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The Uptake of Radioactive Amino Acids by Spermatozoa

THE INTRACELLULAR SITE OF INCORPORATION INTO PROTEINS

BY K. A. ABRAHAM AND P. M. BHARGAVA
Regional Research Laboratory, Hyderabad, 9, India

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It has been shown that bull semen and washed spermatozoa can incorporate radioactive amino acids into their proteins in spite of the absence of any chemically detectable RNA in the spermatozoa (Bhargava, 1957; Bhargava, Bishop & Work, 1959; Martin & Brachet, 1959). We have obtained evidence for the presence of a small amount of metabolically active RNA in bovine spermatozoa (Abraham & Bhargava, 1963); apparently, these spermatozoa can bring about net synthesis of this RNA. If the incorporation of amino acids into the proteins of spermatozoa represents the formation of new protein molecules, it is possible that the information necessary for this synthesis is obtained from the DNA of the cell through this metabolically active RNA; recent work strongly suggests that a special type of RNA, which has a high metabolic activity, is involved in the transfer of hereditary information from DNA to proteins (Gros *et al.* 1961; Brenner, Jacob & Meselson, 1961). Spermatozoa may thus provide a useful system for a study of the exact mechanism of the transfer of information from DNA to protein, since the dilution of the active RNA that may mediate the transfer with non-active RNA would be much less in this system than is usually obtained in most other systems. In view of the possible importance of protein synthesis by spermatozoa, it was considered desirable to gain further information on the nature of the incorporation of radioactive amino acids into the proteins of spermatozoa. In this paper we present evidence that strongly suggests that the above-mentioned incorporation occurs in the acrosomal region of the spermatozoa.

Since so far there have been only two reports on the incorporation of isotopically labelled amino acids into the proteins of spermatozoa, and since both these reports have been confined to bull spermatozoa, it was considered desirable to determine whether other mammalian spermatozoa which contain a normal acrosome, and fowl spermatozoa in which the acrosome is either absent or is strongly modified (P. T. Iype, K. A. Abraham & P. M. Bhargava, unpublished work), are capable of incorporating amino acids into their proteins. We have therefore extended some of the earlier studies, on the incorporation of amino acids into bull spermatozoa, to buffalo, goat and cock spermatozoa; the spermatozoa of buffalo, a species closely related to bull, have been earlier shown to differ from those of bulls in several ways, for example, in their preservability (Perry, 1960). The incorporation of ^{14}C-labelled amino acids into the proteins of buffalo and goat spermatozoa was found to follow the same pattern as is obtained with bull spermatozoa (Bhargava *et al.* 1959), and no significant incorporation was obtained with cock spermatozoa.

EXPERIMENTAL

Materials

Semen. Mammalian spermatozoa used in these studies were obtained as described by Abraham & Bhargava (1963), from two buffaloes (BB1 and BB2), two bulls (CB1 and CB2) and one goat. Fowl semen was collected from 12 White Leghorn cocks by the massage technique of Burrows & Quinn as described by Perry (1960). All the animals were of proven fertility. All semen samples were examined under a phase-contrast microscope and only those which had a good motility and which were free from any visible contamination were used, always within 2 hr. of collection. Spermatozoa counts in the semen or in the washed cell suspensions were made on a haemocytometer.

Radioactive chemicals. A solution (referred to below as ^{14}C-labelled *Chlorella* hydrolysate) containing a mixture of ^{14}C-labelled amino acids was prepared either from generally ^{14}C-labelled *Chlorella* protein (specific activity 100 μc/mg.) as described by Bhargava *et al.* (1959), or directly from freeze-dried ^{14}C-labelled *Chlorella* protein hydrolysate. In the former case, the solution contained about 4·9 μmoles of amino acids and 50 μc/ml., and in the latter case about 1·9 μmoles of amino acids and 22·5 μc/ml. Generally labelled [^{14}C]valine was dissolved in iso-osmotic salt solution to a concentration of 9·3 μmoles and 11·4 μc/ml. All the radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks.

Metabolic inhibitors. The following inhibitors were used: ribonuclease (crystalline, C. F. Boehringer und Soehne), chloramphenicol (Chloromycetin; Parke, Davis & Co. Ltd.), 2,4-dinitrophenol, sodium fluoride and sodium iodoacetate. The addition of inhibitors to the incubation medium was carried out as described by Bhargava *et al.* (1959).

Buffers. Either Krebs–Ringer original bicarbonate buffer or Krebs–Ringer original phosphate buffer, each containing 1% (w/v) of fructose as in the preceding paper (Abraham & Bhargava, 1963), was used for preparation of the spermatozoa suspensions and subsequent incubation.

These buffers are referred to subsequently as the 'fructose–bicarbonate buffer' and the 'fructose–phosphate buffer' respectively.

Methods

Preparation of cell suspensions. Suspensions of washed mammalian spermatozoa were prepared in one of the above-mentioned buffers as described by Bhargava *et al.* (1959). To obtain a suspension of cock spermatozoa, 1 ml. of the semen was diluted immediately after collection with 5 ml. of the fructose–phosphate buffer, and centrifuged at 500*g* for 10 min. within an hour of dilution, to sediment blood cells and other contaminants which are usually present in fowl semen. The supernatant was centrifuged at 1000*g* for 10 min.; the residue was resuspended in 5 ml. of the above-mentioned buffer and centrifuged again at 500*g* for 10 min. The spermatozoa in the supernatant were sedimented by further centrifuging at 1000*g* for 10 min., and resuspended in 3·5 ml. of the fructose–bicarbonate buffer. Spermatozoa suspensions used for incubation contained 300–1000 $\times 10^6$ cells/ml.

Incubations. Whole semen or a suspension of washed spermatozoa (1–2 ml.) was incubated aerobically either with ^{14}C-labelled *Chlorella* hydrolysate (0·1 ml./ml. of the semen or suspension) or with the solution of [^{14}C]valine (0·05 ml./ml. of the semen or suspension) as described by Abraham & Bhargava (1963). Anaerobic incubations were carried out under a stream of moist 100% nitrogen with shaking. In some experiments the spermatozoa, after incubation as described above, were washed and subjected to further incubation at 37°, or were stored at 0° before precipitation with trichloroacetic acid. Details of such treatments are in the text.

Isolation of proteins. Whole semen or a suspension of washed spermatozoa was precipitated with trichloroacetic acid to a final concentration of 20% (w/v), and the total proteins were isolated as described by Bhargava *et al.* (1959). In some experiments the step involving dissolution in 1 N-NaOH and reprecipitation with trichloroacetic acid was omitted.

Radioactivity assay. Protein was made into a slurry in 50% ethanol, plated on tared aluminium planchets (2 cm.^{2}) and dried in an air oven. The radioactivity was determined as described by Abraham & Bhargava (1963). Corrections for self-absorption were made according to Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). Specific activities are expressed as counts/min./mg. of protein.

RESULTS

Incorporation of a mixture of ^{14}C-labelled amino acids into bull, buffalo and goat spermatozoa. There have been only two previous reports on the incorporation of labelled amino acids into the proteins of bull spermatozoa (Bhargava *et al.* 1959; Martin & Brachet, 1959). In both the earlier investigations the animals were maintained under conditions, for example climatic, which are different from those obtaining in India; this is known to result in significant variations in the functional potency of spermatozoa. For example, the seasonal variation in the fertilizing capacity of bull spermatozoa is much more marked in India than in Europe. It was therefore considered advisable first to determine the pattern and the extent of the incorporation of labelled amino acids into the proteins of bull spermatozoa derived from animals of indigenous breed (unknown pedigree), maintained under local conditions, where the animals are exposed to extremes of climate, before proceeding to study the species variation in this regard. The incorporation of ^{14}C-labelled *Chlorella* hydrolysate into the proteins of spermatozoa derived from several batches of semen of two such bulls, and the effect of chloramphenicol and ribonuclease on this incorporation (Table 1), was found to be qualitatively and quantitatively similar to that reported by Bhargava *et al.* (1959). The inhibitions by anaerobiosis and by fluoride, iodoacetate and dinitrophenol under both aerobic and anaerobic conditions (Table 1) were significantly greater than those observed by Bhargava *et al.* (1959). Some of these differences may be related to the fact that air was used as the gas phase for incubation in the present study, in contrast with oxygen used by Bhargava *et al.* (1959). These workers showed that oxygen stimulated the incorporation of amino acids into proteins and could therefore partly counteract the action of the inhibitor. Dinitrophenol was found by them to have a slightly stimulatory effect on the incorporation under oxygen. In the present study, dinitrophenol inhibited incorporation by

Table 1. *Effect of various inhibitors on the incorporation of ^{14}C-labelled* Chlorella *hydrolysate into the proteins of bull spermatozoa*

A portion (2 ml.) of a suspension (in the fructose–bicarbonate buffer) of washed spermatozoa from semen (bull CB 1) was incubated with the inhibitor and 0·2 ml. (0·98 μmole of mixed amino acid and 10 μc) of ^{14}C-labelled *Chlorella* hydrolysate as described in the text. Inhibitors were present in the following final concentrations: ribonuclease, 100 μg./ml.; chloramphenicol, 100 μg./ml.; dinitrophenol, 1 mM; sodium fluoride, 10 mM; sodium iodoacetate, 5 mM. The control was incubated in air. Proteins were isolated as described in the text.

Inhibitor	Gas phase used for incubation	Inhibition of incorporation into protein (% of control)*	
		1½ hr.	3 hr.
None	N_2	46	50
Fluoride	Air	42	7
Fluoride	N_2	48	51
Iodoacetate	Air	28	54
Iodoacetate	N_2	52	66
Dinitrophenol	Air	49	38
Dinitrophenol	N_2	52	63
Ribonuclease	Air	4	2
Chloramphenicol	Air	6	9

* Incorporation in the control was 81 and 134 counts/min./mg. of protein at 1½ and 3 hr. respectively.

50%; under anaerobic conditions, which were not tried previously, the inhibition was even greater (Table 1).

The general pattern and the extent of the incorporation of ^{14}C-labelled *Chlorella* hydrolysate into the proteins of buffalo and goat spermatozoa was similar to that reported earlier for bull spermatozoa (Bhargava *et al.* 1959). The results of two typical experiments, one each with washed buffalo and goat spermatozoa, are given in Fig. 1. Semen from two buffaloes gave nearly the same incorporation values, provided that the initial motility of the spermatozoa was of the same order.

Incorporation of [^{14}C]*valine into buffalo spermatozoa.* The pattern of incorporation of [^{14}C]valine into the proteins of buffalo spermatozoa (Fig. 2) was similar to that obtained with ^{14}C-labelled *Chlorella* hydrolysate. The extent of incorporation was of the same order as obtained earlier with bull spermatozoa (Bhargava *et al.* 1959).

Incorporation of a mixture of ^{14}C*-labelled amino acids into cock spermatozoa.* Cock spermatozoa did not incorporate the radioactive amino acids into their proteins on incubation up to 90 min. (Table 2). The specific activity of the proteins obtained after 3 hr. incubation was 7, in contrast with values of 150–1000 obtained with mammalian spermatozoa under similar conditions.

Metabolic stability of the proteins of spermatozoa labelled with ^{14}C*-labelled amino acids.* Washed buffalo spermatozoa were incubated for various times with ^{14}C-labelled *Chlorella* hydrolysate in the fructose–phosphate buffer, washed twice, each time with 10 ml. of the buffer containing 2 mg. of a mixture of non-radioactive amino acids/ml., and then reincubated in 5 ml. of the buffer for 0–90 min. Trichloroacetic acid was then added and the proteins were isolated. No release of radioactivity from the proteins occurred during the second incubation (Table 3).

In a similar experiment with whole bull semen there was again no significant release of radioactivity from the labelled cells on reincubation in a non-radioactive medium (Table 4). This result supports the finding (Bhargava *et al.* 1959) that there is little incorporation of labelled amino acids into the proteins of whole semen after the first 30 min. of incubation, in contrast with washed spermatozoa, into which the incorporation is linear for nearly 3 hr.

Release of radioactive proteins from spermatozoa after storage at 0° and rewarming. Since it is known

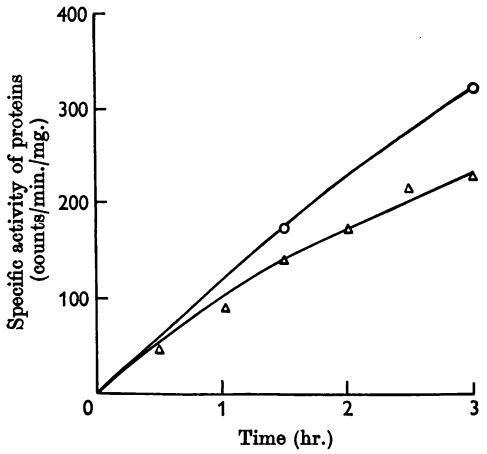

Fig. 1. Incorporation of ^{14}C-labelled *Chlorella* hydrolysate into the proteins of washed goat (○) and buffalo (BB1; △) spermatozoa. A portion (1 ml.) of a suspension of the spermatozoa in the fructose–bicarbonate buffer was incubated with 0·1 ml. (0·49 μmole of mixed amino acids and 5 μc) of ^{14}C-labelled *Chlorella* hydrolysate, as described in the text.

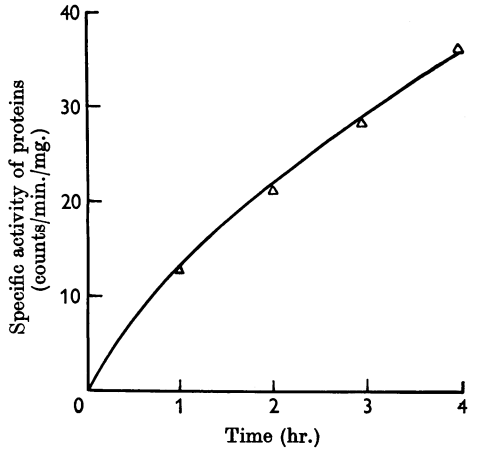

Fig. 2. Incorporation of [^{14}C]valine into the proteins of buffalo (BB2) spermatozoa. A portion (1 ml.) of a suspension of the spermatozoa in the fructose–bicarbonate buffer was incubated with 0·1 ml. (0·93 μmole and 1·14 μc) of the [^{14}C]valine solution, as described in the text.

Table 2. *Incorporation of* ^{14}C*-labelled* Chlorella *hydrolysate into the proteins of washed cock spermatozoa*

A portion (1 ml.) of the suspension of the spermatozoa in the fructose–phosphate buffer was incubated with 0·1 ml. (0·19 μmole of mixed amino acids and 2·25 μc) of ^{14}C-labelled *Chlorella* hydrolysate. Proteins were isolated as described in the text.

Time of incubation (hr.)	Specific activity of proteins (counts/min./mg.)
1½	0
3	7

that certain enzymes, such as hyaluronidase, are released from spermatozoa on storage at temperatures just above 0°, and rewarming (Swyer, 1947), the effect of such treatment on the release of radioactive proteins from spermatozoa preincubated with ^{14}C-labelled amino acids was studied. Bull or buffalo spermatozoa incubated for various time-intervals with ^{14}C-labelled *Chlorella* hydrolysate in the fructose–phosphate buffer were washed twice, each time with 10 ml. of buffer containing 2 mg. of non-radioactive amino acids/ml., and resuspended in 3 ml. of water. The suspensions were left for 18 hr. in ice, and then centrifuged for 5 min. at 900*g* in the cold. Protein was isolated from the supernatant fluid, from the residue and from control samples precipitated with trichloroacetic acid immediately after washing with the buffer containing non-radioactive amino acids, as described in the Experimental section; in one experiment, the protein in the supernatant was precipitated with $(NH_4)_2SO_4$ at 30% saturation. The results of several experiments carried out as described above are given in Table 5. The amount of protein in the supernatant was insignificant (always less than 1% of the protein contained in the cells), and in most experiments it was difficult to get enough protein from the supernatant for radioactivity measurement; whenever sufficient protein could be isolated from this fraction, its specific activity was many times higher than in the control. The specific activity of the proteins isolated from the cells after the cold treatment was always considerably less (20–70%) than that in the control, showing that a significant part of the incorporation of ^{14}C-labelled amino acids into spermatozoa occurs in proteins that are released on cold treatment. In an experiment (Table 5, Expt. 28) in which an attempt was made to recover the proteins released in the supernatant, by precipitation with $(NH_4)_2SO_4$ at 30% saturation, all the radioactivity lost from the spermatozoa during the cold treatment was recovered in the precipitate.

Table 3. *Retention of radioactivity in isotopically labelled proteins of washed spermatozoa on reincubation in a non-radioactive medium*

A portion (1 ml.) of a suspension (in the fructose–phosphate buffer) of washed spermatozoa from mixed semen of two buffaloes (BB1 and BB2) was incubated with 0·1 ml. (0·49 μmole of mixed amino acids and 5 μc) of ^{14}C-labelled *Chlorella* hydrolysate. Details of this incubation, of the reincubation of the labelled spermatozoa in a non-radioactive medium and of the isolation of proteins, are given in the text.

Expt. no.	Time of incubation in radioactive medium (hr.)	Time of second incubation in non-radioactive medium (hr.)	Specific activity of proteins (counts/min./mg.)
22	1½	0	694
	1½	1	674
	1½	2	714
	3	0	1125
	3	½	1102
	3	1	1115
23	1½	0	524
	1½	½	528
	1½	1	505
	1½	1½	536
	3	0	1065
	3	½	1020
	3	1	1031

Table 4. *Retention of radioactivity in isotopically labelled spermatozoa proteins from whole semen on reincubation in a non-radioactive medium*

A portion (1 ml.) of whole semen (bull CB1) was incubated with 0·1 ml. (0·49 μmole of mixed amino acids and 5 μc) of ^{14}C-labelled *Chlorella* hydrolysate. Details of this incubation, of the reincubation of the labelled spermatozoa in a non-radioactive medium, and of the isolation of proteins, are given in the text.

Time of incubation in radioactive medium (hr.)	Time of reincubation in non-radioactive medium (hr.)	Specific activity of proteins (counts/min./mg.)
½	0	161
½	1½	143
1	0	150
1	1½	148
1½	0	160
1½	1½	151
2	0	157
2	1½	149

DISCUSSION

The observations reported here confirm the two previous reports on incorporation of ^{14}C-labelled amino acids into the proteins of bull spermatozoa (Bhargava *et al.* 1959; Martin & Brachet, 1959). Incorporation of ^{14}C-labelled amino acids into the proteins of buffalo and goat spermatozoa suggests that this incorporation may be a general characteristic of mammalian spermatozoa. The incorporation could be due to either a non-specific exchange of amino acids in existing protein molecules, or a synthesis of new protein molecules. The possibility of a non-specific exchange is eliminated by the observed retention of radioactivity on reincubation of the labelled spermatozoa.

We have found that the acrosome is lost from all the cells when a suspension of bull or buffalo spermatozoa is stored at 0° (P. T. Iype, K. A. Abraham & P. M. Bhargava, unpublished work). Since the acrosome as well as a significant part of the incorporated radioactivity are released from

Table 5. *Release of radioactivity from isotopically labelled spermatozoa proteins on storage of spermatozoa at 0°*

A portion (1 ml.) of a suspension (in the fructose–phosphate buffer) of washed spermatozoa was incubated with 0·1 ml. (0·49 μmole of mixed amino acids and 5 μc) of ^{14}C-labelled *Chlorella* hydrolysate; the incubated cells were submitted to cold treatment and the proteins were isolated as described in the text.

Expt. no.	Animal	Time of incubation in radioactive medium (hr.)	Specific activity of spermatozoa proteins (counts/min./mg.)		Release of radioactivity on cold treatment (% of control)
			Control*	Cold-treated	
25	BB1+BB2	1½	188	58	72
		3	186	76	61
26	CB2	1	135	110	19
		2	186	130	42
		3	172	99	54
27	BB1	1½	229	185	19
		3	272	219	20
28†	BB1	3	227	143	37
50	BB2	2	712	513	28

* Precipitated with trichloroacetic acid immediately after incubation and washing (see text).

† In this experiment, the proteins released in the medium during the cold treatment were precipitated with $(NH_4)_2SO_4$ at 30% saturation; all the radioactivity lost from the spermatozoa was recovered in the precipitate.

spermatozoa under conditions that do not cause cell lysis or a loss of the bulk of any major intracellular constituent (such as protein), it seems probable that the incorporation of labelled amino acids into spermatozoa occurs in the acrosomal region. This view is further supported by the observation that cock spermatozoa, which are either devoid of the acrosome or have this structure in a strongly modified form (one that does not exhibit any of the properties characterizing mammalian acrosomes; P. T. Iype, K. A. Abraham & P. M. Bhargava, unpublished work), are unable to incorporate labelled amino acids into their proteins, whereas all the three types of mammalian spermatozoa examined (bull, buffalo and goat), which have a normal acrosome, do so to a significant extent. The variation observed from experiment to experiment in the amount of radioactivity released from spermatozoa on storage at 0°, in contrast with the complete loss of acrosome from almost all the cells in every experiment, may be due to the sedimentation of a varying quantity of acrosome fragments along with the spermatozoa during the centrifuging. Intact acrosomes or stainable acrosome fragments could not be detected in the suspending medium after the cold treatment of spermatozoa. It was therefore difficult to assess, even qualitatively, the amount of protein of acrosomal origin remaining in the supernatant after centrifuging, and the relation between distribution of acrosomes and of radioactivity between supernatant and sedimented fractions could not be studied.

Martin & Brachet (1959), on radioautography of bull spermatozoa labelled with ^{14}C-labelled amino acids, could not detect any radioactivity above the background in the spermatozoa; they suggested that the presence of bacteria or unidentified cell debris in the suspension of spermatozoa might be responsible for the observed incorporation of labelled amino acid into the proteins derived from the suspension. The lack of a significant increase in the initially low bacterial count of semen on incubation (Bhargava *et al.* 1959), and the incorporation of nucleic acid precursors exclusively into RNA and not into DNA in suspensions of spermatozoa (Abraham & Bhargava, 1963), are strong arguments against bacteria being responsible for the observed incorporation of labelled amino acids into the proteins of spermatozoa. Since the cell debris is unlikely to represent more than a small fraction of the total protein content of the spermatozoa preparations, the intrinsic ability of the cell debris to incorporate amino acids will have to be of a magnitude which is not normally obtained with somatic cell preparations, if all the incorporation observed were to be accounted for by the cell debris. We have recently observed that the treatment of spermatozoa with methanol, used by Martin & Brachet (1959) as a fixative during their radioautographic studies, results in a disintegration or modification of the acrosome (P. T. Iype, K. A. Abraham & P. M. Bhargava, unpublished work); this might explain their negative findings.

SUMMARY

1. The incorporation of a mixture of ^{14}C-labelled amino acids into the proteins of spermatozoa derived from bulls of Indian breed, maintained under local conditions, and the effect of several

inhibitors on this incorporation, have been studied and compared with the results of similar investigations carried out earlier.

2. Washed spermatozoa from buffalo and goat have been shown to incorporate radioactive amino acids into their proteins. No such incorporation was observed in cock spermatozoa.

3. The radioactivity of bull- and buffalo-spermatozoa proteins was retained on reincubation of prelabelled spermatozoa with a large excess of unlabelled amino acids for 1½ hr.

4. There was an average 50% loss of radioactivity from the proteins of bull and buffalo spermatozoa on storage at 0° for 18 hr.; under these conditions the spermatozoa are known to lose their acrosome and hyaluronidase.

5. The findings support the view that the incorporation of radioactive amino acids into the proteins of mammalian spermatozoa occurs in the acrosomal region.

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REFERENCES

Abraham, K. A. & Bhargava, P. M. (1963). *Biochem. J.* **86**, 298.
Bhargava, P. M. (1957). *Nature, Lond.*, **179**, 1120.
Bhargava, P. M., Bishop, M. W. H. & Work, T. S. (1959). *Biochem. J.* **73**, 247.
Brenner, S., Jacob, F. & Meselson, M. (1961). *Nature, Lond.*, **190**, 576.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*, p. 317. New York: J. Wiley and Sons Inc.
Gros, F., Hiatt, H., Gilbert, W., Kurland, C. G., Rosebrough, R. W. & Watson, J. D. (1961). *Nature, Lond.*, **190**, 581.
Martin, F. & Brachet, J. (1959). *Exp. Cell Res.* **17**, 399.
Perry, E. J. (1960). *Artificial Insemination in Farm Animals*, 3rd ed. New Jersey: Rutgers University Press.
Swyer, G. I. M. (1947). *Biochem. J.* **41**, 413.

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Purification and Properties of Chlorophyll *c* from *Sargassum flavicans*

By S. W. JEFFREY*
C.S.I.R.O. Marine Laboratory, Cronulla, Sydney, Australia

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Chlorophyll *c* is found together with chlorophyll *a* in many types of marine algae. These include the Bacillariophyceae, Dinophyceae, Phaeophyceae and Cryptophyceae (Strain & Manning, 1942; Strain, Manning & Hardin, 1943; Haxo & Fork, 1959), and some members of the Chrysophyceae (Allen, French & Brown, 1960; Jeffrey, 1961). Although chlorophyll *c* has been obtained sufficiently free from other pigments to enable its spectrum to be determined (Smith & Benitez, 1955; Strain, 1958; Haxo & Fork, 1959) it was not obtained in the pure state. Therefore, little is known of its chemistry, and methods used for the estimation of chlorophyll *c* are indirect (Richards & Thompson, 1952). The present work describes a method which has been developed for the purification and crystallization of chlorophyll *c* from a member of the Phaeophyceae, *Sargassum flavicans*.

* Present address: Kaiser Foundation Research Institute, Laboratory of Comparative Biology, South 14th Street and Cutting Boulevard, Richmond, Calif., U.S.A.

EXPERIMENTAL

Materials and methods

Solvents. Magnesium-free ethanol was obtained by drying a commercial product over K_2CO_3 and distilling twice. All other solvents were analytical reagent grade, and were used without further purification.

Chromatographic adsorbents. The following were used: cellulose powder (Whatman), silicic acid (Mallinckrodt, analytical reagent), aluminium oxide for chromatography (British Drug Houses Ltd.), Hyflo Supercel (Johns Manville) and silicic acid for thin-layer chromatography (Merck).

Source of chlorophyll c. Sargassum flavicans was chosen as the source material for chlorophyll *c* because of its availability and the absence of chlorophyllase activity during extraction. This enzyme converts chlorophyll *a* into chlorophyllide *a*, which is difficult to separate from chlorophyll *c* (Jeffrey, 1961). The seaweed was collected from Port Hacking and Lake Macquarie, New South Wales, and kept in sea water until extracted.

Assay of total phosphorus. This was carried out by the method of Allen (1940).