surrounding medium was observed spectroscopically, in the Zeiss Microspectroscope, by a method outlined in an earlier paper (Mann, 1951*a*).

Surface-active agents. The following substances were used. Hexadecyltrimethylammonium bromide, generally called cetyltrimethylammonium bromide (CTAB), mol.wt. 364; 2-phenoxyethanol (phenoxetol), mol.wt. 138; 'Lubrol W' (a cetyl alcohol/ethylene oxide condensation product manufactured by Imperial Chemical Industries Ltd.), sodium dodecyl sulphate, mol.wt. 288; dodecyl amine hydrochloride, mol.wt. 222; saponin; 'Teepol' (a wetting agent manufactured by the Shell Co. Ltd.), and sodium taurocholate, mol.wt. 538.

RESULTS

Spermicidal effect of surface-active agents

Of all the substances which have been tested, cetyltrimethylammonium bromide (CTAB), a cationic detergent, had the strongest influence on fructo-

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lysis and motility. The response of sperm to CTAB varied somewhat from one sample of ram semen to another; 0.0003–0.0006M CTAB was enough to reduce the rate of fructolysis by 50% and concentrations from 0.0008 to 0.0012M suppressed completely both motility and fructolysis. Sodium dodecyl sulphate used in 0.0005–0.001M concentration produced a 50% and in 0.0015M, a complete inhibition of fructolysis; motility examined 30 min. after the addition of 0.0015M dodecyl sulphate to semen diluted with Ringer-phosphate-fructose was nil. Under similar conditions, 2-phenoxyethanol (phenoxetol) acted in a concentration of 0.02–0.03M. The effects of these three substances on sperm fructolysis are illustrated in Fig. 1.

The oxygen uptake of dilute semen was also affected by these substances but the full effect on respiration usually became obvious somewhat later than on fructolysis.

Of practical interest is the inhibition of sperm motility, fructolysis and respiration by 'Teepol', in view of the common use of this wetting agent for cleaning of glassware. In a dilution 1:750, 'Teepol' produced a 25% inhibition of both fructolysis and respiration; at 1:300 it completely abolished sperm metabolism and motility.

The least active of all substances tested was saponin, of which 0.1% was required to achieve complete inhibition.

Fig. 1. Effect of hexadecyltrimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS) and 2-phenoxyethanol (phenoxetol) on sperm fructolysis; ram semen diluted with 9 vol. Ringer-phosphate-fructose and incubated at 37°; A, no additions; B, 0.0005M CTAB; C, 0.001M CTAB; D, 0.001M SDS; E, 0.01M phenoxetol; F, 0.03M phenoxetol.

Several attempts were made to reverse the inhibition caused by CTAB, dodecyl sulphate and phenoxetol, by washing the detergent-treated sperm with fresh Ringer-phosphate-fructose. However, the inhibition proved to be irreversible in all instances so far examined.

Leakage of cytochrome c

The spermicidal action of all the surface-active agents used in this study, was accompanied by a leakage of cytochrome *c* from the spermatozoa into the external medium. The amount of extracellular cytochrome appeared to be roughly proportional to the concentration of the added agent and increased on incubation. In every case where motility was brought to a standstill by a surface-active agent, the band of cytochrome *c* at 550 mμ. could readily be detected after centrifugation in the sperm-free supernatant fluid, either directly, or better still, after reduction with sodium dithionite.

Effect of sodium succinate on sperm respiration

Fresh semen or once-washed spermatozoa responded to the addition of succinate by a slightly increased rate of oxygen uptake. The response was more pronounced if succinate was used together with cytochrome *c*; it is not improbable, however, that the observed increase was actually due to the moribund cells rather than the intact spermatozoa. On the other hand, a high percentage increase in oxygen consumption was observed when succinate, either alone or with cytochrome *c*, was added to sperm suspensions which had lost their original respiratory activity as a result of treatment with surface-active agents. In order to demonstrate this effect clearly it is essential to add the correct amount of detergent and to avoid an excess. This point is best illustrated by experiments with CTAB. This compound, when present in excessive concentrations, i.e. above 0.002M, led not only to a loss of motility and respiratory as well as fructolytic power, but abolished also the ability of sperm to respond to added succinate; this is not unexpected as it is known that CTAB acts in high concentrations as an inhibitor of the succinic oxidase system (Hockenhull, 1948). When, however, CTAB was present in smaller concentrations, the spermatozoa were deprived of motility as well as of fructolytic and respiratory ability, but became at the same time capable of responding to added succinate with an increased oxygen uptake.

The most convincing results were obtained when semen was first incubated with CTAB alone, then the spermatozoa were separated by centrifugation, suspended in fresh Ringer-phosphate, and finally brought into contact with succinate. The procedure in these experiments was as follows.

Fig. 2. Effect of succinate on the respiration of spermatozoa obtained by centrifugation of (a) semen incubated with Ringer-phosphate, and (b) semen incubated with Ringer-phosphate containing 0.002M CTAB; 2 ml. sperm suspension corresponds to 0.2 ml. semen; for further experimental details see text; ●—●, no addition; ○—○, cytochrome c; ○—○, succinate; x—x, succinate + cytochrome c.

Whole semen (2 ml.) was used; 1 ml. was diluted with an equal volume of Ringer-phosphate, and 1 ml. with an equal volume of a 0.002 M solution of CTAB in Ringer-phosphate. Both samples were incubated in centrifuge tubes for 20 min. at 37° and then centrifuged for 10 min. at 2500 g. The supernatant fluids were used for spectroscopic examination, after addition of sodium dithionite. The two centrifuged sperm samples were suspended in Ringer-phosphate and diluted to 10 ml. From each suspension, four 2 ml. samples were pipetted into manometer vessels and the oxygen uptake was recorded (i) in the absence of any additions, (ii) in the presence of succinate, (iii) in the presence of cytochrome c, and (iv) with succinate and cytochrome c added together.

The spectroscopic examination of the supernatant fluid obtained by centrifugation of semen untreated by CTAB, showed a barely detectable band of cytochrome c; this slight leakage of cytochrome c being due to high-speed centrifugation itself. The supernatant from the CTAB-treated semen on the other hand, was distinctly pink in colour and showed a very strong band of cytochrome c. The measurements of respiratory activity in the untreated and CTAB-treated sperm are recorded in Fig. 2 from which it can be seen that CTAB in the concentration used reduced endogenous respiration and that succinate, particularly if added together with cytochrome c, brought about a higher percentage increase of the oxygen consumption in detergent-treated than in the untreated spermatozoa. Although 'respiring' vigorously in the presence of succinate and cytochrome, the detergent-treated spermatozoa were nevertheless completely immotile and devoid of fructolytic activity.

The oxidation of succinate by the detergent-

Fig. 3. Effect of malonate on the respiratory activity of detergent-treated spermatozoa; ram semen incubated in the presence of 0.001 M CTAB, 20 min., 37°; spermatozoa separated by centrifugation and suspended in Ringer-phosphate; 2 ml. sperm suspension correspond to 0.2 ml. semen; A, no additions; B, with succinate; C, with succinate and cytochrome c; D, with 0.01 M sodium malonate, either with or without succinate; E, with 0.01 M sodium malonate plus succinate plus cytochrome c.

treated spermatozoa was counteracted effectively by malonate (Fig. 3), a fact which is in agreement with the property of malonate to act as a competitive inhibitor of succinic dehydrogenase.

Experiments analogous to those with CTAB, have also been carried out with other substances. In every case where incubation with a given substance rendered the sperm either completely or partly immotile, the reduced rate of oxygen uptake could be increased by the addition of succinate, either alone or with cytochrome *c*. In order to demonstrate clearly the effect of succinate, the following concentrations were used: 0.001M sodium dodecyl sulphate, 0.002M dodecylamine hydrochloride, 0.005–0.01M sodium taurocholate, 0.01M phenoxetol, 0.1% 'Lubrol' or saponin.

A series of experiments was also carried out to examine the possibility that substrates other than succinate might be able to increase the respiration of detergent-treated sperm cells. Two groups of substrates were examined: one comprised various substances known to stimulate the normal respiration of intact spermatozoa, such as fructose, glucose, lactate, pyruvate and acetate; the other was represented by substances which ordinarily are unable to bring about a stimulation of respiration, e.g. ethanol, glycerol and citrate. However, such effects as were obtained, were either small or negligible.

Finally, some experiments were performed to find whether the succinic oxidase activity of detergent-treated spermatozoa was confined to the cells or whether it passed partly also into the extracellular medium together with cytochrome *c*. So far, all attempts to demonstrate succinic oxidase activity in the sperm-surrounding fluid have proved negative.

Structural changes in spermatozoa

Detergent-treated and untreated spermatozoa were examined by Dr J. R. G. Bradfield in the electron microscope, with and without metal shadowing. In contrast to the striking biochemical changes, the structural abnormalities observed after incubation of semen with 0.001M CTAB were exceedingly small. However, incubation with 0.0025M CTAB caused marked disruption of sperm-head membranes in about 50% of spermatozoa and fraying of the tail-ends in about 75%; after treatment with 0.0125M CTAB, about 25% of the middle-pieces became distinctly more 'fuzzy' and cast much shorter shadows than did middle-pieces of untreated spermatozoa. Sections of spermatozoa were not prepared.

DISCUSSION

The literature on the question of whether or not succinate supports the respiration of mammalian spermatozoa is full of conflicting statements (for review see MacLeod, 1943; Mann, 1949). Our own experience, based mainly on observations with ram

sperm, has been that succinate, unlike fructose and the products of fructolysis, is incapable of supporting the respiration of normal intact spermatozoa, but can increase considerably the oxygen uptake of senescent and already partly immotile spermatozoa, without, however, improving their motility. The present investigation demonstrates that certain surface-active substances (i) are spermicidal, in the sense that they inhibit motility, fructolysis and respiration of spermatozoa, (ii) induce a leakage of cytochrome *c*, and (iii) enable the cells, if used in suitable concentrations, to respond to added succinate by an increased oxygen uptake. All these effects are presumably brought about by some change in the permeability of the sperm cell.

There is considerable evidence to show that under physiological conditions, the surface of the spermatozoon is protected by a lipid-containing outer layer, the so-called lipid capsule or 'manteau lipidique', and it is probable that the surface-active substances act directly on the lipoprotein and lipid constituents of that capsule. Thus, the mechanism of the spermicidal activity of detergents on the sperm may be not altogether different from that underlying the haemolytic action of certain surface-active compounds on erythrocytes and the bactericidal effects of such agents on various micro-organisms (cf. Schwartz & Perry, 1949; Salton, 1951; Hugo & Street, 1952; Pethica & Schulman, 1953). Our findings concerning the behaviour of spermatozoa are of special interest in view of the exceptional resistance of these cells to plasmolytic agents in general. Moreover, our results obtained with detergent-treated spermatozoa to which succinate has been added, indicate that an increase in the oxygen consumption of cells *in vitro* need not coincide with the maintenance of their 'vital capacity'. As a matter of fact, we have shown that an increased oxygen uptake of detergent-treated spermatozoa in response to added succinate, was the direct outcome of far-reaching cellular disorganization. The precise mechanism of the phenomena outlined above requires further study, especially as regards the catalytic activity of the sperm cytochrome-succinic oxidase system.

SUMMARY

1. Certain surface-active compounds, including ionic and non-ionic detergents, were found to inhibit motility, fructolysis and respiration of spermatozoa. Among the substances examined, hexadecyl (cetyl)-trimethylammonium bromide and dodecyl sulphate were particularly effective. Their spermicidal power was assessed quantitatively by measuring the inhibitory influence on fructolysis and respiration.

2. Hexadecyl(cetyl)trimethylammonium bromide, 0.0003–0.0006M, inhibited the fructolysis of

ram spermatozoa by 50%; 0.0008–0.0012M was required for complete inhibition of fructolysis and motility. Among similarly active substances were dodecyl sulphate, 2-phenoxyethanol, dodecylamine hydrochloride, sodium taurocholate, 'Lubrol' and 'Teepol'.

3. The surface-active compounds induced a change in the permeability of spermatozoa as indicated by the diffusion of cytochrome *c* from the cells into the surrounding fluid.

4. When succinate was added to detergent-treated immotile spermatozoa, the percentage increase of the oxygen uptake was much greater than in intact motile sperm; thus, the stimulating effect of succinate on the respiration of spermatozoa was an indication of a moribund cell population.

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REFERENCES

- Hockenhull, D. (1948). *Nature, Lond.*, **162**, 850.
 Hugo, W. B. & Street, H. E. (1952). *J. gen. Microbiol.* **6**, 90.
 Humphrey, G. F. & Mann, T. (1949). *Biochem. J.* **44**, 97.
 Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
 MacLeod, J. (1943). *Annu. Rev. Physiol.* **5**, 399.
 Mann, T. (1945). *Biochem. J.* **39**, 451.
 Mann, T. (1946). *Biochem. J.* **40**, 481.
 Mann, T. (1948a). *J. agric. Sci.* **38**, 323.
 Mann, T. (1948b). *Lancet*, **254**, 446.
 Mann, T. (1949). *Advanc. Enzymol.* **9**, 329.
 Mann, T. (1951a). *Biochem. J.* **48**, 386.
 Mann, T. (1951b). *Biochem. Soc. Symp.* **7**, 11.
 Pethica, B. A. & Schulman, J. H. (1953). *Biochem. J.* **53**, 177.
 Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
 Salton, M. R. J. (1951). *J. gen. Microbiol.* **5**, 391.
 Schwartz, A. M. & Perry, J. W. (1949). *Surface Active Agents*. New York and London: Interscience Publishers.
 Walton, A. (1946). *Notes on Artificial Insemination of Sheep, Cattle and Horses*. London: Holborn Surgical Instrument Co.

Two Methods for the Determination of Glycogen in Liver

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Several methods for the determination of glycogen in tissues have been described (Good, Kramer & Somogyi, 1933; Morris, 1948; Boettiger, 1946; Seifter, Dayton, Novic & Muntwyler, 1950; van Wagendonk, Simonsen & Hackett, 1946; van der Kleij, 1951). As we needed a simple technique for the study of the glycogenetic properties of adrenal steroids a survey was made of several techniques often used (see Table 1). For serial determinations the methods of Seifter *et al.* and van der Kleij are attractive, in part because the time-consuming separation of the glycogen by precipitation with alcohol is avoided. However, in Seifter's method this separation can only be omitted if the glycogen content of the liver exceeds 1%, while van der Kleij's technique is not specific for glycogen, since substances like glucose and glucose phosphates, which are known to occur in liver, are also estimated.

The results submitted in this paper show that the process of extracting the glycogen with trichloroacetic acid as proposed by van der Kleij can be used in combination with colorimetric techniques for the estimation of glycogen as described by van Wagendonk *et al.* (with iodine) and Morris (with

anthrone). In this way two methods were developed, which can be used for the serial determination of glycogen in liver. In particular the application of an iodine reagent proved to be very convenient for routine determinations of glycogen. In our hands, the use of this method instead of the more elaborate techniques of Pflüger (1905) and others led to a considerable saving of time and materials. Satisfactory results have been obtained, despite some limitations of the method, which will be discussed in detail.

REAGENTS AND METHODS

Extraction of glycogen from liver with trichloroacetic acid

If the iodine reagent is used for the determination of glycogen in the extracts the concentration of trichloroacetic acid (TCA) should not exceed the limits 4.9–5.1% (w/v). This is checked by titration with 0.1N alkali, and the solution adjusted if necessary by adding water or a concentrated solution of TCA.

Procedure. A 200 mg. sample of liver is weighed on a torsion balance and finely ground with 20 ml. of 5% TCA