Comparative labelling of rat epididymal spermatozoa by intratesticularly administered $^{65}\text{ZnCl}_2$ and [^{35}S]cysteine

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Summary. Spermatozoa of rats injected intratesticularly with 20 μCi $^{65}\text{ZnCl}_2$ and 10 μCi [^{35}S]cysteine were collected from the caput and cauda of the epididymis at 2, 6, 10, 14, 18, 22 and 28 days after injection. The highest specific activities with respect to each isotope were observed in spermatozoa from the caput on Day 10. Maximal levels in spermatozoa from the cauda were obtained on Days 14 and 18 for ^{35}S and Day 18 for ^{65}Zn . Estimation of the relative behaviour of ^{65}Zn and ^{35}S by calculation of $^{65}\text{Zn}/^{35}\text{S}$ ratios suggests that: (1) ^{35}S associated with spermatozoa arrived in the epididymis slightly in advance of ^{65}Zn and was therefore probably incorporated more readily into proteins of very late spermatids; (2) approximately 60% of ^{65}Zn was lost from spermatozoa and 75% from isolated sperm heads during transit from caput to cauda, assuming total retention of ^{35}S ; and (3) retention of ^{65}Zn by the seminiferous epithelium was superior to that of [^{35}S]cysteine, as indicated by increasing $^{65}\text{Zn}/^{35}\text{S}$ ratios following the days of peak specific activity in both caput and cauda epididymal spermatozoa. Only small percentages of either isotope were recovered in isolated sperm heads, suggesting that the primary sites of labelling were in the sperm tail.

Superior retention of ^{65}Zn by testis was confirmed by increasing $^{65}\text{Zn}/^{35}\text{S}$ ratios in individual fractions of testicular homogenates between 2 and 10 days after injection. In addition, both isotopes appeared to be transferred from the testis cytosol to particulate material during this period.

Introduction

The well-established requirement for zinc in mammalian spermatogenesis (Underwood, 1977) has been most extensively investigated in the rat. It has been shown that the impairment of spermatogenesis in rats during zinc deficiency is not preventable by administration of gonadotrophins (Millar, Elcoate, Fischer & Mawson, 1960) and may reflect chiefly the direct requirement for zinc by elongating spermatids, which are selectively depleted during the initial stages of zinc deprivation (Millar *et al.*, 1960; Orgebin-Crist, Freeman & Barney, 1970; Diamond, Swenerton & Hurley, 1971). The uptake of zinc by developing rat spermatids is supported by the coincidental increment in testicular zinc concentrations when these cells first appear during puberty (Parizek, Bournsnel, Hay, Balbicky & Taylor, 1966) and by the short interval between incorporation of subcutaneously or intramuscularly injected ^{65}Zn into testis and its subsequent appearance in the caput epididymidis (Wetterdal, 1958; Gunn & Gould, 1970).

Association of zinc with cysteine-rich sperm proteins in the rat (Calvin & Bleau, 1974; Calvin, Hwang & Wohlrab, 1975; Baccetti, Pallini & Burrini, 1976) and bull (Baccetti *et al.*, 1976) and uptake of [^{35}S]cysteine by both nucleus and cytoplasm in ram spermatids (Loir, 1972)

suggest that incorporation of zinc and cysteine by sperm proteins may be approximately concurrent events which both take place during spermatid differentiation. The incorporation of cysteine into the spermatid proteins is no doubt a prerequisite for the development of the unusual variety of highly differentiated sperm structures which are stabilized by half-cystine bridges in mammalian spermatozoa (Bedford & Calvin, 1974a, b).

Studies of the concentration of ^{65}Zn in rat epididymal spermatozoa 2–20 days after intratesticular administration indicate maximal levels in spermatozoa in the caput at 9–11 days after injection, followed by a more modest peak of activity in the cauda approximately 1 week later. In the present study, these findings have been extended by comparing ^{65}Zn and [^{35}S]cysteine incorporation into rat epididymal spermatozoa between 2 and 28 days after intratesticular administration.

Materials and Methods

Injection of tracers

The tracers were 1.0 mCi ^{65}Zn (sp. act. 370–430 mCi/mmol: New England Nuclear), dissolved in 0.5 M-HCl, and 0.5 mCi [^{35}S]cysteine hydrochloride (sp. act. 70–110 mCi/mmol: Amersham). They were mixed and diluted to a final volume of 5 ml in a solution which also included 0.02 M-Tris, 0.01 M-HCl and 0.15 M-NaCl. Male Sprague–Dawley rats (350–400 g) were anaesthetized with ether and 0.10 ml of the above tracer solution containing 20 μCi ^{65}Zn and 10 μCi [^{35}S]cysteine was injected into each testis. The 0.5-ml syringe was fitted with a $\frac{3}{8}$ -inch, 26-gauge needle and the path of needle entry was approximately equatorial and orientated to avoid contact with the testicular vasculature or the epididymis.

Preparation of sperm samples for assay of specific activities

Animals were killed by cervical dislocation after ether anaesthesia at 2, 6, 10, 14, 18, 22 and 28 days after injection. Each testis was examined and the tiny injection scar of less than 1-mm diameter was noted. In those rare instances where greater damage was evident, the associated epididymis was not included in the study. Spermatozoa were collected separately from each caput and cauda epididymidis by procedures described previously (Calvin, Yu & Bedford, 1973). The segments chosen as caput and cauda corresponded approximately to zones 1–3 and zone 6, respectively, of the zones described by Reid & Cleland (1957). The spermatozoa, suspended in 0.02 M-sodium phosphate, pH 6.0 (Buffer P), were collected by centrifugation at 1500 g for 5 min, washed once with Buffer P and resuspended in this medium for assay of radioactivity. Suspensions of caput spermatozoa (2 ml) and cauda spermatozoa (4 ml) were sonicated until more than 99% of the spermatozoa had been decapitated (Calvin *et al.*, 1973). Six aliquots of 100 μl were removed from each sample for the determination of specific activities as described below. The remainder of each sperm suspension was usually then processed for the isolation of sperm heads by sucrose density gradient centrifugation (Calvin, 1976). For this purpose, spermatozoa derived from the left and right caput epididymidis of a single animal were pooled, whereas the corresponding cauda samples were treated separately. Each pellet of isolated sperm heads was suspended in 1 ml 0.02% sodium dodecyl sulphate (SDS), a single 0.3-ml aliquot was removed for assay of radioactivity, and triplicate 100- μl aliquots were taken for determination of sperm head concentration with a haemocytometer. Contamination by tail fragments was consistently less than 1 per 100 sperm heads.

Preparation of testicular fractions

Testes were removed from rats 2, 6 and 10 days after injection of the usual combined dose of ^{65}Zn and [^{35}S]cysteine and were fractionated separately. Each testis was decapsulated, placed in

0.25 M-sucrose–0.02 M-Tris–HCl (pH 7.5)–3 mM-MgCl₂ and homogenized with a glass-Teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 750 $g_{\max}$ for 15 min and the resulting supernatant recentrifuged at 10 000 $g_{\max}$ for 15 min. Finally, the 10 000 g supernatant was centrifuged at 140 000 $g_{\max}$ for 60 min. The three successive residues obtained have been designated respectively as Residues I, II, and III (see Table 2). Each of these was resuspended in 3–5 ml 1% SDS. Triplicate aliquots of 0.3 ml were removed from each suspension and from the final supernatant for assay of radioactivity.

Determination of radioactive content and specific activities

The amounts of ^{65}Zn and ^{35}S in doubly-labelled samples were determined simultaneously with a Packard Model 3375 liquid scintillation spectrometer which had been adjusted for the simultaneous measurement of ^3H and ^{14}C . Aliquots to be assayed were transferred to 4-ml plastic Wheaton Omni-Vials and diluted to 3 ml in a final mixture which included 0.3 ml of water and 2.7 ml of Hydrofluor (National Diagnostics). Standards of ^{65}Zn and [^{35}S]cysteine, prepared in the same medium on the day of injection and stored at 2–8°C, were assayed in parallel to facilitate correction for radioactive decay during the course of the experiments and establish the counting behaviour of each isotope under the prevailing conditions. ^{65}Zn displayed counting efficiencies of 35–38% in the lower energy (^3H) channel and 1.7–1.9% in the higher energy (^{14}C) channel. Efficiencies for ^{35}S were 11–13% in the ^3H channel and 30–35% in the ^{14}C channel. The results presented are therefore based on net c.p.m. of ^{65}Zn in the ^3H channel and of ^{35}S in the ^{14}C channel. The procedure of Okita, Kabara, Richardson & LeRoy (1957) was employed to correct for overlapping behaviour of the two isotopes. In order to determine the specific activities of spermatozoa or sperm heads, their concentrations in suspensions submitted to radioactive assay were established by dilution of aliquots with 0.02% SDS and scoring of heads with a haemocytometer.

Results were submitted to Student's t test for determination of statistical significance.

Results

Labelling of epididymal spermatozoa

Before injecting mixtures of $^{65}\text{ZnCl}_2$ and [^{35}S]cysteine to compare their uptakes into epididymal spermatozoa, preliminary studies were conducted which verified that the incorporation of either isotope into caput spermatozoa between 6 and 11 days after injection was unaffected by such mixing. It was therefore possible to conduct the present studies by simultaneous administration of the two tracers.

The approximate schedules of ^{65}Zn and [^{35}S]cysteine incorporation into epididymal spermatozoa, established by assay at 4-day intervals, are depicted in Text-figs 1(a) and 1(b), respectively. The specific activities in caput spermatozoa with respect to both isotopes, which were very low on Day 2, rose dramatically by Day 6, with ^{35}S displaying the sharper increase. The maximal activities in caput spermatozoa were recorded on Day 10, followed by a steep decline between Days 10 and 14. Substantial labelling with ^{35}S was first observed in cauda spermatozoa on Day 10 and clearly preceded that with respect to ^{65}Zn . Although maximal ^{65}Zn and ^{35}S levels in cauda spermatozoa both occurred on Day 18, only ^{65}Zn was present in significantly higher concentration on Day 18 than on Day 14 ($P < 0.01$), suggesting that the true peak of ^{35}S activity in cauda spermatozoa may have occurred slightly earlier than that of ^{65}Zn .

The comparative behaviour of ^{65}Zn and ^{35}S was also assessed by determination of $^{65}\text{Zn}/^{35}\text{S}$ in caput and cauda spermatozoa between Days 2 and 28 (Text-fig. 2). This ratio was minimal on Days 6 and 10, respectively, in the two populations, no doubt reflecting the earlier appearance of ^{35}S relative to that of ^{65}Zn in epididymal spermatozoa (Text-figs 1a and 1b). The high $^{65}\text{Zn}/^{35}\text{S}$ ratios in cauda spermatozoa before Day 10 are based upon minute levels of activity and have

Text-fig. 1. Mean $\pm$ s.e.m. specific activities of (a) ^{65}Zn and (b) ^{35}S in caput (—○) and cauda (—●) epididymal spermatozoa after intratesticular administration of $20\text{ }\mu\text{Ci } ^{65}\text{ZnCl}_2$ and $10\text{ }\mu\text{Ci } [^{35}\text{S}]\text{cysteine}$. The numbers of epididymal segments sampled are indicated in parentheses in (a). An equal number of caput and cauda segments were assayed on each of Days 10, 18, 22 and 28. Standard errors not shown are within the symbols represented and were $< \pm 50$ c.p.m. for (a) and $< \pm 10$ c.p.m. for (b).

therefore been plotted as a dashed line in Text-fig. 2, although there is no question of the validity of the decrease shown between Days 2, 6 and 10 ($P < 0.01$ for each step). $^{65}\text{Zn}/^{35}\text{S}$ increased substantially in cauda spermatozoa between Days 10 and 22, then remained approximately constant between Days 22 and 28. In the caput samples, this ratio increased rapidly between Days 6 and 14, changed very little between Days 14 and 22, and then began to decline.

Text-fig. 2. Mean $\pm$ s.e.m. $^{65}\text{Zn}/^{35}\text{S}$ ratios in the samples described in Text-fig. 1(a). Standard errors below ± 0.10 are not indicated.

The higher $^{65}\text{Zn}/^{35}\text{S}$ ratios in caput samples between Days 10 and 22, as compared with those in cauda spermatozoa collected 4–8 days later indicate that ^{65}Zn is lost during sperm transport through the epididymis. Comparison of $^{65}\text{Zn}/^{35}\text{S}$ in cauda spermatozoa on Day 18 (1.89 ± 0.08) with the ratio in caput spermatozoa on Day 10 (4.52 ± 0.16) suggests that approximately 60% of the ^{65}Zn was lost from the spermatozoa during epididymal transit, assuming total retention of ^{35}S .

Specific activities with respect to ^{65}Zn and ^{35}S in sperm heads isolated following sonication are presented in Table 1. The highest specific activities observed with respect to either isotope in the heads of caput spermatozoa occurred on Days 6 and 10, but these were highly variable and much lower than the specific activities of the parent sperm samples (Text-figs 1a and 1b). Activity of ^{35}S reached its highest level on Day 18 in cauda sperm heads ($P < 0.01$ compared with Day 14) and began to decrease sharply after Day 22. Very little ^{65}Zn was detected in cauda sperm heads, suggesting that most of it had been lost during epididymal transit. Nevertheless, it is probable that the highest specific activity observed in these samples occurred on Day 18 (higher than Day 14, $P < 0.01$, and Day 22, $P < 0.05$). $^{65}\text{Zn}/^{35}\text{S}$ in cauda sperm heads on Days 14–22 ranged from 0.21 to 0.25, suggesting a loss of about 75% of the ^{65}Zn present in caput heads 4–8 days earlier.

Table 1. Specific activities of rat epididymal sperm heads following intratesticular injection of $^{65}\text{ZnCl}_2$ and $[^{35}\text{S}]\text{cysteine}$

Day	Caput			Cauda		
	No. of samples	^{65}Zn	^{35}S	No. of samples	^{65}Zn	^{35}S
2	3	6.27 ± 2.17	1.69 ± 0.52	6	0.42 ± 0.05	0.28 ± 0.07
6	7	156 ± 41	118 ± 22	8	0.74 ± 0.06	0.86 ± 0.08
10	6	131 ± 28	135 ± 36	8	1.42 ± 0.16	4.24 ± 0.69
14	8	58.0 ± 2.6	68.5 ± 8.3	15	17.1 ± 2.7	69.2 ± 6.6
18	6	40.2 ± 2.5	36.9 ± 1.9	12	25.9 ± 1.3	101.9 ± 4.3
22	5	40.0 ± 2.4	31.9 ± 3.3	10	18.9 ± 2.3	89.5 ± 9.1
28	4	13.5 ± 1.1	14.3 ± 0.9	7	12.2 ± 2.3	34.0 ± 2.5

The values represent mean $\pm$ s.e.m. specific activities (c.p.m./ 10^6 sperm heads) of caput or cauda epididymal sperm heads.

Labelling of testis fractions

The incorporations of ^{65}Zn and $[^{35}\text{S}]\text{cysteine}$ into subfractions of testicular homogenates were tested in a preliminary study performed on 9 rats. The rats were killed in groups of 3 at 2, 6 and 10 days after injection and the left and right testes of each animal were fractionated separately to yield 6 homogenates on each of these days. However, since the assays were designed to determine total recovery of label in each fraction, only those subcellular fractions obtained without appreciable losses were included in the data.

The results, summarized in Table 2, show clearly that on all assay days both isotopes were found mainly in 2 of the 4 testicular fractions, the 750 g residue (Residue I) and the final 140 000 g supernatant. However, the specific activities of the four fractions were not determined and may well have been comparable, especially since Residues II and III were very small in comparison with Residue I.

Table 2. Distribution of radioactive label among fractions of rat testicular homogenate following intratesticular injections of $^{65}\text{ZnCl}_2$ and $[^{35}\text{S}]\text{cysteine}$

Fraction	Day	No. of samples	^{65}Zn		^{35}S		$^{65}\text{Zn}/^{35}\text{S}$
			c.p.m.	%*	c.p.m.	%*	
Residue I (750 g)	2	4	841 $\pm$ 33	51	339 $\pm$ 23	45	2.50 $\pm$ 0.07
	6	6	896 $\pm$ 29	58	302 $\pm$ 8.0	53	2.98 $\pm$ 0.07
	10	6	737 $\pm$ 80	63	209 $\pm$ 24	58	3.55 $\pm$ 0.18
Residue II (10 000 g)	2	4	109.4 $\pm$ 1.2	6.7	71.2 $\pm$ 3.6	9.4	1.55 $\pm$ 0.09
	6	4	59.4 $\pm$ 5.1	3.8	37.1 $\pm$ 4.2	6.6	1.63 $\pm$ 0.08
	10	6	65.8 $\pm$ 7.2	5.6	35.7 $\pm$ 2.6	9.9	1.83 $\pm$ 0.11
Residue III (140 000 g)	2	4	74.6 $\pm$ 2.8	4.5	34.0 $\pm$ 1.8	4.5	2.20 $\pm$ 0.06
	6	4	71.5 $\pm$ 3.6	4.6	26.6 $\pm$ 1.9	4.7	2.71 $\pm$ 0.15
	10	6	83.0 $\pm$ 8.0	7.1	25.4 $\pm$ 1.8	7.0	3.25 $\pm$ 0.15
Supernatant (140 000 g)	2	4	620 $\pm$ 17	38	314 $\pm$ 14	41	1.99 $\pm$ 0.10
	6	4	517 $\pm$ 19	33	200 $\pm$ 11	35	2.61 $\pm$ 0.15
	10	6	281 $\pm$ 27	24	92.1 $\pm$ 5.5	25	3.02 $\pm$ 0.18
Total			1645		758		2.17
			1544		566		2.73
			1167		362		3.22

The values represent mean $\pm$ s.e.m. total recoveries in each fraction.

* Total c.p.m. in homogenates were calculated by summation of the average c.p.m. in each fraction.

The % values were then calculated.

The transfer of both ^{65}Zn and ^{35}S from soluble to particulate material is very clearly indicated by comparison of their relative contents in Residue I and the supernatant during the period examined. Total ^{65}Zn c.p.m. in Residue I remained nearly constant between Days 2 and 10, but dropped sharply in the supernatant during this time. Thus, the percentage of total ^{65}Zn associated with Residue I increased from 51 to 63% between Days 2 and 10, while its percentage in the supernatant fraction had declined from 38 to 24%. Similarly, the percentage of ^{35}S recovered in Residue I increased from 45% on Day 2 to 58% on Day 10, whereas its percentage in the supernatant had decreased from 41 to 25%. The increasing ratio of ^{65}Zn to ^{35}S between Days 2 and 10 in Residues I and II and the supernatant ($P < 0.01$ for all 3 fractions) indicates superior retention of ^{65}Zn relative to that of ^{35}S during this period.

Discussion

The timing of ^{65}Zn incorporation into epididymal spermatozoa after intratesticular administration of $^{65}\text{ZnCl}_2$ mixed with $[^{35}\text{S}]\text{cysteine}$ agrees with results obtained earlier (Calvin,

1979), in which the respective peaks of ^{65}Zn specific activity in caput and cauda epididymal spermatozoa, following intratesticular injection of $^{65}\text{ZnCl}_2$ alone, occurred at 9–11 days and at 16–18 days. However, when the tracer was administered subcutaneously or intramuscularly, its maximal concentration in testis, caput epididymidis and cauda epididymidis occurred at 1, 2 and 3 weeks respectively after injection (Wetterdal, 1958; Gunn & Gould, 1970). The administration of a pulse of ^{65}Zn directly into the testis therefore shortens its time of appearance in the epididymis by about 3–5 days, in comparison with its behaviour after peripheral administration.

The timing of its arrival in a given segment of the epididymis can be used to determine when a radioactive label was incorporated during spermatogenesis. The arrangement of 19 spermatid stages within the 14 stages of cellular association in the rat and the percentage of the total cycle occupied by each spermatogenic stage, based on the data of Clermont & Harvey (1965), are illustrated in Text-fig. 3. Also included in this figure is a scale of time; approximately 3 days are required for spermatozoa released from the testis to pass entirely through Zones 1–3 of Reid & Cleland (1957) in the caput epididymidis (M. C. Orgebin-Crist, personal communication).

Text-fig. 3. Schedule of spermiogenesis in the Sprague-Dawley rat, based on the data of Clermont & Harvey (1965). The width of each box is proportional to the relative duration of each of Stages I–XIV in the seminiferous epithelium.

Comparison of Text-fig. 3 with Text-fig. 1(b) suggests that the major fraction of administered [^{35}S]cysteine incorporated into spermatozoa enters structural proteins between Stages 15 and 17, first appearing in caput spermatozoa between 6 and 10 days after intratesticular injection, assuming that there is essentially no lag between the time of injection and availability for protein synthesis. Additional monitoring would be needed to establish more precisely the day of maximal ^{35}S activity in caput spermatozoa following intratesticular injection of [^{35}S]cysteine, which probably occurs at some time between Days 6 and 10 (e.g. on Day 8). Moreover, assay of ^{35}S in caput spermatozoa on Days 3 and 4 after injection would be needed to verify whether Stage 18 or 19 spermatids take up significant amounts of [^{35}S]cysteine into stable sperm structures. This is not unlikely, considering the steep increase in ^{35}S between Days 2 and 6 in these cells and the significant rise of ^{35}S in cauda spermatozoa as early as Day 10 (Text-fig. 1b). Since about 6 days are required for passage from Zone 3 of Reid & Cleland (1957) in the caput to Zone 6 in the cauda epididymis (M. C. Orgebin-Crist, personal communication), the spermatozoa which are labelled with ^{35}S on Day 10 in the cauda had probably passed through Zone 3 of the caput region around Day 4.

The present findings resemble those of Grimes, Meistrich, Platz & Hnilica (1977), who estimated, by intratesticular administration of [^3H]arginine, that the cysteine-rich protamine of the sperm nucleus is synthesized between Stages 16 and 19. However, from comparison of Text-fig. 1(b) and Table 1, it is clear that the protamine of the sperm head accounts for only a minor fraction of the total ^{35}S incorporation on the days assayed. Instead, the bulk of the ^{35}S label appears to be associated with proteins of the sperm tail, which also account for the major fraction of assayable -SH in the cell (Calvin *et al.*, 1975).

Within the sperm tail, the dense fibres are the most prominent –S– cross-linked structures (Bedford & Calvin, 1974a) and account for the majority of cysteine-rich protein (Baccetti, Pallini & Burrini, 1973; Calvin *et al.*, 1975). It seems likely, therefore, that the bulk of ^{35}S incorporated into caput spermatozoa on Days 6 and 10 is associated with dense fibre proteins, the major components of which are a pair of cysteine-rich polypeptides whose molecular weight is approximately in the range of 30 000–35 000 (Calvin, 1979). Like the cysteine-rich protamine of the sperm head (Grimes *et al.*, 1977; Loir & Lanneau, 1978), these polypeptides are evidently synthesized in spermatids immediately before their assembly in sperm structures. The formation of dense fibres in the late stages of spermiogenesis over a prolonged period is indicated by morphological observations (Phillips, 1974; Clermont & Rambourg, 1978). However, the latest stages in which dense fibre material is formed have not been reported. Further tracer studies would be needed to determine whether the proteins which make up these structures continue to be elaborated as late as Stage 19.

In contrast to ^{35}S , a relatively minor fraction of intratesticularly administered ^{65}Zn reached the caput sperm population by Day 6. From this, it may be concluded that there is [^{35}S]cysteine-labelled material synthesized in the most mature spermatids (i.e. those of Stage 17 and beyond) which incorporates relatively little injected ^{65}Zn . Since there is reason to believe that cysteine and Zn are primarily associated with the same proteins and that the binding of Zn to such proteins is mediated by cysteine residues (Calvin, 1979), this result is at first surprising. As one possible explanation, it is suggested that extraneous ^{65}Zn may have relatively poor access to the most advanced spermatids (i.e. Stages 17–19), although it is incorporated readily by some of the earlier stages (e.g. Stage 15). Spermatids at the early stages might retain the tracer throughout subsequent stages of development, during which it would continue to bind to newly formed cysteine-rich proteins. Specific information on the kinetics of either zinc or cysteine penetration of the blood–testis barrier does not appear to be available, although published data suggest that other ions and amino acids penetrate this barrier relatively slowly (Setchell, 1978).

The increases in $^{65}\text{Zn}/^{35}\text{S}$ observed in testicular fractions between Days 2 and 10 (Table 2) and subsequently in epididymal spermatozoa (Text-fig. 2) suggest that some mechanisms exist which favour the retention of zinc for relatively long periods by the seminiferous epithelium. This results in preferential accumulation of ^{65}Zn by sperm tail structures, as indicated by continuously increasing $^{65}\text{Zn}/^{35}\text{S}$ ratios in caput spermatozoa between Day 6 and Day 18 (Text-fig. 2) and probably also by the increase of $^{65}\text{Zn}/^{35}\text{S}$ in Residue I between Day 2 and Day 10 (Table 2). On the other hand, the subsequent drop in $^{65}\text{Zn}/^{35}\text{S}$ in caput spermatozoa after Day 18 implies that a minor fraction of ^{35}S -labelled material was retained by testis even more tenaciously than ^{65}Zn .

The changes in $^{65}\text{Zn}/^{35}\text{S}$ in cauda spermatozoa between Days 10 and 28 reflected those which had occurred in the caput population 4–8 days earlier. However, $^{65}\text{Zn}/^{35}\text{S}$ in the cauda population was consistently lower than in caput spermatozoa examined 4–8 days previously and this correlates well with the observation that spermatozoa isolated from the cauda epididymidis contain about 50% as much zinc as those from the caput (Calvin *et al.*, 1975). This net loss of Zn, originally suggested by Gunn & Gould (1970), may be related to the oxidation of –SH groups with which this trace metal is complexed (Calvin *et al.*, 1975). By contrast, turnover of zinc in rat epididymal spermatozoa appears to be of relatively minor importance. Although a rapid but transient incorporation of ^{65}Zn by rat epididymal spermatozoa has been detected following intraperitoneal administration (Adams, Neathery & Johnson, 1975), this does not signify exchange of the main complement of zinc incorporated during spermiogenesis, which is not removable by challenge with exogenous, unlabelled zinc (Gunn & Gould, 1970). This point of view is also supported by the relatively minute levels of ^{65}Zn in epididymal spermatozoa on Day 2, following intratesticular administration (Text-fig. 1a).

The identification of proteins or other molecules in testis responsible for retention of ^{65}Zn and [^{35}S]cysteine requires further investigation. Apart from the sperm proteins, at least one other cysteine-rich protein with a great affinity for zinc, metallothionein, has been demonstrated in

substantial concentrations in testis (Chen & Ganther, 1975), although its localization within the seminiferous tubules remains unknown. In addition, the level of reduced glutathione in testis has been reported to be unusually high (Kochakian, 1975). More studies are needed to clarify the relationships between cysteine-containing proteins or peptides and zinc in the testis.

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