4. The spermatozoa contain only traces of a few free amino acids. The seminal plasma contains 5.72 μ moles of total amino acids/ml.

5. The spermatozoon proteins were found to undergo slow degradation to free amino acids on incubation at 37°.

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Incorporation of [¹⁴C]Amino Acids into the Proteins of Bull Spermatozoa

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During the last two decades, a considerable amount of evidence has accumulated to suggest that ribonucleic acid plays an essential role in protein biosynthesis (see Brachet, 1955). Since bull spermatozoa are virtually devoid of ribonucleic acid (Bhargava, Bishop & Work, 1959), they would be expected to be incapable of protein synthesis. It has already been shown, however, that washed bull spermatozoa can incorporate radioactive amino acids into their proteins (Bhargava, 1957); these observations are extended and evaluated in the

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present paper. We have also studied amino acid incorporation by unwashed spermatozoa in seminal plasma since this system more closely resembles that met with *in vivo*. Marked differences were observed between the washed and the unwashed cells in both the rates and the patterns of incorporation of amino acid into protein. The effect of several metabolic inhibitors on incorporation by both washed and unwashed cells was also investigated. For comparative purposes, the rate of incorporation of amino acids into proteins of reticulocytes has been measured under similar conditions to those used for washed spermatozoa.

EXPERIMENTAL

Materials and methods

Semen. The bull spermatozoa used were obtained from five normal fertile bulls. These bulls (N_1 , N_2 , N_3 , N_4 and N_5) together with the method of collecting the semen and the method of determining the concentration of spermatozoa, have been described in the preceding paper (Bhargava *et al.* 1959). Bull N_1 was the animal used in an earlier investigation (Bhargava, 1957).

Radioactive amino acids. Dried *Chlorella* protein generally labelled with ^{14}C (4.8 mg., specific activity $102\ \mu\text{C}/\text{mg.}$; the Radiochemical Centre, Amersham, Bucks.), was hydrolysed with 2 ml. of 6N-HCl. The hydrolysate was evaporated to dryness, extracted with water and the extract made up to 10.6 ml.; it was then stirred with 0.5 g. of Celite (grade 519A, Johns-Manville Co. Ltd.) to remove suspended material and centrifuged. The supernatant, subsequently referred to as [^{14}C]Chlorella hydrolysate, contained about $4.5\ \mu\text{moles}$ of mixed amino acids and $46\ \mu\text{C}$ of radioactive carbon/ml. In a few experiments, Chlorella protein of half this specific activity was used; in these experiments the observed counts have been doubled so as to facilitate comparison between experiments.

Radioactive valine (specific activity $8\ \mu\text{C}/\mu\text{mole}$; The Radiochemical Centre, Amersham, Bucks.) was dissolved in water to a concentration of $4.5\ \mu\text{moles}/\text{ml.}$ All data obtained with [^{14}C]valine have been adjusted to correspond to a value of $10.2\ \mu\text{C}/\mu\text{mole}$ (the same specific activity as the standard Chlorella hydrolysate). In this way, direct comparison can be made between results with [^{14}C]Chlorella hydrolysate and those with [^{14}C]valine.

Metabolic inhibitors. The following inhibitors were used: chloramphenicol, 2,4-dinitrophenol, sodium fluoride, iodoacetic acid (neutralized to pH 7.0), 8-azaguanine, *p*-fluorophenylalanine and ribonuclease. Solutions to be tested were prepared in water (dinitrophenol was in 1% NaHCO_3 soln.) and the appropriate amount was evaporated to dryness in the tubes in which the cells were to be incubated. *p*-Fluorophenylalanine was synthesized in this Laboratory; ribonuclease was prepared by Dr R. R. Porter; all other inhibitors were commercial preparations.

Spermatozoon motility. Estimates of spermatozoon motility were made on the fresh undiluted semen, on the washed cell suspensions just before starting incubation and on samples taken out after incubation for various periods of time. The ratings were made by the method of Bishop & Salisbury (1955a). All samples were rated by the same person.

Bacterial counts. In some experiments estimations were made of the number of bacteria present before incubation and after incubation of semen or washed spermatozoa for various lengths of time. Bacterial counts were determined, after suitable dilution, by plating on blood-agar plates and incubating aerobically at 34° for 20 hr.

Preparation of cell suspensions

Suspensions of washed spermatozoa. Portions of semen (0.6–1.4 ml.) were pipetted into 15 ml. round-bottomed centrifuge tubes, diluted with 3–5 vol. of Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1957; referred to below as bicarbonate buffer), or Krebs-Ringer bicarbonate buffer containing 5% (w/v) of fructose

(subsequently called fructose-bicarbonate buffer), and centrifuged for 5–20 min. at 2500 rev./min. (MSE Major centrifuge). Different batches of semen differed in the rate and extent of sedimentation of the spermatozoa. The cell sediment was resuspended in fructose-bicarbonate buffer (3–9 ml.). When the effect of omitting fructose was studied, the cells were washed with and resuspended in bicarbonate buffer. The concentration of spermatozoa was determined in the combined supernatants; in some experiments the first supernatant, consisting of diluted seminal plasma, was also used for incubation. In those experiments in which the washed cells had to be divided into a large number of samples, the cells were washed in one lot, resuspended in buffer and samples of the suspension were dispensed into the tubes used for incubation. In experiments in which the effect of varying cell concentration on the incorporation of [^{14}C]Chlorella hydrolysate was studied, 1, 2, 3 and 5 ml. volumes of the cell suspension were pipetted into separate tubes and each was made up to 5 ml. with fructose-bicarbonate buffer.

Suspensions of reticulocytes. A rabbit (3 kg.) was injected intravenously with 50 mg. of *N*-acetylphenylhydrazine in 5 ml. of 0.9% NaCl soln. each day for 5 days (Cruz, 1941). On the second day after the last injection, the reticulocyte content, as determined by the method of Cruz (1941), was 80–90% of all blood cells. Next day the rabbit was bled into sterile 0.9% NaCl soln. containing heparin. The cells were washed twice with 0.9% NaCl, twice with sterile bicarbonate buffer and then suspended in a volume of bicarbonate buffer equal to the original volume of blood. After determination of the cell count, the suspension was further diluted with the buffer to give the desired cell concentrations.

Incubations

Whole semen, spermatozoa and seminal plasma. Samples of semen, washed cell suspension or diluted seminal plasma (for quantities see individual Tables) were dispensed into 15 ml. round-bottomed centrifuge tubes and [^{14}C]Chlorella hydrolysate or [^{14}C]valine solution was added. The tubes were incubated at 37° with shaking (100 cyc./min.). Unless otherwise stated, all incubations were carried out in air or $\text{O}_2 + \text{CO}_2$ (95:5), the results being substantially the same in either case. At the appropriate time, samples were taken out for motility determinations and for bacterial counts, and to the remainder a sufficient volume of 30% (w/v) trichloroacetic acid was added to give a final concentration of 10%.

To check that incorporation was taking place solely in intracellular proteins, some semen samples (1.2 ml.) were diluted, after incubation, with 7.5 ml. of fructose-bicarbonate buffer. The diluted samples were centrifuged for 30 min. (regarded as part of the incubation period) and the supernatant was decanted and mixed with 4 ml. of 30% (w/v) trichloroacetic acid; the sedimented cells were suspended in 3 ml. of fructose-bicarbonate buffer and precipitated with 2 ml. of 30% trichloroacetic acid.

In other experiments, the effect of replacing seminal plasma with an equal volume of fructose-bicarbonate buffer was studied. The semen (0.75 ml.) was diluted with 5 ml. of fructose-bicarbonate buffer, centrifuged and the supernatant decanted. The inside of the tube was wiped with filter paper and the cells were resuspended in 0.55 ml. of fresh buffer together with 0.15 ml. of [^{14}C]Chlorella

hydrolysate and incubated at 37°. Proteins were precipitated by the method described for semen.

Reticulocytes. For experiments on the incorporation of [^{14}C]Chlorella hydrolysate, 2 and 8 ml. samples of the reticulocyte suspension were diluted to 24 ml. with bicarbonate buffer and each was incubated at 37° with 0.24 ml. of [^{14}C]Chlorella hydrolysate in an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5). At appropriate times, 5 ml. samples were taken out of each flask and mixed with 2 ml. of 30% (w/v) trichloroacetic acid. Finally, a 2 ml. sample was taken out for determination of protein content.

For the experiment on the incorporation of [^{14}C]valine, three samples (2 ml.) of the reticulocyte suspension were diluted to 18 ml. with bicarbonate buffer in three flasks, and 2 mg. and 20 mg. of non-radioactive valine was added to flasks 2 and 3, followed by 0.29 ml. of the [^{14}C]valine solution in all flasks. The mixture was incubated at 37° under $\text{O}_2 + \text{CO}_2$ (95:5) and, at appropriate time intervals, 4 ml. samples were removed from each flask and mixed with trichloroacetic acid.

In all experiments, samples for the zero-time point were precipitated with trichloroacetic acid immediately after addition of the radioactive amino acid.

Isolation of proteins

Semen, spermatozoa and seminal plasma. After precipitation with 30% (w/v) trichloroacetic acid, the mixture was left to stand for at least 15 min. It was then centrifuged, washed once with 5% trichloroacetic acid and once with 5% (w/v) NaCl. (With spermatozoa, the supernatants were invariably clear, but with seminal plasma or whole semen protein, the washes were often turbid. The finely suspended material in these washes could be removed by prolonged centrifuging at 5000 rev./min., but in general this was not done.) The residue obtained after the NaCl wash was dissolved in *N*-NaOH (1–3 ml., depending on the volume of semen used). The seminal plasma proteins went into solution easily at room temperature. The spermatozoon proteins dissolved in alkali solution only on heating at 70° for 1–2 min. Non-radioactive bovine serum-albumin hydrolysate (0.2 ml., corresponding to approx. 4 mg. of mixed amino acids) or valine (4 mg. in 0.2 ml.), as appropriate, was added and the mixture centrifuged to remove alkali-insoluble material, normally present in trace amounts. The alkali solution was treated with an equal volume of 30% (w/v) trichloroacetic acid. The precipitate was washed once with 5% trichloroacetic acid, then heated with 5% trichloroacetic acid at 90° for 20 min., washed once again with 5% trichloroacetic acid at room temperature and, to remove lipids, was washed twice with ethanol, twice with acetone, twice with ethanol-ether (3:1, v/v) and finally once with ether. All washings were done with 4–5 ml. of the wash fluid.

Reticulocytes. The precipitate obtained on addition of trichloroacetic acid to the incubation mixture was washed once with 5% (w/v) trichloroacetic acid and then with 5 ml. of acetone containing a few drops of 30% trichloroacetic acid at the boiling point of the mixture (to remove porphyrin). The residue was heated with 5% trichloroacetic acid at 80° for 20 min. and then dissolved in *N*-NaOH containing an excess of serum-albumin hydrolysate. The alkaline solution was mixed with an equal volume of 30% (w/v) trichloroacetic acid and the precipitate was washed twice with 5% trichloroacetic acid, once with ethanol and once with

ethanol-ether (3:1, v/v). The entire process from dissolution in alkali onwards was then repeated, and the proteins obtained were finally washed with ether.

Measurement of radioactivity

Protein was plated as a slurry in 30% (v/v) ethanol (ether for reticulocyte protein) on preweighed 2 cm.² aluminium planchets. After drying over CaCl_2 the plates were reweighed. Radioactivity was determined in a thin-window Geiger-Müller counter. A standard Perspex disk of infinite thickness, 1 cm.² area and specific activity $10^{-3} \mu\text{Ci}/\text{mg.}$, gave 1000 counts/min. on this counter. Corrections for self-absorption were made with the data given for wax by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). Specific activities are expressed as counts/min./mg. of protein. A small variable amount of radioactivity was always found associated with proteins obtained at zero time (i.e. after addition of radioactive amino acids followed by immediate precipitation). Unless otherwise stated, specific activity at zero time has been subtracted from the values obtained at other times.

With [^{14}C]valine, a specific activity of 1000 counts/mg. of protein corresponds to an incorporation of $0.275 \mu\text{mole}$ of radioactive amino acid/g. of protein.

Treatment of proteins with performic acid. Performic acid (1 ml.; prepared by the method of Hirs, 1956) was added to labelled spermatozoon protein in solution in formic acid at 0°. The mixture was held at 0° for 2 hr. and then mixed with 0.5 ml. of either casein hydrolysate solution or valine solution (both containing about 10 mg. of amino acid) as appropriate. It was then dried *in vacuo* at 0° over P_2O_5 ; the residue was dissolved in *N*-NaOH, precipitated with trichloroacetic acid and the precipitate was washed and dried in the usual way.

RESULTS

Incorporation of mixed [^{14}C]amino acids into bull spermatozoa

The incorporation of [^{14}C]Chlorella hydrolysate into the proteins of washed spermatozoa was usually linear for about 2 hr. The results of a typical experiment are given in Fig. 1. Considerable variations in incorporation rate were observed from experiment to experiment, and the results suggest (Table 1) that there may have been a seasonal change in rate. By contrast, the incorporation of amino acids from [^{14}C]Chlorella hydrolysate into whole semen proceeded linearly for only 20 min. and then ceased. Fig. 2 gives the results of a typical experiment with bull N_1 . Variations between experiments with bull N_1 over a period of 6 months are given in Table 2, along with results of experiments with semen from four other bulls (N_2 , N_3 , N_4 and N_5). As can be seen from Table 2, there was only one exceptional experiment, during which incorporation continued for 2 hr.

There was no incorporation in diluted seminal plasma (Fig. 1). Since seminal plasma freed from spermatozoa did not incorporate radioactive amino acids, it seemed likely that incorporation of amino

Table 1. Variation in the rate of incorporation of [^{14}C]Chlorella hydrolysate into the proteins of washed bull spermatozoa

For details of incubation and the method of isolating proteins see Fig. 1 and text. In all experiments except no. 28 the initial motility was good, and was maintained nearly unchanged for 3 hr. In Expt. 28 the initial motility was poor, and was nil at 3 hr.

Expt. no.	Date	Cell concentration (millions/ml.)	Concn. of [^{14}C]amino acid (mM)	Specific activity of proteins (counts/min./mg.)				
				1 hr.	1½ hr.	3 hr.	3½ hr.	4½ hr.
1	20. xii. 56	Not counted	0.050	422	—	—	—	—
2	10. i. 57	210	0.050	254	—	583	—	708 (5 hr.)
28	9. v. 57	235	0.075	183	—	294	—	—
34	29. v. 57	155	0.075	—	173	240	—	—
44	3. vii. 57	268	0.075	—	70	—	153	162
59	7. viii. 57	188	0.075	—	52	89	—	—
60	7. viii. 57	188	0.075	—	52	—	129	142
61	23. i. 58	Not counted	0.075	86	—	135	—	—
62	27. iii. 58	157	0.075	51	103 (2 hr.)	—	—	—

Fig. 1. Incorporation of [^{14}C]Chlorella hydrolysate into the proteins of washed spermatozoa (Δ) and diluted seminal plasma (○) from bull N₁. For each time point, 4.5 ml. of a suspension of washed cells (210×10^6 cells/ml.) or diluted seminal plasma was incubated with 0.05 ml. of the [^{14}C]Chlorella hydrolysate. Proteins were isolated as described in the text. Specific activities have not been corrected for radioactivity at zero incubation time.

Fig. 2. Incorporation of [^{14}C]Chlorella hydrolysate into the proteins of bull semen (○, ethanol-insoluble proteins; Δ, ethanol-soluble proteins). Semen (1.2 ml. for each time point; 1220×10^6 cells/ml.) from bull N₁ was incubated with 0.3 ml. of [^{14}C]Chlorella hydrolysate and proteins were isolated as described in the text.

acids into whole semen was also exclusively intracellular. To confirm this, spermatozoa and seminal plasma were separated by centrifuging after various periods of incubation and the two kinds of protein examined separately. Radioactivity was found in proteins from spermatozoa whereas no appreciable uptake was observed in those from seminal plasma (Table 3).

In one experiment (Fig. 3) with semen from bull N₁, the seminal plasma was removed and the spermatozoa were incubated with [^{14}C]Chlorella hydrolysate in a volume of fructose buffer equal to that of the original semen. The results, which

resemble those with washed cells, show that the differences in the pattern of incorporation observed between whole semen and washed spermatozoa are not caused by differences in cell concentration. It was found in other experiments that dilution of semen with buffer shifted the pattern of incorporation from that characteristic of whole semen towards that characteristic of the washed cells.

Incorporation of valine

Since studies on protein synthesis in other systems have usually been carried out with a single labelled amino acid, we have studied also the incorporation of valine. The incorporation of [^{14}C]valine (Fig. 4) into the proteins of washed spermatozoa, was similar (except for an initial lag) to that

Table 2. Variations between experiments in the incorporation of [¹⁴C]Chlorella hydrolysate into the proteins of bull semen

Semen (0.3–1.2 ml.) was incubated with 25 % (in Expt. 11, 33 %) of its own volume of [¹⁴C]Chlorella hydrolysate. The proteins were isolated as described in the text. All experiments with bull N₁ were started within 1½ hr. of collection of the semen. The experiments with bulls N₃–N₆ were begun about 4 hr. after collection.

Expt. no.	Date	Bull	Cell concentration (millions/ml. of semen)	Specific activity of proteins (counts/min./mg.)					
				15 min.	30 min.	1 hr.	2 hr.	3 hr.	4 hr.
5	28. ii. 57	N ₁	1220	284*	288	282	—	—	—
7	18. iii. 57	N ₁	1100	—	46	48	—	—	—
11	29. iii. 57	N ₁	Not counted	—	210	—	—	—	297
12	4. iv. 57	N ₁	1280	—	171	—	168†	—	191
24	2. v. 57	N ₁	1670	—	51	56	—	48‡	—
30	22. v. 57	N ₁	2430	—	81	138	186	181	191
37	1. vi. 57	N ₁	1340	—	—	51	—	57	—
42	1. vii. 57	N ₂	760	45	49	53	—	—	—
42	1. vii. 57	N ₃	570	58	58	56	—	—	—
42	1. vii. 57	N ₄	1650	30	42	41	—	—	—
42	1. vii. 57	N ₆	1470	29	32	—	—	—	—

*

20 min.

†

1½ hr.

‡

2½ hr.

Table 3. Radioactivity in the proteins of spermatozoa and of seminal plasma after incubation of normal semen with [¹⁴C]Chlorella hydrolysate

Semen from bull N₁ (1.2 ml. for each time point, 1540 × 10⁶ cells/ml.) was incubated with 0.3 ml. of [¹⁴C]Chlorella hydrolysate and the spermatozoa were separated by centrifuging. Proteins were isolated as described in the text.

Time of incubation (hr.)	Specific activity of proteins (counts/min./mg.)	
	Spermatozoa	Seminal plasma*
0	0	0
½	600	0
1½	593	49
3½	618	70
5	520	70
6½	579	70
8	583	70

* It is difficult to obtain a plasma sample entirely free from spermatozoa and the small residual radioactivity found is due at least in part to contamination of the plasma with cells (cf. Fig. 1).

obtained with [¹⁴C]Chlorella hydrolysate. Variation in the rate of valine incorporation in three experiments is shown in Table 4. The incorporation of [¹⁴C]valine into proteins of whole semen was almost negligible, as shown in Table 5.

Effect of storage of semen

During the course of this work, it occurred to us that some of the variation encountered from experiment to experiment in the extent of incorporation of radioactive amino acids into spermatozoa might be caused by differences in the time elapsing between the collection of the semen and the beginning of the experiment, or by differences in the concentration of the cells in the

Fig. 3. Effect of replacing seminal plasma with an equal volume of fructose–bicarbonate buffer on the incorporation of [¹⁴C]Chlorella hydrolysate into the proteins of bull spermatozoa. For each time point, cells from 0.6 ml. of semen (1840 × 10⁶ cells/ml.) were suspended in 0.55 ml. of fructose–bicarbonate buffer and incubated with 0.15 ml. of [¹⁴C]Chlorella hydrolysate. Proteins were isolated as described in the text.

suspensions used. Table 6 shows fluctuations in the rate of amino acid incorporation after different periods of storage of semen at room temperature. Fig. 5 shows that the concentration of the cells in the incubation mixture is one factor influencing the rate of uptake of radioactive amino acids, the two parameters being inversely related.

Ethanol-soluble proteins

The recovery of protein per cell, after precipitation with trichloroacetic acid and removal of lipids and DNA, varied from experiment to experiment. These differences were caused by extraction of varying amounts of protein by organic solvents

Table 4. Variation in the rate of incorporation of [^{14}C]valine into the proteins of washed spermatozoa

For details of incubation and method of isolating proteins see text.

Expt. no.	Date	Cell concentration (millions/ml.)	Concn. of [^{14}C]valine (mM)	Specific activity of proteins (counts/min./mg.)				
				$\frac{1}{2}$ hr.	1 hr.	2 hr.	3 hr.	4 hr.
15	10. iv. 57	—	0.150	7	150	231	—	—
20	23. iv. 57	113	0.100	8	100	160	—	—
45	9. vii. 57	148	0.075	7	24	42	65	73

Fig. 4. Incorporation of [^{14}C]valine into the proteins of washed spermatozoa. For each time point, 3 ml. of the cell suspension (148×10^6 cells/ml.) and 0.05 ml. of [^{14}C]valine solution was used. Proteins were isolated as described in the text.Table 5. Incorporation of [^{14}C]valine into the proteins of normal semen (bull N_1)

In Expts. 14 and 46, 0.6 and 0.3 ml. of semen was used respectively. After incubation with [^{14}C]valine, the proteins were isolated as described in the text. In Expt. 14, 0.1 ml. of [^{14}C]valine solution was used; in Expt. 46, 0.075 ml. was used.

Time (hr.)	Specific activity of proteins (counts/min./mg.)	
	Expt. 14	Expt. 46
0	0	0
$\frac{1}{2}$	2.7	6.7
1	3.9	—
$1\frac{1}{2}$	—	6.2
3	4.9	8.7

(mostly by ethanol) used for removing lipids (cf. Bhargava *et al.* 1959). The protein extracted in this way could be rendered insoluble in ethanol by repeated dissolution in ethanol and evaporation of the solvent at 80° . The protein had much the same specific activity as the ethanol-insoluble protein (Fig. 2). In whole semen the seminal plasma usually accounted for about half the total protein.

Table 6. Effect of storage of whole semen on the incorporation of [^{14}C]Chlorella hydrolysate into proteins of washed normal spermatozoa

Spermatozoa were stored for the indicated times in whole semen at room temperature, then washed and incubated for 2 hr. with [^{14}C]Chlorella hydrolysate (0.1 ml.) and the proteins isolated as described in the text. In Expt. 1, 1570×10^6 cells were suspended in 3.3 ml. of fructose-bicarbonate buffer for each time point; in Expt. 2, 3.6 ml. of buffer contained 1656×10^6 cells.

Storage time (min.)	Specific activity of proteins (counts/min./mg.)		
	Expt. 1	Expt. 2	
	Ethanol-insoluble proteins	Ethanol-insoluble proteins	Ethanol-soluble proteins*
10	—	180	—
30	156	—	—
75	219	—	—
90	—	129	142
120	153	—	—
180	—	304	283
210	142	—	—
360	93	—	—

* From the combined lipid washes (see text). This material was shown to be protein by nitrogen estimation and estimation of total amino acids after hydrolysis.

Incorporation of [^{14}C]amino acids into reticulocytes

Reticulocytes are known to have a high RNA content and (as measured by radioactive amino acid incorporation) a high rate of protein turnover (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1952).

Our observations on uptake of [^{14}C]Chlorella hydrolysate and of [^{14}C]valine by rabbit reticulocytes under conditions similar to those used for washed spermatozoa are shown in Figs. 6 and 7. For reticulocytes suspended in buffer at a total protein concentration of 3 mg./ml. we obtained valine incorporation rates of 0.36, 0.43 and $0.47 \mu\text{mole}$ of amino acid/g. of protein/hr., with 0.095, 0.996 and $9.94 \mu\text{moles}$ of valine/ml., respectively. This is in fair agreement with results obtained by the above-mentioned workers. With [^{14}C]Chlorella hydrolysate ($0.045 \mu\text{mole}$ of labelled amino acids/ml. and with a reticulocyte suspension

containing 2.35 mg. of protein/ml.), an incorporation rate of 0.75 μ mole of labelled amino acids/g. of protein/hr. was observed. Reticulocytes resembled washed spermatozoa in that a decrease in cell concentration was accompanied by an increased rate of incorporation (Fig. 6).

Effect of inhibitors

Since protein synthesis by spermatozoa in the absence of RNA was unexpected, the effects of known inhibitors of protein synthesis were studied, in an effort to evaluate the significance of the observed amino acid incorporation. As can be seen from Table 7, ribonuclease and azaguanine were without effect, whereas fluoride had a slight inhibitory effect on incorporation of mixed [¹⁴C]-amino acids into the proteins of washed cells but no effect in whole semen. Iodoacetate had relatively little effect under aerobic conditions but when incubations were in nitrogen it was an effective inhibitor. Chloramphenicol showed a limited inhibitory effect on both whole semen and washed cells.

p-Fluorophenylalanine, 2:4-dinitrophenol, sodium fluoride and iodoacetate inhibited the motility of spermatozoa whereas the other compounds had

Fig. 5. Effect of cell concentration on the incorporation of [¹⁴C]Chlorella hydrolysate into the proteins of washed spermatozoa. All incubations were for 2 hr. in a volume of 3 ml., with 0.05 ml. of [¹⁴C]Chlorella hydrolysate. For details, see text. Specific activities of proteins have not been corrected for radioactivity at zero incubation time. A cell concentration of one unit is equivalent to 80 $\times 10^6$ /ml.

Table 7. Effect of various metabolic inhibitors and inhibiting conditions on the incorporation of [¹⁴C]Chlorella hydrolysate into the proteins of bull spermatozoa

Inhibitor	Gas phase	Whole semen					
		1 $\frac{1}{2}$ hr.		1 hr.		3 hr.	
		Change in specific activity of proteins (%)	Change in motility (%)	Change in specific activity of proteins (%)	Change in motility (%)	Change in specific activity of proteins (%)	Change in motility (%)
None	O ₂	+22	None	—	—	—	—
None	N ₂	-14	None	None	-40	None	None
Ribonuclease	O ₂	None	None	None	-17	None	None
8-Azaguanine	O ₂	None	-33	None	-17	None	None
Chloramphenicol	O ₂	None	-14	-35	None	-27	-50
p-Fluorophenylalanine	O ₂	-13	-100	None	-70	None	-100
Dinitrophenol	O ₂	None	-100	+20	-17	None	-100
Fluoride	O ₂	None	-86	None	-100	None	-100
Fluoride	N ₂	-17	-100	None	-100	None	-85
Iodoacetate	O ₂	None	-100	+24	-100	+70	-100
Iodoacetate	N ₂	-52	-100	-41	-100	-16	-100

The whole semen (0.3 ml.) or washed cell suspension in fructose-bicarbonate buffer (3 ml.) was incubated with inhibitor and 0.05 ml. of the Chlorella hydrolysate as described in the text. Inhibitors were present in the following final concentrations: ribonuclease, 100 μ g./ml.; 8-azaguanine, 50 μ g./ml. (0.33 mm); chloramphenicol, 50 μ g./ml. (0.15 mm); p-fluorophenylalanine, 1.83 mg./ml. (10 mm); dinitrophenol, 184 μ g./ml. (1 mm); fluoride, 10 mm; iodoacetate, 5 mm. Anaerobic incubations were done under nitrogen, controls were incubated in air. Inhibition (—) or stimulation (+) is given as a percentage of the control at the appropriate time.

little effect. The inhibiting effect of fluoride and iodoacetate on the motility of spermatozoa has been described earlier (Lardy & Phillips, 1943; Mann & Lutwak-Mann, 1948). Anaerobic conditions did not affect motility, which also is in accord with earlier observations (Ivanov, 1931; Redenz, 1933). In the presence of inhibitors there appeared to be no correlation between the degree of motility and the rate of incorporation of amino acids.

Bacterial contamination

Since bull semen usually contains small numbers of bacteria (Dott & Glover, 1954), it was necessary to preclude the possibility that the incorporation of radioactive amino acids observed in our experiments with spermatozoa was caused by growth of bacteria. In several experiments the number of bacteria present initially, and after various periods of incubation of the washed cell suspension, was determined. The semen of bull N₁ was found to contain about 5000 bacteria/ml., and there was no detectable increase in this number during incubation for periods up to 2 hr. Since 1 ml. of semen contains about 1000 million spermatozoa and since the rate of incorporation was initially fairly high and fell throughout the incubation period, it is very unlikely that the observed incorporation can be due to the small number of bacteria present.

Fig. 6. Incorporation of [¹⁴C]Chlorella hydrolysate into the proteins of suspensions of rabbit reticulocytes at two levels of cell concentration; ○, 22×10^6 cells/ml. (2.35 mg. of protein/ml.); △, 88×10^6 cells/ml. (9.4 mg. of protein/ml.); 0.1 ml. of [¹⁴C]Chlorella hydrolysate was added for each 10 ml. of cell suspension. For details, see text.

DISCUSSION

The experiments described in this paper continued over about 1 year. During that time, the rate of amino acid incorporation varied within fairly wide limits but the value derived from nine experiments with washed spermatozoa and [¹⁴C]Chlorella hydrolysate as source of radioactive amino acid was about 0.035 μ mole/g. of protein/hr. This is about one-twentieth of the rate of incorporation obtained with washed reticulocytes under similar conditions. When whole semen was used instead of washed cells, the initial rate of incorporation was greater (after allowance for the weight of inert seminal plasma protein) but incorporation continued for only 20 min. (Fig. 2). We cannot at present offer any explanation of this abrupt cessation of incorporation into the cell fraction of whole semen. With washed spermatozoa and [¹⁴C]valine, the mean rate of incorporation was again between one-tenth and one-twentieth (Table 4) of that obtained with reticulocytes under similar conditions. In whole semen, however, the rate of incorporation of valine was much lower than in

Fig. 7. Incorporation of [¹⁴C]valine into the proteins of rabbit reticulocytes at three levels of valine concentration (○, 0.095 mM; △, 0.996 mM; □, 9.94 mM). Each flask contained 0.11 ml. of the [¹⁴C]valine solution, the appropriate amount of [¹²C]valine and 270×10^6 cells in 10 ml. Specific activity values have been adjusted for the dilution by [¹²C]valine (see text).

washed cells. We are unable to offer any explanation for the failure of spermatozoa in whole semen to incorporate valine at a significant rate.

The amino acid incorporation observed in these experiments is clearly not caused by physical adsorption, since there was no reduction in the specific activity of protein after treatment with either performic acid or sodium hydroxide. Although this indicates that the radioactive amino acids are linked to the protein fraction by covalent bonds, it in no way proves that the process is one of protein synthesis. Additional evidence on the nature of the incorporation process was sought by the use of compounds known to act in other biological systems as effective inhibitors of protein synthesis. Of the inhibitors tried, only iodoacetate under anaerobic conditions produced substantial inhibition of incorporation. The inhibitor tests thus suggest that although the incorporation is a true metabolic reaction, it is not the usual process of protein synthesis. Spermatozoa differ in many respects from other mammalian cells, especially in their nucleic acid, nucleotide and amino acid composition (Bhargava *et al.* 1959); thus the incorporation of amino acids into the proteins of spermatozoa may represent a process not normally detected in other mammalian cells. Carboxyl group activation of amino acids by enzymes from rat liver is not inhibited by either fluoride or by dinitrophenol (Rendi, Di Milia & Fronticelli, 1958). If similar enzymes function in spermatozoa, an inhibitor-resistant amino acid activation could lead to labelling of reactive sites on spermatozoon proteins.

In both spermatozoa and reticulocytes the rate of amino acid incorporation is inversely related to the concentration of cells. In each case the amount of radioactive amino acid incorporated represented a very small fraction of the total amino acids present in the medium. The observed effect of cell concentration cannot therefore be attributed to competition for amino acids. There is, however, a variable dilution of added [^{14}C]amino acid by [^{12}C]amino acids of the cells and also appreciable breakdown of spermatozoon protein during incubation (Bhargava *et al.* 1959). These two factors together could explain the inverse relationship between cell concentration and uptake of [^{14}C]amino acid, but since both respiration and fructolysis of bull spermatozoa are also inversely related to cell concentration the situation is obviously very complex (cf. Bishop & Salisbury, 1955*a, b*).

In conclusion, we wish to mention some additional experiments on amino acid incorporation that were carried out with decapitate spermatozoa. The adjective decapitate is used to describe spermatozoa in which the head is disconnected from the tail at the neck (Bishop & Walton, 1959). This con-

dition occurs spontaneously in all the spermatozoa of certain sterile Guernsey bulls (Hancock & Rollinson, 1949; Hancock, 1955). Most of the heads, as judged by their staining reactions, appear to be dead at ejaculation, but most of the tails remain actively motile for several hours *in vitro*. The tails can be separated from the heads by differential centrifuging, and are devoid of either DNA or RNA. Experiments similar to those described earlier in this paper were performed with decapitate spermatozoon tails from three Guernsey bulls. The rates of incorporation of amino acids into protein in these preparations were substantial but with all three bulls the bacterial content of the 'decapitate'-sperm semen was much greater than that of normal bull semen and the interpretation of our results therefore remains equivocal. These results are mentioned here because they have obtained some currency through oral communications and because the 'decapitate' condition is rather rare, and unless bulls can be found producing 'decapitate'-sperm semen which is free of bacteria further progress along these lines will not be possible.

SUMMARY

1. The incorporation of radioactive amino acids into the proteins of bull semen has been measured.
2. In whole semen, the incorporation is exclusively into the spermatozoon protein.
3. In suspensions of washed spermatozoa, incorporation continues for several hours but in whole semen incorporation stops after about 20 min.
4. The incorporation reaction has been compared with that observed in reticulocytes incubated under similar conditions. The rate of incorporation into spermatozoon protein is about one-twentieth of that into reticulocyte protein.
5. Small numbers of bacteria were always present in semen but their presence did not account adequately for the observed incorporation.
6. The rate of incorporation into both spermatozoa and reticulocytes was inversely related to the concentration of cells present.
7. The effect of various inhibitors on the incorporation reaction was examined.
8. It is concluded that the incorporation reaction in spermatozoa is unlikely to be a true measure of protein synthesis.

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Incorporation of ^{65}Zn in the Sub-cellular Fractions of the Liver and Spontaneously Occurring Mammary Tumours of Mice after the Injection of Zinc-Glycine Containing ^{65}Zn

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Relatively little is known about the intracellular distribution of zinc in normal and tumour tissues. Heath (1949) and Heath & Liquier-Milward (1950) obtained evidence that zinc in tumour tissue is associated with nucleoprotein. Their interpretation of the results was, however, complicated by the fact that, since they adopted the method of Dounce (1943), citric acid was used in the isolation of the cell nuclei and it is certain that much or all of the zinc present would be leached out by this acid. Rosenfeld & Tobias (1951) could obtain no evidence for the association of zinc with nucleoprotein in the cell nucleus, but these workers also used a citric acid method (that described by Mirsky & Pollister, 1946) for the isolation of cell nuclei. In investigations on normal rat liver Thiers & Vallee (1957) found that the zinc was present in the nuclear, mitochondrial and 'supernatant' fractions, the lowest concentration being in the mitochondria and the highest in the supernatant and microsomal fractions.

An increased uptake of ^{65}Zn by spontaneous mammary tumours compared with normal mammary tissue was found in female mice injected with the isotope as a zinc-glycine complex (Tupper, Watts & Wormall, 1955). A similar increased

uptake of ^{65}Zn has been found in skin tumours induced in mice by 3:4-benzpyrene if the tumours are compared with normal skin (M. E. Bartholomew & R. Tupper, unpublished work). This difference in zinc metabolism between tumours and their normal tissue of origin led us to investigate more fully the binding of zinc in spontaneous mammary tumours of mice, and this paper records the results of our studies on the distribution of injected zinc in the various sub-cellular fractions separated from spontaneous mammary tumours of mice injected with zinc-glycine containing ^{65}Zn . For comparison, similar measurements have been made in the corresponding fractions of the livers of the same mice. The choice of the liver for this purpose was influenced by the fact that there is available a considerable amount of information about the intracellular particles of this organ, and furthermore the liver contains at least two zinc-containing enzymes in which we are particularly interested; at least one of these, glutamic dehydrogenase, is present in the mitochondria.

A preliminary study has also been made on the stability of the zinc compounds in these tissues, and the extent to which the zinc can be removed by prolonged dialysis at different pH values has been