The Dependence of the Effect of Azide, Amytal, and Rotenone on the Respiratory Rate Studied in Epididymal Bull Spermatozoa

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The concentrations of azide, amytal, and rotenone causing 50 % inhibition of endogenous and fructose supported respiration in epididymal bull spermatozoa have been determined. This concentration was found in every case to be inversely proportional to a power of the rate of the normal respiration.

There exist early observations^{24,21,22,14,11,27} to the effect that a higher rate of respiration enhances the efficiency of substances, like carbon monoxide and cyanide, that inhibit cellular respiration by the blocking of the terminal link of the carrier system. From a kinetic point of view this phenomenon was elucidated for CO by Klein and Runnström¹⁰. In the course of study of some metabolic properties of epididymal bull spermatozoa the present authors confirmed the results of Henle and Zittle,⁸ *viz.*, that the rate of respiration of this material is rather varying already without exogenous substrate, and can be furthermore increased by addition of fructose. This kind of mammalian cells seems thus suitable for studies on the relationship between the rate of respiration and the degree of inhibition caused by respiratory inhibitors. For inhibitors we have chosen such which develop their blocking effect at widely different concentrations, *viz.* azide, amytal, and rotenone. The first-mentioned substance was shown by Keilin and Hartree⁹ to block respiration by reacting with cytochrome oxidase. Neilands and Stumpf¹⁸ state that azide reacts with metalloporphyrins and metals, which implies a lower specificity. The blocking of respiration by amytal was thought at first to be localized between the flavin adenin dinucleotide (FAD) of diaphorase and Slater's factor⁴, but was later traced to some link between the reduced diphosphopyridine nucleotide and FAD². Rotenone has recently been shown^{12,13,20,5} to block a link in the electron transporting system situated at the diaphorase level. Neither amytal nor rotenone have any effect on the oxidation of succinate in isolated mitochondria.

The general aim of the present investigation was to determine at different levels of respiration the concentration of the inhibitors causing 50 % inhibition.

MATERIALS AND METHODS

Epididymal spermatozoa have been obtained according to Henle⁷ from slaughtered bulls of Swedish White and Red Breed aged 2–10 (chiefly 2–3) years. They were prepared from the testes within one hour after the death of the animal. The suspension was then stored at room temperature for about 2 h. Its density was brought to 150–200 millions of sperms per ml just before the start of the experiments. This density rather than that recommended by Henle and Zittle⁸, which was first tried, was found to give maximal rate of respiration. The medium was the phosphate Ringer's solution of Mann¹⁷. The pH of the sperm suspension was 7.1.

Respiration was measured by the direct Warburg method in cups of about 10 ml volume, provided with 1.5 ml suspension, 0.1 ml 0.17 M fructose (alt. Ringer's solution), 0.1 ml solution of the relevant inhibitor in varied concentration (alt. Ringer's solution), and 0.2 ml KOH. The fructose and inhibitor solutions were poured into the chief compartment after a period of equilibrium of 20 min. NaN_3 was dissolved in Ringer's solution. The final concentration varied between 2.5×10^{-4} and 2.5×10^{-1} M. Stock solutions of amytal according to Ref.⁴ were freshly prepared before use. We used final concentrations within the range 0.6×10^{-3} – 7.2×10^{-3} M. A stock solution of rotenone (1.27×10^{-3} M) in ethanol was diluted with Ringer's solution¹⁴. The final concentration of ethanol never exceeded 8.4×10^{-3} M, and was accordingly of no influence upon respiration. The final concentrations of rotenone fell within the range 6×10^{-9} – 6×10^{-5} M. The pH of all solutions of the inhibitors was brought to 7.1. The rate of rocking the manometers was 90 oscillations/min. Readings were taken every 10 min during one hour. The oxygen consumption is expressed in $\mu\text{l O}_2$ per 100 millions of sperms in one hour ($\mu\text{l O}_2 \times 10^{-8}$ sperms $\times \text{h}^{-1}$). The sperm density was obtained by countings in a Buerker chamber. All determinations of the respiratory rate were duplicated. The difference between duplicates never exceeded 2 %.

For each experiment the inhibition is plotted against the logarithm of the concentration of the inhibitor, the logarithm of the concentration giving 50 % inhibition ($C_{50\%}$) being interpolated.

EXPERIMENTS

From Fig. 1, curve a, can be seen that in the presence of fructose the relationship between inhibition and the concentration of azide is not a simple one. With increasing concentration of azide the inhibition at 2.5×10^{-3} M reaches a value not far from 50 %. Then follows an inconsiderable increase up to 2.5×10^{-2} M after which concentration the curve has a steeper course. In many experiments, however, the inhibition is lower at 2.5×10^{-2} than at 2.5×10^{-3} M as demonstrated in Fig. 1, curve b. Also the curves obtained with azide on endogenous respiration show a strange course, the extension of their initial (left) part being remarkable in combination with the great steepness of their main slope (cf. Fig. 1, curve c). At 2.5×10^{-1} M azide the inhibition is generally 80–90 %. At this concentration the osmotic pressure of the medium is already essentially increased. It is thus not possible to decide, whether azide can block respiration altogether or not.

The general course of the curves indicates that, especially at the higher rate of respiration induced by fructose (cf. Table 1), some reaction takes place, when the concentration of azide is raised above some critical value between 2.5×10^{-3} and 2.5×10^{-2} M that brings about an apparent diminution of the inhibition. The concentration of azide necessary to cause 50 % inhibition is

Fig. 1. Inhibition of respiration in epididymal spermatozoa as a function of $\log_{10}$ molar concentration of sodium azide. $\square$ exogenous, $\blacksquare$ endogenous respiration. Curves a and c obtained with the same sample of spermatozoa.

Fig. 2. Inhibition of respiration in epididymal spermatozoa as a function of $\log_{10}$ molar concentration of amytal. $\triangle$ exogenous, $\blacktriangle$ endogenous respiration. The curves in A obtained with the same, those in B with different samples of spermatozoa.

thus higher than could be expected from the low-concentration part of the curve. Different samples of semen show this phenomenon to a varying extent.

In the case of exogenous respiration our curves relating inhibition to concentration of amytal do not extend sufficiently far into the low-concentration range (Fig. 2). For endogenous respiration the curves again show a rather extended initial part which leads abruptly into the steeply rising part of the curve (Fig. 2). The concentration causing 50 % inhibition always falls within the steep part of the curve. Maximum inhibition attained in every single experiment takes place near 80 %. As several of our curves do not extend far enough to make possible the estimation of maximum inhibition we cannot carry out a desirable correction of the $C_{50\%}$ -values.

Table 1. Log $C_{50\%}$ and N.R. (normal respiration, $\mu\text{l O}_2 \times 10^{-8}$ spermatozoa $\times \text{h}^{-1}$) from experiments with azide, amytal, and rotenone. Experiments linked with brackets performed with the same sample of spermatozoa.

Azide			Amytal			Rotenone		
Fructose	log $C_{50\%}$	N.R.	Fructose	log $C_{50\%}$	N.R.	Fructose	log $C_{50\%}$	N.R.
{ -	-1.100	13.8	{ -	-2.900	21.3	{ -	-6.419	15.2
{ +	-1.550	27.5	{ +	-2.080	15.6	{ +	-6.350	18.5
{ -	-1.360	13.1	{ -	-3.910	26.4	{ -	-6.020	16.2
{ +	-1.560	28.1	{ +	-2.160	14.7	{ +	-7.520	27.2
{ -	-1.040	16.2	-	-2.350	16.5	{ -	-6.180	18.1
{ +	-1.360	26.9	-	-2.140	15.9	{ +	-7.839	26.9
+	-1.180	19.1	-	-2.150	13.6	{ -	-6.400	12.0
+	-1.175	19.3	-	-2.140	13.8	{ +	-8.018	36.8
+	-1.475	30.0	+	-2.939	21.7	{ -	-6.300	12.9
+	-1.330	28.6				{ +	-7.991	36.1

Fig. 3. Inhibition of respiration in epididymal spermatozoa as a function of $\log_{10}$ molar concentration of rotenone. $\circ$ exogenous, $\bullet$ endogenous respiration. Both curves obtained with the same sample of spermatozoa.

Fig. 4. $\log_{10}$ molar concentration of inhibitor giving 50% inhibition ($C_{50\%}$) plotted against $\log_{10}$ rate of normal respiration (N.R.). $\square$, $\blacksquare$, azide; $\triangle$, $\blacktriangle$, amytal; $\circ$, $\bullet$, rotenone; open marks exogenous, filled marks endogenous respiration.

Plotting inhibition of the endogenous respiration against concentration of rotenone (Fig. 3) gives curves which are more or less symmetrically S-shaped. With addition of glucose no sufficiently low concentrations of rotenone were used to give complete curves. In most experiments maximum inhibition was obtained already with 6.0×10^{-6} M. Nevertheless, in some experiments the concentration of rotenone was raised to 6.0×10^{-5} M. However, at slightly higher concentrations the rotenone "solutions" are not stable. The maximum varies between 80 and 100 %, and is on the whole somewhat higher for exogenous than for endogenous respiration. Here we are able to introduce corrections according to the level of maximum inhibition, the corrected $C_{50\%}$ -values being in each case referred to that part of the respiration which is suppressed at maximum inhibition. Also a second correction was tried by which the normal respiration was reduced in proportion to the maximum inhibition.

The $C_{50\%}$ -values for rotenone, only the uncorrected ones, and the corresponding rates of normal respiration (N.R.) have been collected in Table 1.

DISCUSSION

Starting from the hypothesis that the degree of inhibition depends upon the height of normal respiration $\log C_{50\%}$ is plotted against $\log$ (N.R.). For all three inhibitors this suggests a fairly good linear correlation (Fig. 4). A statistical treatment of the data is presented in Table 2.

In the experiments with rotenone there exists a high probability for correlation between $\log C_{50\%}$ and $\log$ (N.R.). This association is only insignificantly

improved by a correction of the $C_{50\%}$ -values in relation to the incomplete maximum inhibition (quotient of residual by total variance decreased from 0.201 to 0.165), but is then essentially impaired by a correction of the rate of the normal respiration also (quotient of residual by total variance 2.549). For this reason all corrected values have been omitted from Table 1. The curves relating inhibition to concentration of rotenone (Fig. 3) approach rather closely the symmetrical S-form of the ideal curve of reversible inhibition of a single enzyme. There is always a more or less pronounced shortening of the low-concentration part of the curves. This could depend on a seemingly non-reversible binding of rotenone to some other constituent than the inhibited enzyme, a constituent which would soon be saturated. In this connection the very low solubility of rotenone in water and its high solubility in lipid solvents should be stressed. In our experimental samples it occurs as a suspension of micelles.

Whereas 6×10^{-7} M rotenone inhibits 93 % of the respiration of isolated mouse liver mitochondria with pyruvate as a substrate¹⁴, 6×10^{-6} to 6×10^{-5} M is necessary in order to produce maximum inhibition in epididymal spermatozoa.

Our incomplete curves of exogenous respiration inhibited with amytal suggest S-form. The deviation from this form in the absence of exogenous substrate (Fig. 2) indicates some secondary action of amytal in addition to its main effect. The resulting picture may be rather different from one sperm sample to another. In spite of this the correlation between $\log C_{50\%}$ and $\log$ (N.R.) with amytal is fairly good (Table 2). This side effect does not appear in the curves which Gonse⁶ has obtained with ejaculated dog or bull spermatozoa on exogenous substrate. It seems probable that amytal interferes with the respiratory system in two different ways. This becomes apparent only, when the system is working at a low rate. In addition to the electron transport amytal is known to inhibit transphosphorylation reactions in mitochondria, e.g. the exchange between inorganic phosphate and adenosintriphosphate¹⁵, the dinitrophenol-induced adenosintriphosphatase²⁵.

Whilst maximum inhibition caused by 8×10^{-3} – 10×10^{-3} M amytal in our experiments (bull spermatozoa) amounts to 80 to 90 % as in those of

Table 2. Statistical treatment of correlation between $\log C_{50\%}$ and $\log$ (N.R.). n number of determinations. The correlation coefficient (r) is tested with $t = r\sqrt{n-2}/\sqrt{1-r^2}$, the

regression coefficient (b) with $t = b \cdot \sqrt{\frac{(n-2)S(x-\bar{x})^2}{S(y-\bar{Y})^2}}$. R.V. residual variance.

Inhibitor	n	Correlation			Regression			
		r	t	P	b	t	P	R.V.
Azide	10	−0.692	2.712	0.02	−0.891	2.455	0.05	0.024
Amytal	9	−0.862	4.499	0.01	−3.395	2.567	0.05	0.047
Rotenone	10	−0.909	6.169	0.001	−4.261	6.102	0.001	0.137

Gonse ⁶ on dog spermatozoa, total inhibition of respiration in isolated rat liver mitochondria supported by L-glutamate and other substrates ⁴ is attained already with 1.8×10^{-3} M.

Azide has been shown to be an inhibitor of oxidative phosphorylation,¹⁶ and is known as an inhibitor of Mg^{2+} -activated and 2,4-dinitrophenol-activated adenosintriphosphatase ^{19,23,26,25}. Besides its well-known effect on cytochrome oxidase Gonse ⁶ discovered a further effect on bull spermatozoa expressed *i.a.* by a failing reduction or, at low turnover, a reoxidation of cytochrome b. Gonse interprets this effect as the results of an uncoupling action of azide which is further supported by his data obtained on *Spisula* spermatozoa in which respiration can be activated by azide. Seen against this background the apparent partial release of the respiration from inhibition in the concentration range of 2.5×10^{-3} to 2.5×10^{-2} in the present experiments could be interpreted as a result of uncoupling. In this case, however, this phenomenon should be expected to occur also in spermatozoa on endogenous substrate. As it appears mainly in the presence of fructose it might be due primarily to interference of azide with fructolysis. An action of azide on glycolysis has been suggested by other investigations^{3,1}. The fact that the respiration in dog spermatozoa on lactate does not exhibit any similar release from inhibition caused by azide ((Ref.²⁰), Fig. 14) may be taken as a further support of this view.

Gonse ⁶ arrives at the conclusion that in bull spermatozoa the inhibition caused by the addition of azide is dependent on the rate of respiration (turnover) of the cell sample in question. He furthermore finds an increase in the sensitivity to amytal of the endogenous respiration in *Spisula* spermatozoa, when it is activated with dinitrophenol. The above is well in keeping with the general results of the present investigation.

Also with azide there exists a significant negative correlation between $\log C_{50\%}$ and $\log (N.R.)$, of Table 2. Obviously this association is not influenced by secondary effects of the inhibitor upon the degree of inhibition.

From Fig. 4 it appears that for rotenone the dispersion of the points around the regression line is greater than those for the other two inhibitors. This is also reflected by the residual variances in Table 2 which increase in the order: azide, amytal, rotenone.

The equations of the regression lines,
for azide

$$\log C_{50\%} = -0.891 \times \log (N.R.) - 0.125$$

for amytal

$$\log C_{50\%} = -3.395 \times \log (N.R.) + 1.773$$

and for rotenone

$$\log C_{50\%} = -4.261 \times \log (N.R.) - 1.322$$

can be written in the form

$$C_{50\%} = \frac{1}{P (N.R.)^k}$$

in which the proportionality constant, P , is the reciprocal of the antilogarithm of the intercept and k the "slope" of the regression line. Thus in every case the concentration of the inhibitor giving an inhibition of 50 % is inversely proportional to a power of the rate of normal respiration. The influence of the variation in this rate upon $C_{50\%}$ depends upon the values of k and P for each of the three inhibitors. In this context k obviously has a much greater effect than P . With azide k is near unity (0.891) when the rate of respiration is expressed in the units used here, and therefore does not to any appreciable degree influence upon the relation between $C_{50\%}$ and normal respiration. The effect of the variation of the rate of the normal respiration is not very much strengthened by P ($= 1.33$). We here observe a situation similar to that deduced by Klein and Runnström¹⁰ for carbon monoxide which reacts with the same enzyme as azide. With rotenone on the other hand the rate of the normal respiration comes into action with its fourth dignity ($k = 4.261$), being further multiplied by a relatively high value of P ($= 21.0$). In the case of amytal the value of k is nearly as high as with rotenone (3.395). Resulting from the low value of P (0.0169) the effect of the variation in the rate of normal respiration is here nevertheless considerably smaller.

REFERENCES

1. Chance, B. *Harvey Lectures* 49 (1955) 145.
2. Chance, B. in *Enzymes: Units of Biological Structure and Function*. Academic Press, New York 1956.
3. Clifton, C. E. *Advan. Enzymol.* 6 (1946) 296.
4. Ernster, L., Jalling, O., Löw, H. and Lindberg, O. *Exptl. Cell Res. Suppl.* 3 (1955) 124.
5. Fukami, J. *Bull. Natl. Inst. Agr. Sci. (Japan), Ser. C*, No. 13 (1961) 33.
6. Gonse, P. H. in *Spermatozoan Motility*, Washington 1962.
7. Henle, W. *J. Immunol.* 34 (1938) 325.
8. Henle, W. and Zittle, G. A. *Am. J. Physiol.* 136 (1942) 70.
9. Keilin, D. and Hartree, E. F. *Proc. Roy. Soc. (London)* B 127 (1939) 167.
10. Klein, O. and Runnström, J. *Arkiv Kemi, Mineral. Geol.* 19 (1940/41) No. 4.
11. Lindahl, P. E. *Z. vergleich. Physiol.* 27 (1939) 136.
12. Lindahl, P. E. and Öberg, K. E. *Nature* 183 (1960) 784.
13. Lindahl, P. E. and Öberg, K. E. *Exptl. Cell. Res.* 23 (1961) 228.
14. Lindahl, P. E. and Öhman, L. O. *Naturwiss.* 24 (1936) 157.
15. Löw, H., Siekewitz, P., Ernster, L. and Lindberg, O. *Biochim. Biophys. Acta* 29 (1958) 392.
16. Loomis, W. F. and Lipmann, F. *J. Biol. Chem.* 179 (1949) 503.
17. Mann, T. *The Biochemistry of Semen*, London 1954.
18. Neilands, J. B. and Stumpf, P. K. *Enzyme Chemistry*, New York 1958.
19. Novicoff, A. B., Hecht, L., Podber, E. and Ryan, J. *J. Biol. Chem.* 194 (1952) 153.
20. Öberg, K. E. *Exptl. Cell Res.* 24 (1961) 163.
21. Öström, Å. *Protoplasma* 15 (1932) 566.
22. Öström, Å. *Protoplasma* 24 (1935) 157.
23. Robertson, H. E. and Boyer, P. D. *J. Biol. Chem.* 214 (1955) 295.
24. Runnström, J. *Protoplasma* 10 (1930) 106.
25. Siekewitz, P., Löw, H., Ernster, L. and Lindberg, O. *Biochim. Biophys. Acta* 29 (1958) 378.
26. Swansson, M. A. *Biochim. Biophys. Acta* 20 (1956) 85.
27. Tamiya, H. and Kubo, H. *Acta Phytochim. (Japan)* 9 (1939) 123.

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